

# An Extracellular Protein Microdomain Controls Up-regulation of Neuronal Nicotinic Acetylcholine Receptors by Nicotine\*

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**In smoker's brain, rodent brain, and in cultured cells expressing nicotinic receptors, chronic nicotine treatment induces an increase in the total number of high affinity receptors for acetylcholine and nicotine, a process referred to as up-regulation. Up-regulation induced by 1 mM nicotine reaches 6-fold for  $\alpha 3\beta 2$  nicotinic receptors transiently expressed in HEK 293 cells, whereas it is much smaller for  $\alpha 3\beta 4$  receptors, offering a rationale to investigate the molecular mechanism underlying up-regulation. In this expression system binding sites are mainly intracellular, as shown by [ $^3\text{H}$ ]epibatidine binding experiments and competition with the impermeant ligand carbamylcholine. Systematic analysis of  $\beta 2/\beta 4$  chimeras demonstrates the following. (i) The extracellular domain critically contributes to up-regulation. (ii) Only residues belonging to two  $\beta 2$  segments, 74–89 and 106–115, confer up-regulation to  $\beta 4$ , mainly by decreasing the amount of binding sites in the absence of nicotine; on an atomic three-dimensional model of the  $\alpha 3\beta 2$  receptor these amino acids form a compact microdomain that mainly contributes to the subunit interface and also faces the acetylcholine binding site. (iii) The  $\beta 4$  microdomain is sufficient to confer to  $\beta 2$  a  $\beta 4$ -like up-regulation. (iv) This microdomain makes an equivalent contribution to the up-regulation differences between  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$ . We propose that nicotine, by binding to immature oligomers, elicits a conformational reorganization of the microdomain, strengthening the interaction between adjacent subunits and, thus, facilitating maturation processes toward high affinity receptors. This mechanism may be central to nicotine addiction, since  $\alpha 4\beta 2$  is the subtype exhibiting the highest degree of up-regulation in the brain.**

Nicotine is the primary substance responsible for tobacco addiction, a major cause of death in western societies (1, 2). Chronic exposure to nicotine causes a strong addiction, which is mediated by the interaction of nicotine with neuronal nicotinic acetylcholine receptors (nAChRs),<sup>1</sup> a class of pentameric allosteric ligand-gated ion channels engaged in cholinergic nicotinic transmission in the brain (3, 4).

Post-mortem analysis of brain slices from smokers (5, 6) and from rats or mice chronically treated with nicotine (7–10) reveals large increases in the number of high affinity nicotinic binding sites, a phenomenon termed up-regulation. Nicotine up-regulates the  $\alpha 3\beta 2$  (11, 12),  $\alpha 4\beta 2$  (13–18),  $\alpha 7$  (11, 19), and ( $\alpha 1$ ) $2\beta 1\gamma\delta$  (19) nAChR subtypes reconstituted in cell lines or in *Xenopus* oocytes.

In addition to the increased number of sites, modulation of the magnitude of the nicotine-elicited electrophysiological response is also observed upon chronic nicotine incubation of cell lines expressing recombinant nAChRs. This functional potentiation of the response is observed with nAChR oligomers  $\alpha 4\beta 2$  and  $\alpha 3\beta 2$  reconstituted in mammalian cell lines (12, 20). But functional depression almost systematically takes place with the same combination of subunits reconstituted in *Xenopus* oocytes (13, 21, 22). The functional potentiation of nAChRs by chronic nicotine treatment *in vivo* (23, 24) is thought to contribute to tobacco addiction (4, 25).

Yet the molecular mechanisms underlying these plasticity phenomena remain to be elucidated. Transcriptional processes (15) have so far been disregarded in favor of post-translational recruitment of preexisting subunits (12) or stabilization of already assembled pentamers and protection against turnover (15) or both. Equilibrium binding and electrophysiological recordings reveal that upon stable expression in HEK 293 cell lines,  $\alpha 3\beta 2$  receptors are both strongly up-regulated and potentiated by chronic nicotine exposure. In contrast,  $\alpha 3\beta 4$  receptors were unaffected by chronic nicotine treatment (12). This observation offers a rationale to investigate the molecular mechanisms underlying up-regulation by identifying the protein regions controlling this process.

In the present study, we focus our analysis on up-regulation of the number of binding sites for nicotine. By constructing  $\beta 2/\beta 4$  chimeras, we identified key amino acids that form a compact microdomain, contributing to the differences in up-regulation between  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$ . This microdomain also accounts for the up-regulation differences between  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$  at nicotine concentrations compatible with those found in the blood of tobacco smokers. We propose a molecular mechanism of up-regulation where nicotine, binding to immature receptor precursors, causes a conformational reorganization of this microdomain.

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<sup>1</sup> The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ECD, extracellular domain; HEK cells, human embryonic kidney cells; nH, Hill number; PBS, phosphate-buffered saline.

## MATERIALS AND METHODS

**Construction of Chimeras between  $\beta 2$  and  $\beta 4$  Subunits**—cDNAs encoding the  $\beta 2$  and the  $\beta 4$  rat subunits were excised as a NotI/XhoI fragment from the clones kindly supplied by J. Boulter and introduced into the pMT<sub>3</sub> vector for expression (26). Synthetic genes coding for the extracellular domain (ECD) of  $\beta 2$  and  $\beta 4$  were constructed by PCR amplification (pwo-polymerase, Roche Applied Science) using 60-mer oligonucleotides with an overlap of 20 base pairs, containing the minimum set of mutations allowing adequately distributed common restriction sites. We then fused those synthetic genes to the C-terminal SpeI/XhoI-amplified  $\beta 4$  (pwo-polymerase, Roche Applied Science). Chimeras were then constructed by simple subcloning between the two synthetic constructs. Single amino acid mutations were performed by subcloning hybridized pairs of oligonucleotides containing the appropriate mutation into the  $\beta 4$  synthetic gene. Synthetic genes and restriction sites are shown on Fig. 2.

**Cell Culture and Transient Transfection of Human HEK 293 Cells**—Human HEK 293 cells, an embryonic kidney cell line, were maintained in minimum essential medium (Invitrogen) supplemented with 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM glutamine, and 10% fetal calf serum (Invitrogen) in a CO<sub>2</sub> (5%) incubator at 37 °C. Cells were grown in 10-cm dishes and transfected using the calcium phosphate method (27). The transfectant was rinsed 24 h after transfection and replaced by fresh medium to allow expression for an additional 24 h.

**[<sup>3</sup>H]Epibatidine Binding, Nicotine Up-regulation**—Up-regulation of  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  receptors transiently transfected in the HEK 293 cell line was examined by allowing expression of the receptors to reach a near steady-state level for 1 day and then incubating the cells with culture medium supplemented with (up-regulation conditions) or without (control conditions) the concentrations of nicotine given in the corresponding sections of the text (0.01–1000  $\mu$ M) for 24 h. Nicotine was then eliminated from the culture medium for 30 min 2 times at 37 °C. Cells were then rinsed 3 times with PBS (137 mM NaCl, 2.7 mM KCl, 5.3 mM Na<sub>2</sub>HPO<sub>4</sub>, and 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2) and harvested using 5 mM EDTA in PBS. After 5 min of centrifugation at 1500 rpm, the cells were resuspended in 1 ml of PBS. [<sup>3</sup>H]Epibatidine binding was carried out for 30 min at 37 °C at a concentration of 5 nM in 250  $\mu$ l of the cell suspension. This mixture was then filtered 2 times on GF/C filters (Whatman) with ice-cold PBS. The filters were then counted using biodegradable counting scintillant (BCS, Amersham) in a 1209 Rack-beta counter (LKB Wallak). Nonspecific binding was assessed using 5 mM nicotine. For  $\alpha 3\beta 4$ , a significant up-regulation took place at 1 mM nicotine and reached more than 2-fold at 10 mM nicotine but sometimes resulted in a large decrease in binding sites due to the toxicity of nicotine at this concentration.

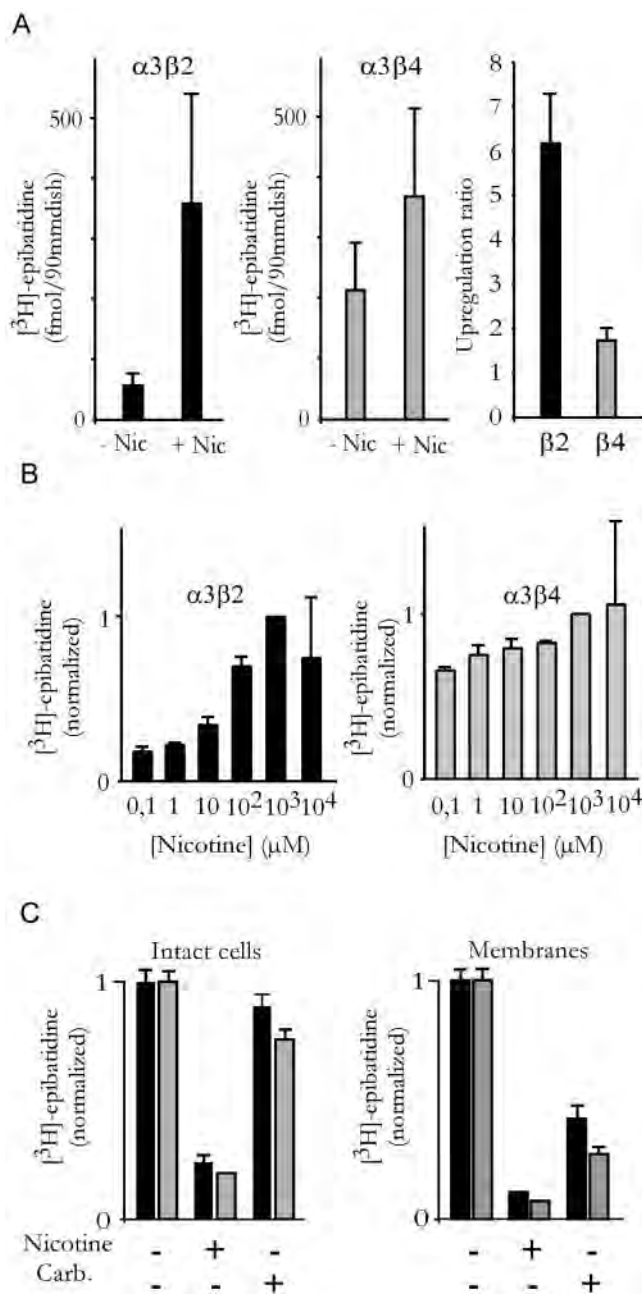
**Membrane Preparation**—The cells were pelleted at 1500 rpm for 5 min, resuspended in 1 ml of homogenization buffer (ice-cold PBS containing 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride). Cell disruption was performed through sonication (3 times for 10 s) as previously described (17), and binding was performed in the usual conditions.

**Molecular Modeling**—Molecular models were constructed with Modeller (28) and Autodock (29) as described elsewhere (30). Handling of models and generation of pictures were performed with Deep-View (31), RasMol (32), and ViewerLite (Accelrys).

## RESULTS

**Up-regulation Properties of  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  Receptors Transiently Transfected in HEK 293 Cells**—The identification of the amino acids responsible for the differences in up-regulation between  $\beta 2$  and  $\beta 4$  subunits by a chimeric approach required the generation and screening of numerous chimeras. To do so, we designed an up-regulation procedure after transient transfection in HEK 293 cells. Cells expressing  $\alpha 3$  along with  $\beta 2$ ,  $\beta 4$ , or a  $\beta 2/\beta 4$  chimera were cultured for 24 h without (control condition) or with nicotine at a given concentration. After 1 h of washing to remove the nicotine bound to the receptor, the number of nicotinic binding sites was measured by equilibrium binding with [<sup>3</sup>H]epibatidine, a high affinity agonist. In all experiments, subunits from rat, previously designed to dissect the molecular determinant of desensitization (33), were used.

Fig 1A shows the average [<sup>3</sup>H]epibatidine binding site expression of the wild-type  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  receptors in control condition and upon up-regulation by 1 mM nicotine. Data are



**FIG. 1. Up-regulation of  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  receptors transiently expressed in HEK 293 cells.** A,  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  panels, [<sup>3</sup>H]epibatidine binding to HEK 293 cells transfected with the corresponding cDNAs cultured in control conditions or in medium supplemented with 1 mM nicotine (Nic). Data are taken from eight independent experiments, and error bars correspond to  $\pm$  S.D., illustrating the variability in receptor expression. Right panel, mean and S.D. values of the up-regulation ratios for the same eight experiments. Ratios are defined as the number of high affinity binding sites in up-regulated conditions divided by the number of high affinity binding sites in control conditions and were calculated within each individual experiment. This illustrates the good reproducibility of up-regulation, independent of the absolute expression levels. In all the figures,  $\alpha 3\beta 2$  is represented in black, and  $\alpha 3\beta 4$  is in gray. B, dose dependence of nicotine-elicited up-regulation for  $\alpha 3\beta 2$  (left, black) and  $\alpha 3\beta 4$  (right, gray). Data are  $\pm$  S.D. from three different experiments individually normalized to the 1 mM nicotine value. C, displacement of [<sup>3</sup>H]epibatidine binding by nicotine or carbamylcholine (Carb.) on intact cells (left) or cell membranes (right) transfected with  $\alpha 3\beta 2$  (black) or  $\alpha 3\beta 4$  (gray). Data are  $\pm$  S.D. from three different experiments.

taken from a series of eight independent experiments. Each experiment consisted of expressing both receptors in control and up-regulated conditions the same day on the same batch of

cells. Fig. 1A indicates that the high affinity binding site expression is variable, with S.D. around 50% in the four conditions. However, we found that this variability was mainly dependent on the state of the cells at the time of the transfection. Indeed, within each experiment the relative expression level between both receptors is stable. For instance, the ratio of the expression of up-regulated  $\alpha 3\beta 4$  to up-regulated  $\alpha 3\beta 2$  is  $1.00 \pm 0.17$ .

Furthermore, we found that up-regulation was independent of the absolute expression level. Indeed, for each independent experiment the up-regulation ratio ( $r$ ) was calculated by dividing the number of binding sites in the up-regulated condition by the number of binding sites in the control condition. This ratio remained stable ( $r = 1.73 \pm 0.29$  and  $6.15 \pm 1.19$  for  $\alpha 3\beta 4$  and  $\alpha 3\beta 2$ , respectively; Fig 1A, right panel), independent of the absolute expression level inherent to the state of the cells.

Thus, in our system,  $\alpha 3\beta 2$  up-regulates strongly, whereas  $\alpha 3\beta 4$  displays relatively weak but significant up-regulation. Because  $\alpha 3\beta 2$  expression is much lower than  $\alpha 3\beta 4$  in the absence of nicotine up-regulation and since both receptors display nearly identical expression in up-regulated conditions, the difference in up-regulation is the consequence of different levels of expression in control conditions. Further pharmacological analysis in our system shows the following. (i) Performing a saturation analysis of [ $^3\text{H}$ ]epibatidine binding for each combination of subunits did not reveal differences in the affinity for [ $^3\text{H}$ ]epibatidine between control and up-regulated receptors. Fitting the data points according to the empirical Hill equation yielded  $K_d$  values of  $0.4 \pm 0.1$  nM ( $n\text{H} = 1.5 \pm 0.2$ ) and  $0.35 \pm 0.1$  nM ( $n\text{H} = 1.6 \pm 0.1$ ) for  $\alpha 3\beta 2$  and  $K_d$  values of  $0.25 \pm 0.15$  nM ( $n\text{H} = 1.7 \pm 0.1$ ) and  $0.25 \pm 0.2$  nM ( $n\text{H} = 1.6 \pm 0.1$ ) for  $\alpha 3\beta 4$ , respectively, after exposure to control medium or to nicotine-supplemented medium. A concentration of 5 nM [ $^3\text{H}$ ]epibatidine was, thus, selected to measure the total amount of ACh binding sites in the subsequent study. (ii) The up-regulation of  $\alpha 3\beta 2$  was concentration-dependent, with a half-maximal effect obtained around 30  $\mu\text{M}$  nicotine and a maximal effect around 1 mM nicotine (Fig. 1B). For  $\alpha 3\beta 4$  the  $\text{EC}_{50}$  was more difficult to evaluate due to the lower up-regulation level and to the observation that nicotine starts to be toxic for the cells at 10 mM concentration, preventing a reliable evaluation of up-regulation at this concentration. Still, high concentrations of nicotine were required, apparently with an  $\text{EC}_{50}$  in the range of 100  $\mu\text{M}$ . (iii) None of the antagonists tested (mecamylamine (1 mM), d-tubocurarine (1 mM), dihydro- $\beta$ -erythroidine (0.1 mM)) was found to cause significant up-regulation for both  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$ , and none of them significantly inhibited up-regulation elicited by 1 mM nicotine (data not shown).

Altogether, these data are consistent with those previously reported (12) but with a lower up-regulation ratio for  $\alpha 3\beta 2$  (6-fold as compared with 24-fold) and a higher  $\text{EC}_{50}$  of up-regulation (30  $\mu\text{M}$  as compared 3  $\mu\text{M}$ ). Furthermore, we found that  $\alpha 3\beta 4$  exhibited a weak but significant up-regulation. These differences possibly reflect different phenotypes between the rat and human  $\alpha 3$ -containing nAChRs or, alternatively, differences in the expression systems; that is, transient transfection in HEK 293 cells *versus* stable expression in tsA210 cells.

**Up-regulation Involves an Increase in Intracellular Binding Sites**—Nicotine and epibatidine are small organic tertiary amines that cross cell membranes in their uncharged form. To distinguish between binding to surface or to intracellular nAChRs, we investigated the ability of carbamylcholine, an impermeant quaternary amine, to compete with [ $^3\text{H}$ ]epibatidine binding. For both the  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  receptors, we found that a high concentration (5 mM) of carbamylcholine did not

significantly inhibit [ $^3\text{H}$ ]epibatidine binding (Fig. 1C). On the other hand, after disruption of the cells by sonication, the same concentration of carbamylcholine was found to displace a large fraction of bound [ $^3\text{H}$ ]epibatidine. Thus, in the intact cell, [ $^3\text{H}$ ]epibatidine primarily binds to intracellular  $\alpha 3\beta 2$  or  $\alpha 3\beta 4$  nAChR, as previously reported for the  $\alpha 4\beta 2$  receptor expressed in M10 cells (17). The residual carbamylcholine insensitive [ $^3\text{H}$ ]epibatidine binding observed after sonication (30–40% of total [ $^3\text{H}$ ]epibatidine binding) could correspond to sites that are still inaccessible to carbamylcholine after homogenization.

**The ECD of the  $\beta$ -Subunit Controls Nicotine Up-regulation**—In the subsequent study, mutant receptors were compared both on the basis of their up-regulation ratio (after treatment by 1 mM nicotine) and of their absolute expression level. Although the up-regulation ratios displayed good reproducibility, we have seen that the absolute expression levels were much more variable. To reduce this variability, we systematically included in each experiment the wild-type  $\alpha 3\beta 4$  receptor. Absolute expression levels of the various constructs were then normalized to the expression of  $\alpha 3\beta 4$  in up-regulated conditions.

Heteromeric neuronal  $\alpha 3(\alpha 4)\beta 2(\beta 4)$  nAChRs are pentameric transmembrane channel receptors. All subunits possess a similar transmembrane organization; that is, a large extracellular N-terminal domain followed by three transmembrane segments, a cytoplasmic domain, a fourth transmembrane segment, and an extracellular C terminus. To investigate which domain(s) of the  $\beta$  subunit is responsible for the differences in up-regulation between  $\beta 2$ - and  $\beta 4$ -containing receptors, we constructed chimeras  $\beta 2$ -(1–212) consisting of the ECD of  $\beta 2$  and the transmembrane and cytoplasmic domains of  $\beta 4$  (all chimeras are named according to the  $\beta 2$  primary sequence they contain, the balance of the primary sequence belonging to  $\beta 4$ ) and  $\beta 2$ -(212–407), possessing the complementary organization. The up-regulation ratio of  $\beta 2$ -(1–212) indicates a  $\beta 2$ -like phenotype ( $r = 7.26$ ), and that of  $\beta 2$ -(212–407) indicates a  $\beta 4$ -like phenotype ( $r = 1.43$ ) (Fig. 3), showing that the ECD confers up-regulation when inserted from  $\beta 2$  into  $\beta 4$  and conversely inhibits up-regulation when transferred from  $\beta 4$  into  $\beta 2$ . In addition, chimeras  $\beta 2$ -(1–212) and  $\beta 2$ -(212–407) displayed expression levels nearly 2-fold higher than that of  $\beta 2$  and  $\beta 4$ , respectively. This suggests that specific interactions between the ECD of  $\beta 2$  (respectively,  $\beta 4$ ) and the transmembrane domain of  $\beta 4$  (respectively,  $\beta 2$ ) increase absolute high affinity protein expression.

**A Compact Microdomain of Two  $\beta 2$  Segments,  $\beta 2$ -(74–89) and  $\beta 2$ -(106–115), Confers Up-regulation to  $\beta 4$** —Further dissection of the contribution of the ECD to the up-regulation process was achieved by taking  $\alpha 3\beta 4$  as a reference and introducing progressively larger portions of the  $\beta 2$  subunit at both the N- and C-terminal ends of the  $\beta 4$  ECD. To achieve this goal we constructed synthetic genes encoding the  $\beta 2$  and  $\beta 4$  subunits, with common restriction sites within their ECD (Fig. 2). We verified that the gene encoding synthetic and wild-type  $\beta 4$  displayed comparable expression and electrophysiological functional properties (data not shown).

This approach showed that segment 38–115 of  $\beta 2$  contains the critical elements conferring up-regulation to  $\beta 4$ . Indeed,  $\beta 2$ -(1–37) and  $\beta 2$ -(116–212) exhibited a  $\beta 4$ -like phenotype ( $r = 1.83$  and 1.76, respectively), whereas  $\beta 2$ -(1–89) and  $\beta 2$ -(90–212) displayed an up-regulation ratio higher than the one of  $\alpha 3\beta 4$  ( $r = 2.86$  and 5.26, respectively) (Fig. 3). It is noteworthy that chimeras  $\beta 2$ -(1–37),  $\beta 2$ -(1–89), and  $\beta 2$ -(90–212) displayed absolute expression levels much lower than the wild-type receptors, indicating that these macrochimeras may experience destabilizing interactions within their structure or during their maturation.

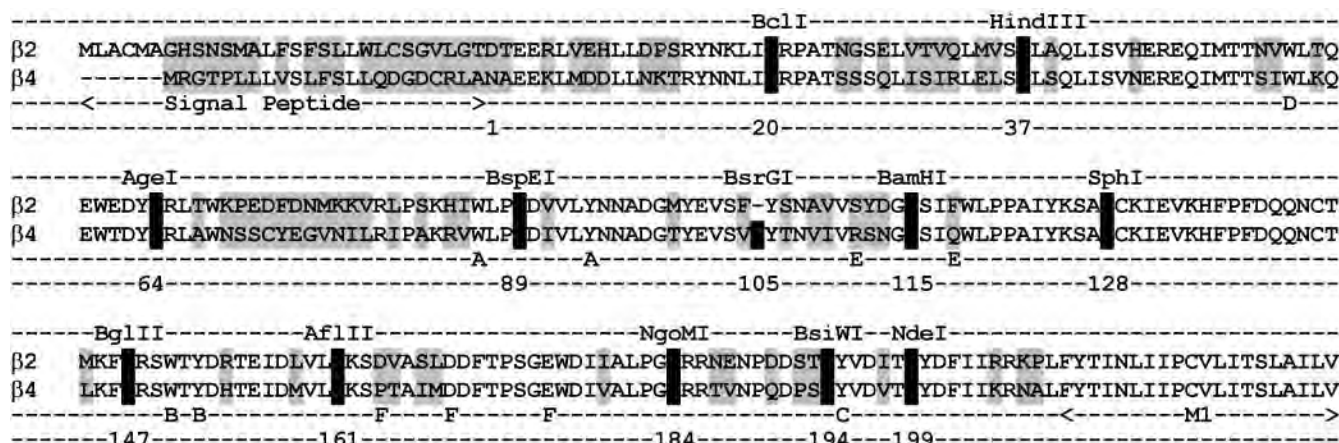


FIG. 2. Amino acid sequences of  $\beta 2$  and  $\beta 4$  extracellular domains. The restriction sites inserted in our synthetic genes are indicated, and numbers correspond to both the  $\beta 2$  and  $\beta 4$  sequence; gray boxes highlight non-conserved residues between  $\beta 2$  and  $\beta 4$ .

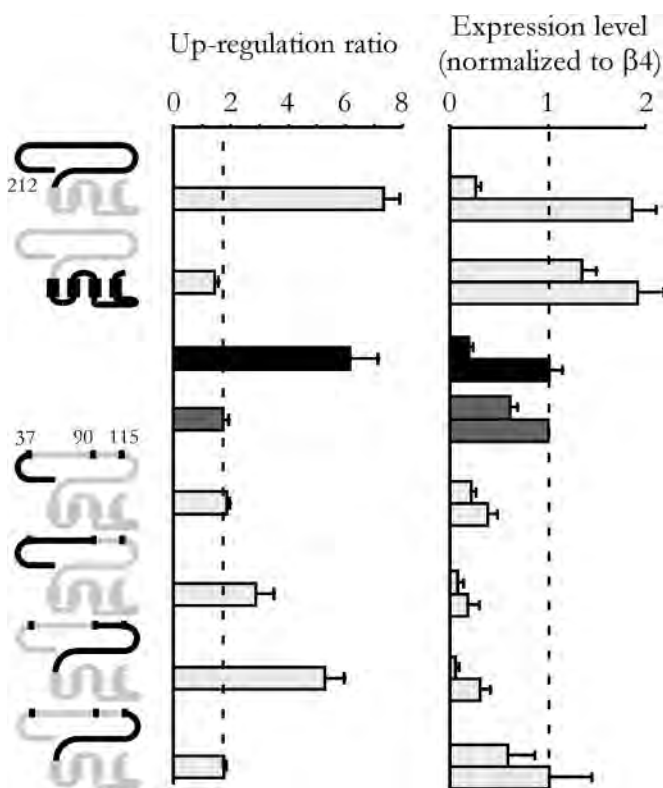


FIG. 3. The N-terminal  $\beta 2$ -(37–115) segment contains the critical elements conferring up-regulation to  $\alpha 3\beta 4$ . Up-regulation ratio (left panel) and expression levels normalized to up-regulated  $\alpha 3\beta 4$  (right panel), for each construct the upper bar corresponds to control conditions, and the lower bar corresponds to 1 mM nicotine up-regulated conditions) of N-terminal  $\beta 2/\beta 4$  chimeras co-expressed with  $\alpha 3$ . Wild-type  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  are shown in black and gray, respectively. Schematics represent the transmembrane topology of the  $\beta$  subunit, with an N-terminal extracellular domain followed by a C-terminal transmembrane and cytoplasmic domain composing four transmembrane segments, represented as rectangles. Black and gray lines represent, respectively, the  $\beta 2$  and  $\beta 4$  sequences. Data are  $\pm$  S.D. from three different experiments.

In a second step segment 38–115 was dissected in three cassettes that were tested individually (Fig. 4). First,  $\beta 2$ -(38–64) had a  $\beta 4$ -like phenotype both for the up-regulation ratio and the expression level. Second,  $\beta 2$ -(65–89) was directly split into four cassettes;  $\beta 2$ -(65–73) displayed a  $\beta 4$ -like phenotype ( $r = 1.63$ ),  $\beta 2$ -(74–79) up-regulated strongly ( $r = 4.94$ ), and  $\beta 2$ -(81) and  $\beta 2$ -(82–89) exhibited a weak but significantly increased up-regulation compared with  $\beta 4$  ( $r = 2.27$  and 2.60,

respectively). Furthermore, these four chimeras displayed expression levels similar to that of  $\beta 4$ . Third,  $\beta 2$ -(90–115) presented a very weak expression, preventing an accurate measurement of the number of binding sites in control conditions. Still, the evaluated up-regulation ratio ( $3.48 \pm 1.11$ , data not shown) suggested that this cassette may confer strong up-regulation to  $\beta 4$ . We split this cassette into  $\beta 2$ -(90–105) and  $\beta 2$ -(106–115) with  $r = 1.82$  and 4.43, respectively. The segment  $\beta 2$ -(106–115) was finally separated into 2 microchimeras,  $\beta 2$ -(106–110) and  $\beta 2$ -(111–115), with respective up-regulation ratios of 3.34 and 2.87 and expression levels similar to that of  $\beta 4$ .

Altogether, the critical residues of  $\beta 2$  conferring up-regulation to  $\alpha 3\beta 4$  belong to two separate segments,  $\beta 2$ -(74–89) and  $\beta 2$ -(106–115). Within  $\beta 2$ -(74–89),  $\beta 2$ -(74–79) played a major role in up-regulation, whereas  $\beta 2$ -(81) and  $\beta 2$ -(82–89) produced weaker effects. Within  $\beta 2$ -(106–115), the two half-cassettes,  $\beta 2$ -(106–110) and  $\beta 2$ -(111–115) nearly equally contributed to the up-regulation. We also performed single amino acid mutants  $\beta 2$ -(74),  $\beta 2$ -(75),  $\beta 2$ -(76),  $\beta 2$ -(77),  $\beta 2$ -(78),  $\beta 2$ -(79),  $\beta 2$ -(107),  $\beta 2$ -(109),  $\beta 2$ -(110),  $\beta 2$ -(112),  $\beta 2$ -(113), and  $\beta 2$ -(114). All of them exhibited up-regulation ratios and absolute expression levels similar to that of  $\beta 4$  (data not shown), indicating that within the sub-domains  $\beta 2$ -(74–79),  $\beta 2$ -(106–110), and  $\beta 2$ -(111–115) a combination of amino acids generates the phenotypes.

Visualization of the  $\beta 2$  residues conferring up-regulation to  $\beta 4$  on an atomic model of the  $\alpha 3\beta 2$  receptor extracellular domain derived from AChBP (30, 34) (Fig. 5) revealed that these amino acids form a compact microdomain. This “up-regulation domain” is located in the upper part of each subunit and is composed of 1) two  $\beta$  strands ( $\beta 3$  and  $\beta 5$ ) interacting together from the main  $\beta$ -sheet of the structure ( $\beta 3$ : Val-78 and Leu-80;  $\beta 5$ : Ser-107, Ala-109, and Val-110) and 2) loops flanking these  $\beta$  strands (loop 1 (Asp-73, Asn-74, Met-75, Lys-76, Lys-77, Val-78), loop 2 (Ser-82, His-84, and Ile-85), and loop 3 (Ser-112, Tyr-113, and Asp-114)). The up-regulation domain, thus, encompasses a large part of the interface between  $\beta$  and  $\alpha$ . Moreover, its lower part is adjacent to the agonist binding site.

**The Up-regulation Microdomain of  $\beta 4$  Abolishes  $\beta 2$ -Like Up-regulation**—After demonstrating that the insertion of the  $\beta 2$  up-regulation microdomain into  $\beta 4$  confers to  $\beta 4$  a  $\beta 2$ -like up-regulation, we tested the converse process by inserting the  $\beta 4$  74–89 and 106–115 segments into the  $\beta 2$ -(1–212) chimera. Insertion of each segment independently resulted in a large decrease in the absolute expression level but no significant changes in the up-regulation ratios (Fig. 6). Insertion of both segments strongly decreased the up-regulation of the  $\beta 2$ -(1–212) chimera to the  $\beta 4$  level, still with a large decrease in

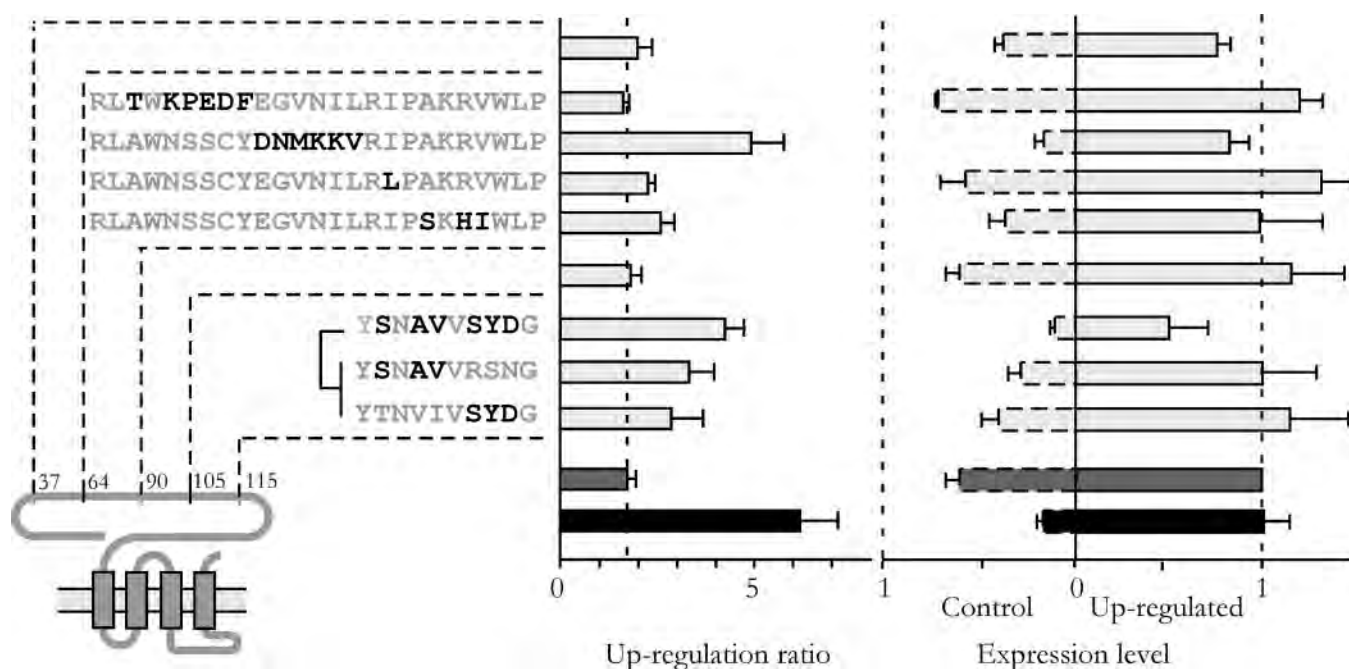


FIG. 4. The two  $\beta 2$  segments 74–89 and 106–115 confer up-regulation to  $\alpha 3\beta 4$ . Up-regulation ratio (left panel) and expression levels normalized to up-regulated  $\alpha 3\beta 4$  (right panel, for each construct, the left bar corresponds to control conditions, and the right bar corresponds to 1 mM nicotine-up-regulated conditions) of N-terminal  $\beta 2/\beta 4$  chimeras co expressed with  $\alpha 3$ . Up-regulation ratios for  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  are shown in black and gray, respectively. Data are  $\pm$  S.D. from three different experiments.

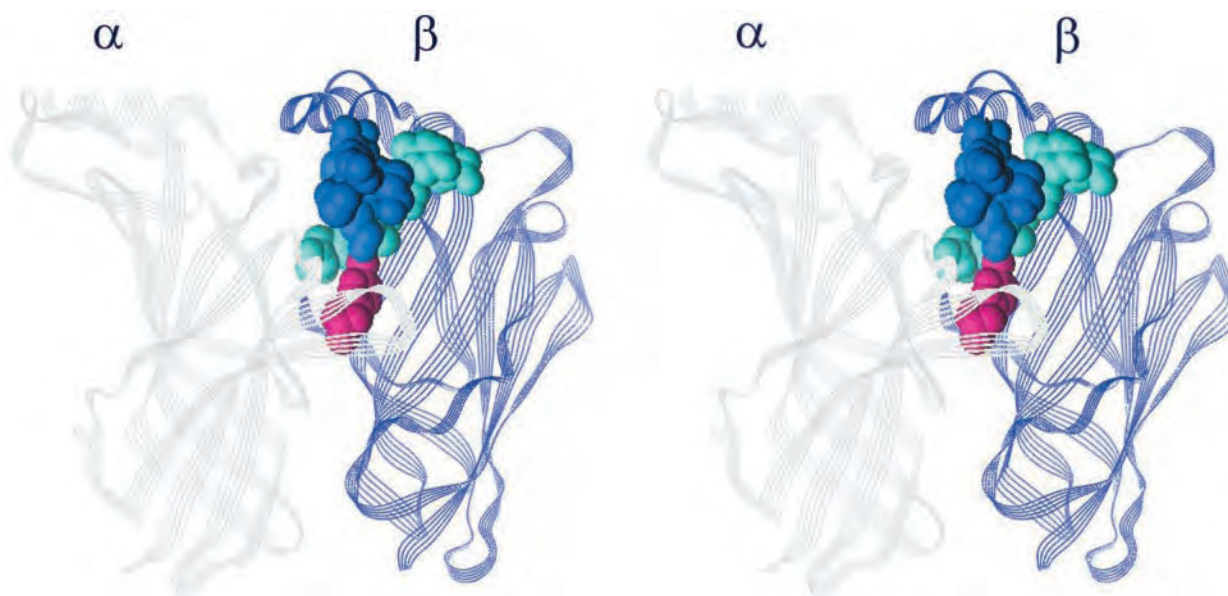
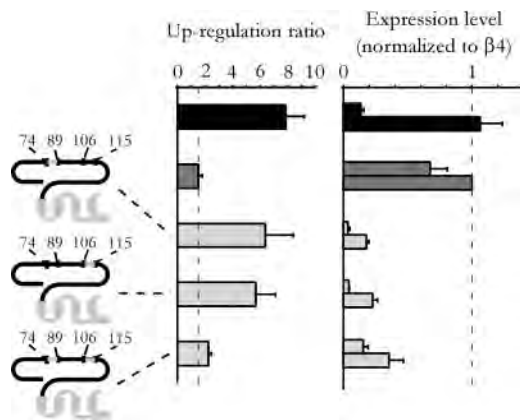


FIG. 5. Stereoscopic representation of the  $\beta 2$  residues conferring up-regulation to  $\alpha 3\beta 4$  receptor. The model of the extracellular domain of  $\alpha 3\beta 2$  is based on the crystal structure of AChBP (34). Residues belonging to the  $\beta 2$  segment (74–89) are shown in blue and belonging to the  $\beta 2$  segment (106–115) are in cyan. These residues form a compact microdomain located at the interface between  $\alpha$  (light gray) and  $\beta 2$  (dark blue) and contact the upper part of the nicotinic site. Epibatidine is shown in pink, according to previous docking calculations (30).

absolute expression level. Thus, the up-regulation microdomain confers up-regulation when inserted from  $\beta 2$  into  $\beta 4$  and, conversely, inhibits up-regulation when inserted from  $\beta 4$  into  $\beta 2$ .

*The Microdomain Contributes to the Different Up-regulations of  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$* —In the rat brain, up-regulation of nicotine binding sites is primarily attributed to  $\alpha 4\beta 2$  receptors (35). To investigate the possible contribution of the microdomain to the up-regulation of  $\alpha 4$  containing receptors, we investigated the up-regulation pattern of  $\alpha 4$  in combination with  $\beta 2$ ,  $\beta 4$ , and with selected critical  $\beta 2/\beta 4$  chimeras. Fig. 7 shows that similar results are obtained with  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$  as compared with

$\alpha 3\beta 2$  and  $\alpha 3\beta 4$  but with much lower concentrations of nicotine required for up-regulation. (i) On  $\alpha 4\beta 2$  receptors, up-regulation reaches 4.02- and 6.85-fold at 1  $\mu$ M and 100  $\mu$ M nicotine concentration, respectively, indicating an  $EC_{50}$  in the micromolar range; in contrast,  $\alpha 4\beta 4$  receptors up-regulated to a much lower degree, with a maximal up-regulation of 1.14- and 2.09-fold at 1 and 100  $\mu$ M nicotine concentration. (ii) Analysis of  $\beta 2$ -(1–212) and  $\beta 2$ -(212–407) chimeras demonstrate that the extracellular domain controls the different up-regulations of  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$ . (iii) Introduction of either the  $\beta 2$  segments 1–37 or 90–115 within  $\beta 4$  did not alter the up-regulation significantly, pointing to a critical role of the 38–114 segment. (iv)



**FIG. 6. The microdomain of  $\beta 4$  inhibits up-regulation when inserted into  $\beta 2$ .** Up-regulation ratio (left panel) and expression levels normalized to up-regulated  $\alpha 3\beta 4$  (right panel, for each construct, the upper bar corresponds to control conditions, and the lower bar corresponds to 1 mM nicotine-up-regulated conditions) of N-terminal  $\beta 2/\beta 4$  chimeras co expressed with  $\alpha 3$ . Up-regulation ratios for wild-type  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  are shown in black and gray, respectively. For the subunit schematics, black lines represent the  $\beta 2$  sequence, and gray lines represent  $\beta 4$ . Data are  $\pm$  S.D. from three different experiments.

Within this segment introduction of the critical  $\beta 2$  cassettes 74–79 and 106–115 produced strong phenotypes, characterized by a large decrease in expression level as compared with  $\beta 4$ , no significant change in the up-regulation at 1  $\mu$ M nicotine, but a large increase in up-regulation by 100  $\mu$ M nicotine. Therefore, we show here that the critical segments of the microdomain confer up-regulation to  $\alpha 4\beta 4$  but with an apparent lower sensitivity as compared with  $\alpha 4\beta 2$ .

**Correlation between Up-regulation Ratios and Absolute Expression Levels**—The data presented in Fig. 1A concerning wild-type nAChRs show that the different up-regulation ratios between  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  apparently correspond to a difference in binding site expression in the absence of nicotine, with no significant change in the number of binding sites in up-regulation conditions. This observation might have important implications regarding the actual mechanism of up-regulation but is difficult to interpret since  $\beta 4$  and  $\beta 2$  may intrinsically produce different expression levels due to their large difference in primary sequence.

Our study produced numerous chimeras, some of them displaying higher and lower expression levels as compared with  $\beta 4$ , probably as a consequence of structural interactions between the  $\beta 2$  and  $\beta 4$  portions. Yet in the  $\alpha 3\beta 4$  context we found that the minimal mutations conferring up-regulation to  $\beta 4$  (namely  $\beta 2$ -(74–79),  $\beta 2$ -(81),  $\beta 2$ -(82–89),  $\beta 2$ -(106–110), and  $\beta 2$ -(111–115)) caused in all cases absolute expression levels in up-regulated conditions close to those of  $\beta 4$  (Fig. 4). Thus, in these chimeras, up-regulation looks to be associated with the lowering of  $\beta 4$  expression in control conditions, nicotine in the culture medium thus rescuing the expression of the high affinity binding sites to  $\beta 4$  levels. However, when the microdomain was transferred from  $\beta 2$  to  $\beta 4$  in the  $\alpha 4\beta 4$  context or when transferred from  $\beta 4$  to  $\beta 2$  in the  $\alpha 3\beta 2$  context more complex phenotypes were observed. In these cases the transfer of the up-regulation phenotypes are associated with large decreases in absolute expression levels, suggesting an additional role of the microdomain in protein maturation and/or metabolic stability.

#### DISCUSSION

**Different Contributions of the  $\alpha$  and  $\beta$  Subunits to the Up-regulation of Neuronal Heteromeric Receptors**—The present study supports the notion that up-regulation is a general property of heteromeric receptors since we found that even  $\beta 4$

produces weak but significant up-regulation when co-expressed with either  $\alpha 3$  and  $\alpha 4$ . Comparison of the up-regulation patterns of  $\alpha 3\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 4\beta 2$  documents the contribution of the “principal” ( $\alpha 3$ ,  $\alpha 4$ ) and “complementary” ( $\beta 2$ ,  $\beta 4$ ) subunits to the up-regulation phenotypes.

First, the principal subunits play a major role in the sensitivity of the receptor to up-regulation. Indeed,  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  display similar amplitude of up-regulation at supra-maximal nicotine concentrations, but the  $EC_{50}$  of up-regulation is 2 orders of magnitude lower for  $\alpha 4\beta 2$  than for  $\alpha 3\beta 2$ .

Second, comparison of  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  reveals that the complementary subunits primarily modulate the amplitude of up-regulation. However, the relative sensitivity of these nAChR subtypes to nicotine up-regulation cannot be measured since it is not possible to evaluate the  $EC_{50}$  of  $\alpha 3\beta 4$  for technical reasons. Identification of the minimal protein segments from  $\beta 2$  conferring up-regulation to the  $\alpha 3\beta 4$  receptor also provides clues on the up-regulation mechanism. All mutations within the microdomain produced up-regulation by lowering high affinity binding site expression in control conditions without significantly changing the expression in up-regulated conditions. This suggests that  $\alpha 3\beta 4$  receptors are constitutively up-regulated, whereas  $\alpha 3\beta 2$  receptors are weakly expressed, a feature surmounted by nicotine action.

**A Microdomain within the Extracellular Domain Controls the Different Up-regulation of  $\beta 2$ - and  $\beta 4$ -containing Receptors**—Chimeras in which the extracellular domains were exchanged between  $\beta 2$  and  $\beta 4$  demonstrate that the ECD of the  $\beta$  subunit critically contributes to up-regulation. This indicates that the transmembrane and intracellular domains of the protein are not responsible for the difference in phenotypes between  $\alpha 3(\alpha 4)\beta 2$  and  $\alpha 3(\alpha 4)\beta 4$ . In particular, specific sequences for phosphorylation or interaction with intracellular factors can be discarded.

The critical residues we identified as conferring an  $\alpha 3\beta 2$ -like up-regulation to  $\alpha 3\beta 4$  and, conversely, as conferring an  $\alpha 3\beta 4$ -like up-regulation to  $\alpha 3\beta 2$  form a compact microdomain when visualized in the  $\alpha 3\beta 4$  ECD model derived from AChBP. This microdomain comprises a significant amount of the interface with the adjacent subunit and faces the agonist binding site (Fig. 5).

The microdomain largely contributes to the interface with the adjacent subunit, suggesting a critical role in subunit-subunit interaction and/or assembly. In support of this notion, several residues of the microdomain were previously found to play a major role in both the efficacy and specificity of subunit-subunit interaction for other ligand-gated ion channels. (i)  $\beta 4$ -(Thr-106) and  $\beta 4$ -(Ser-115) correspond to amino acids responsible for the differences in assembly efficacy between rat/mouse  $\epsilon$  and  $\alpha$  subunits to form heterodimers (36); (ii)  $\beta 4$ -(Gly-74) corresponds to an asparagine on the mouse  $\delta$  subunit, which provides a crucial glycosylation site for the formation of  $\alpha\delta$  heterodimers (37); (iii)  $\beta 4$ -(Ile-77) corresponds to an amino acid of the  $\alpha 1$  subunit of the glycine receptor shown to play an important role in subunit homooligomerization (38); and (iv)  $\beta$ -(473–485) is included in a 20 amino acids zone of the  $\alpha 1$  subunit of the  $\gamma$ -aminobutyric acid, type A receptor identified as crucial for the assembly specificity with the  $\gamma 2$  subunit (39). Furthermore, the  $\beta 4$ -(106–109) stretch interacts directly with  $\alpha 3S148$  within our model, a residue homologous to  $\gamma T150$  known to critically contribute to  $\alpha\gamma\alpha\gamma$  tetramer formation (40). It is noteworthy that the contribution of some of those amino acids (36, 37, 40) occurs at early assembly steps (typically from monomers to dimers formation), indicating a putative maturational role of the microdomain in early assembly of subunits during nAChRs formation.

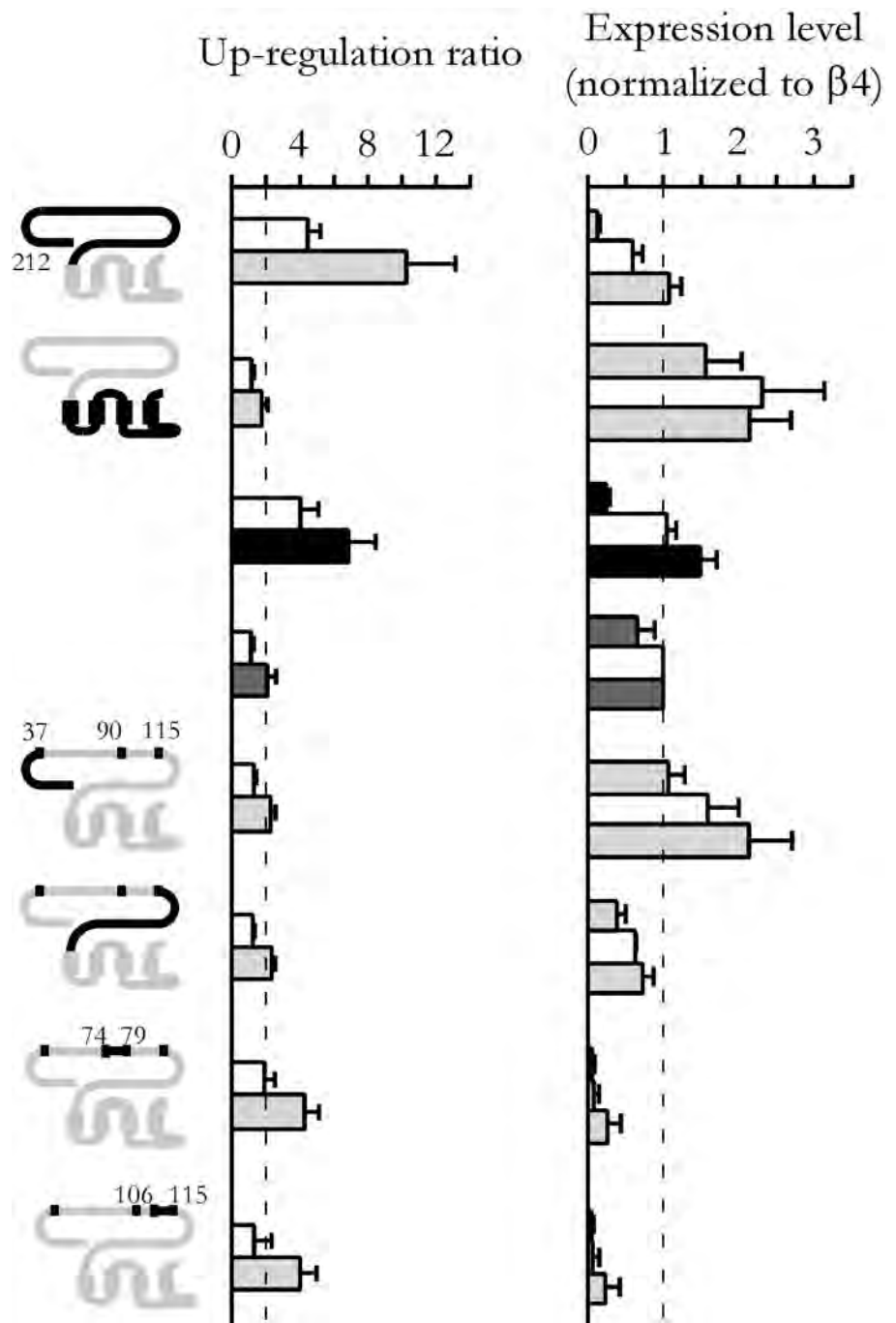


FIG. 7. The microdomain contributes to the differences in up-regulation between  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$  receptors. Up-regulation ratio (left panel) and expression levels normalized to up-regulated  $\alpha 4\beta 4$  (right panel). On the right panel, for each construct the upper bar corresponds to control conditions, the middle white bar corresponds to 1  $\mu\text{M}$  nicotine-up-regulated conditions, and the lower bar corresponds to 100  $\mu\text{M}$  nicotine-up-regulated conditions of N-terminal  $\beta 2/\beta 4$  chimeras co expressed with  $\alpha 4$ . On the left panel, the up-regulation ratio at 1  $\mu\text{M}$  nicotine is represented by the white upper bar, and the up-regulation ratio at 100  $\mu\text{M}$  is represented by the lower bar. Up-regulation ratios for wild-type  $\alpha 4\beta 2$  and  $\alpha 4\beta 4$  are shown in black and gray, respectively. For the subunit schematics, black lines represent  $\beta 2$  sequence, and gray lines represent  $\beta 4$ . Data are  $\pm$  S.D. from three different experiments.

In addition, in its lower part, the microdomain faces the binding site for agonists and competitive antagonists. Indeed, affinity labeling experiments performed on the *Torpedo* nAChR demonstrated that the ACh binding sites were located at the interface between the  $\alpha$  and  $\gamma$  or  $\delta$  subunits, particularly through 4 loops of binding homologous to  $\alpha 3$ -(Tyr-91),  $\alpha 3$ -(Trp-147),  $\alpha 3$ -(Tyr-188), and  $\beta 4$ -(Trp-55). An additional binding loop, although not labeled by small ligands of the size of nicotine and epibatidine, was found to contribute directly to d-tubocurarine binding, a macrocyclic competitive antagonist of ACh (41, 42). This loop carries  $\delta$ -(Ser-110) that is homologous to  $\beta 4$ -(Arg-111) belonging to the up-regulation microdomain. Thus, the microdomain is close but may not contribute directly to the agonist binding site.

**Increased Maturation by Nicotine Contributes to Up-regulation**—It is well established that nicotine does not modify levels of mRNA coding for nAChRs and, thus, acts on post-transcrip-

tional processes (9, 11, 15, 19). The more straightforward molecular mechanism of up-regulation would be an action of nicotine on the functional receptors promoting up-regulation either through receptor activation or desensitization. The first hypothesis can be discarded since we showed that competitive antagonists do not inhibit up-regulation, in agreement with previous work (11, 17). The second hypothesis has been nicely demonstrated in the case of the  $\alpha 4\beta 2$  receptors expressed in the *Xenopus* oocytes, where the nicotine concentrations producing up-regulation are identical to those required to occupy the high affinity binding sites and to desensitize the receptors. In this case, up-regulation would be associated with a total inactivation of the receptors, caused by an irreversible desensitization (13). This mechanism also does not apply to our case since the concentrations of nicotine eliciting up-regulation are several orders of magnitude higher than those necessary to occupy the high affinity binding site of the functional receptor (17), *i.e.* for

$\alpha 3\beta 2$  and  $\alpha 3\beta 4$ , we find a nicotine  $EC_{50}$  around 30 and 100  $\mu M$ , respectively, for up-regulation compared with intrinsic  $K_I$  of 16 and 300 nM (43). In addition up-regulation in cell lines is in all cases associated with a strong functional potentiation of the responses (12, 14, 20). It is noteworthy that the  $EC_{50}$  of up-regulation in our case on  $\alpha 4\beta 2$  receptors (1  $\mu M$ ) as compared with the  $EC_{50}$  in *Xenopus* oocyte (10 nM) (13) matches well those observed in the rat brain (around 1  $\mu M$ ) (10), suggesting that the cell lines constitute an expression system much closer to physiological conditions than *Xenopus* oocytes.

Among the numerous studies dedicated to the up-regulation of heteromeric neuronal receptors expressed in cell lines, the  $\alpha 3\beta 2$  receptors was investigated in great detail upon stable expression in tsA201 cells (12). The 24-fold up-regulation of these receptors mainly results from an increase in receptor maturation, since a 12-fold up-regulation was observed in the absence of protein synthesis at a time scale (2–3 h) where the receptors do not undergo any significant degradation due to turnover. This demonstrates that nicotine converted pre-existing pools of unmaturing subunits/oligomers toward high affinity receptors. In addition, nicotine was found to decrease the turnover rate of the high affinity receptors in the presence of cycloheximide, but its quantitative contribution to the up-regulation process in standard conditions remains to be established. Furthermore, such maturational mechanism is fully consistent with the pharmacology of up-regulation, which is elicited by much higher concentrations than those required to occupy the high affinity binding sites (this study; see also Refs. 11 and 19). For  $\alpha 4\beta 2$  receptors, more fragmentary data are available in the literature. Nicotine was found to decrease the turnover rate of  $\alpha 4\beta 2$  receptors expressed in M10 cells (15), but maturational processes were not investigated in this system (16, 17). Upon expression in HEK 293 cells, no accumulation of subunit protein is associated with the increase in high affinity binding sites (44), suggesting that up-regulation corresponds to a conversion of precursors from low to high affinity entities rather than to the stabilization and accumulation of high affinity receptors.

Therefore, it appears premature to propose a complete up-regulation mechanism from the available literature. Although maturational processes, at least for  $\alpha 3\beta 2$ , were shown to critically contribute to up-regulation, the cellular location of the pools of precursors is still a matter of controversy, since impermeant ligands such as carbamylcholine are found to promote up-regulation, raising the possibility that the immature precursors might actually be located at the membrane surface (17).

**A Plausible Molecular Mechanism for Up-regulation**—Our observation that the same protein region contributes to up-regulation of  $\alpha 3\beta 2$  and  $\alpha 4\beta 2$  receptors suggests that similar mechanisms occur in both cases. From the above discussion, we will limit our analysis to the maturational mechanism that was convincingly shown to contribute to up-regulation of  $\alpha 3\beta 2$  receptors, but a modulation of the receptor turnover by the mentioned microdomain may also contribute to this up-regulation process.

We have seen that the microdomain largely contributes to the subunit interface and carries amino acids previously shown to contribute to subunit assembly. It is noteworthy that subunit assembly has been shown to be rate-limiting in the formation of muscle-type receptors (45). Furthermore, up-regulation of  $\alpha 3\beta 2$  was found to be associated with a dramatic increase in co-immunoprecipitation between  $\beta 2$  and  $\alpha 3$  (12), indicating that nicotine increased the interaction between subunits. An increase in subunit interaction was also observed for  $\alpha 4\beta 2$  receptors, since  $\alpha 4$  subunits from the endoplasmic reticulum were shown to co-assemble tightly and consequently retain intracellularly a  $\beta 2$  chimera in the presence of nicotine (44). These data are consistent with a scheme where the microdo-

main contributes to an  $\alpha/\beta$  assembly-limiting step for the maturation toward high affinity receptors. The  $\beta 2$  microdomain would impair some key molecular interaction between adjacent subunits, causing incomplete maturation, whereas for  $\beta 4$ , efficient  $\alpha/\beta$  and/or  $\beta/\beta$  oligomerization would allow subsequent conformational reorganizations leading to high affinity receptors.

The high concentrations necessary to produce up-regulation suggest that the immature precursors display low affinity for nicotine. A wide range of agonists specific for the binding site, such as nicotine, epibatidine, carbamylcholine, cytosine, dimethyl phenyl piperazinium (12, 15, 16, 46, 47), and even in some cases antagonists such as d-tubocurarine and dihydro- $\beta$ -erythroidine (14, 46) have been shown to promote up-regulation of heteromeric receptors expressed in cell lines. Thus, a common pharmacological spectrum for up-regulation and high affinity binding is observed. Moreover, a correlation between the ligand binding concentrations and those required to up-regulate has been observed (16, 17, 46, 48). In our opinion, this suggests that the binding site for up-regulation corresponds to a low affinity conformation of the classical binding site that bridges the interface between the  $\alpha$  and  $\beta$  subunits. A plausible molecular mechanism providing the simplest interpretation of our data is that nicotine, by binding to the  $\alpha/\beta$  interface within pools of precursors, would allosterically stabilize a conformation of the  $\beta 2$  microdomain similar to the one of  $\beta 4$ . This would result in an efficient  $\alpha/\beta$  interaction, allowing further maturation. The up-regulation microdomain would act as a transduction element, converting the binding of nicotine into an efficient assembly signal between neighboring subunits.

This scheme postulates that up-regulation is primarily associated with an alteration of subunit-subunit interactions. It is thus plausible that, in addition to simply rescuing immature precursors, nicotine might favor specific inter-subunit interactions and modulate the final receptor stoichiometry. Such an idea would provide a rational explanation of previous electrophysiological experiments, associating up-regulation of  $\alpha 4\beta 2$  receptors with significant changes in receptor activation and desensitization (20). Moreover, recent overexpression and electrophysiological experiments suggest that up-regulation of  $\alpha 4\beta 2$  receptors is associated with an enrichment of  $\beta 2$  subunits within pentamers (49).

**Conclusion**—In the central nervous system,  $\beta 2$ -containing nAChRs are critical for nicotine-evoked enhancement of striatal dopamine release and nicotine autoadministration (50). Because chronic nicotine has been shown both to up-regulate  $\beta 2$ -containing heteromeric receptors and potentiate striatal dopamine release (24), it is likely that up-regulation contributes to the long term effect of chronic nicotine exposure, including its addictive properties.

It is noteworthy that the pharmacology of up-regulation observed in our expression system, namely that  $\alpha 4\beta 2$  receptors are readily up-regulated by micromolar concentrations of nicotine, whereas  $\alpha 3$ - or  $\beta 4$ -containing receptor requires much higher concentrations, matches previous studies performed in the rat brain. Indeed, increases in nAChR binding density in various rat brain areas was found to start at 0.1  $\mu M$  nicotine, with a stronger effect at 1  $\mu M$  (10). A majority of the up-regulated receptors were of the  $\alpha 4\beta 2$  subtype, with relatively little effects on  $\alpha 3\beta 2$ -like and  $\alpha 3\beta 4$ -like receptors (8, 35). Therefore, the up-regulation microdomain is likely to contribute to nicotine up-regulation in the brain. In addition, the  $\beta 2$  and  $\beta 4$  microdomains are highly conserved among rat, mouse, and human species. Our molecular dissection, thus, paves the way to an analysis of the functional consequences of up-regulation through *in vivo* expression in the mice of  $\beta$  subunits endowed with different up-regulation properties.



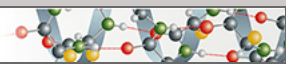
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**Protein Structure and Folding:**  
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**Nicotinic Acetylcholine Receptors by**  
**Nicotine**

PROTEIN STRUCTURE  
AND FOLDING



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