Biochemical Pharmacology 78 (2009) 756–765

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Review

Nicotine-induced upregulation of nicotinic receptors: Underlying mechanisms and relevance to nicotine addiction

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ARTICLE INFO

Article history:
Received 10 April 2009
Accepted 9 June 2009

Keywords:
Nicotinic
Receptors
Subtypes
Upregulation
Sensitization
Addiction

ABSTRACT

A major hurdle in defining the molecular biology of nicotine addiction has been characterizing the different nicotinic acetylcholine receptor (nAChR) subtypes in the brain and how nicotine alters their function. Mounting evidence suggests that the addictive effects of nicotine, like other drugs of abuse, occur through interactions with its receptors in the mesolimbic dopamine system, particularly ventral tegmental area (VTA) neurons, where nicotinic receptors act to modulate the release of dopamine. The molecular identity of the nicotinic receptors responsible for drug seeking behavior, their cellular and subcellular location and the mechanisms by which these receptors initiate and maintain addiction are poorly defined. In this commentary, we review how nicotinic acetylcholine receptors (nAChRs) are upregulated by nicotine exposure, the potential posttranslational events that appear to cause it and how upregulation is linked to nicotine addiction.

Contents

1. Introduction

Significant evidence indicates that nicotine is the component in tobacco leading to abuse and addiction. Addiction is initiated by nicotine binding to nicotinic receptors in the brain. A phenomenon

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doi:10.1016/j.bcp.2009.06.011

linked to nicotine addiction is nicotine-induced upregulation. Nicotine exposure for several hours to days increases or upregulates the number of high-affinity nicotine binding sites, measured by radio-labeled nicotine or epibatidine. Understanding the mechanism of upregulation in the brain has been difficult since various nicotinic receptor subtypes coexist in the brain. A comprehensive review into the various nicotinic receptor subtypes, their significance in mediating physiological response to nicotine and possible mechanisms of upregulation are provided below.

1.1. Nicotinic acetylcholine receptor (nAChR) subtypes

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of a family of neurotransmitter-gated ion channels that includes muscle nAChRs, $GABA_A$ receptors, glycine receptors and $5HT_3$ receptors (for reviews see [1,2]). The 11 neuronal subunits are closely related to each other with amino acid homology in the range of 40–55% [3]. There are eight different subunits, α2–α7, α9–α10, homologous to muscle α subunits, and three different subunits, β2–β4, homologous to muscle β subunits. Studies have established that combinations of different α and β subunits result in the pharmacological and functional diversity of the neuronal nAChRs *in vivo* [3,4] and that neuronal receptors are oligomeric, integral membrane proteins like the muscle receptor. The different neuronal subtypes are generally composed of two or more different subunits with the exception of the α-bungarotoxin (αBgt) binding subtype, which is a homomeric receptor of α7 subunits [5]. In addition, neuronal nAChRs are pentameric complexes [5–7].

Neuronal nicotinic receptors are broadly classified into two pharmacological types. One subtype selectively binds α-bungarotoxin, the peptide antagonist of the muscle nicotinic receptors that is different from neuronal (nBgt; also known as Bgt 3.1, kappa-Bgt, and toxin F). αBgt-binding receptors found in PC12 cells and rat brain are pentamers composed solely of α7 subunits [5]. αBgt-binding is blocked only by relatively high concentrations of nicotine, and ^{3}H-nicotine does not bind with high-affinity to these sites in brain [8–10]. The second pharmacological subtype binds nicotine and other agonists with high-affinity. This subtype was first identified by ^{3}H-nicotine and ^{3}H-ACh autoradiography and binding in brain preparations. There is almost total overlap between the binding of the nicotinic agonists, ^{3}H-nicotine, ^{3}H-ACh and ^{3}H-epibatidine in the brain [11,12], with no apparent overlap with αBgt-binding. Accumulating evidence indicates that these high-affinity nicotine binding sites represent virtually all of the nicotinic receptors different from the αBgt-binding subtype. In addition, other studies indicate that these receptors initiate and are involved in addiction. The most compelling evidence linking high-affinity nicotine binding sites to addiction are studies of mice where β2 subunits were "knocked out" by homologous recombination methods. These mice, which lack all high-affinity nicotine binding sites, largely fail to self-administer nicotine, and dopamine release from mesolimbic neurons is no longer stimulated by nicotine [13].

The majority (~90%) of high-affinity nicotine binding sites in the brain are composed of α4 and β2 subunits [14]. In addition to the "α4β2" receptors, there are other high-affinity nicotine binding sites that are less well characterized [15]. These include nicotinic receptors composed of α4, β2, and α5 subunits [16] and receptors that contain α3, β2 and β4 [17,18]. More recently, there is growing evidence that high-affinity nicotine binding sites in the brain can also contain α6 and β2 in addition to the other subunits [18–20]. Because of nicotinic receptor heterogeneity, it has been difficult to study nicotinic receptors in the brain or to purify them from brain preparations. Much has been learned from studies of receptors of defined subunit compositions obtained by heterologous expression of different combinations of the subunits. These studies have found that high-affinity nicotine binding sites result from various subunit combinations, such as α4β2, α3β2, α4β2α5, α3β2α5, α3β4α5 [21,22], α6β2, α6β4, α6β2β3, α6β4β3 [23,24] and α6β4β3α5 [25]. The different subunit combinations give rise to large differences in their affinities for different ligands, their electrophysiological properties, Ca^{2+} permeability and their ability to be "upregulated" by nicotine (see below). Thus, defining nicotinic receptor subunit composition *in vivo* remains a critical issue to be addressed in characterizing the mechanisms underlying addiction.

1.2. Dopamine, nicotine-induced behaviors and sensitization

Considerable evidence indicates that nicotine, like other drugs of abuse, produces its effects on behavior by activating the mesocorticolimbic dopamine (DA) system, a pathway originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc) and other forebrain sites. Activation of this system has also been shown to be critical for the drug's rewarding properties (see [26] for a review). In experimental animals, nicotine increases the firing of midbrain DA neurons [27,28] and increases DA overflow in the NAcc [29,30]. These actions likely mediate nicotine's locomotor activating effects and its ability to support self-administration as both are prevented by DA receptor blockade [31,32] and lesions of the mesoaccumbens DA system [33,34], and all nicotine effects are blocked by nicotine receptor antagonists [35,36]. Nicotine receptors in the VTA are particularly important as selectively blocking these receptors, but not those in other sites like the NAcc, prevents nicotine-induced NAcc DA release, locomotion as well as nicotine self-administration [37,38].

As with other stimulants like amphetamine and cocaine, when nicotine injections are repeated, sensitization of the drug's locomotor activating effects is observed [35,39]. Similarly, there is evidence that exposure to repeated nicotine injections also sensitizes this drug's ability to increase NAcc DA release [40–43]. Interestingly, again as with other stimulants [44], the sensitized NAcc DA response to nicotine may become more enhanced with longer withdrawal times [42,43].

It has been reported that when administered non-contingently acute nicotine selectively increases DA overflow in the shell subregion of the NAcc and that after these injections are repeated, enhanced DA overflow is observed selectively in the NAcc core [45,46]. In contrast, when nicotine is self-administered over a prolonged period of time, enhanced DA overflow is observed in both subregions with more pronounced increases found in the NAcc shell [47]. Such findings indicating complex, injection mode dependent, and anatomically specific changes produced by exposure to nicotine have been incorporated into models of drug addiction that attribute different functions to these two subnuclei (e.g., [48–50]. Nonetheless, the mechanisms underlying the differential effects observed and the nAChR subtypes involved remain to be elucidated.

High-affinity nicotine binding sites are localized in discrete regions throughout the brain. The regions of highest density include sites associated with the mesolimbic DA pathways, in particular the VTA and the NAcc. *In situ* hybridization experiments have demonstrated the presence of α4, α6, β2, β3, α3 and α5 mRNA in the VTA and NAcc that corresponds to high-affinity nicotine [51,52] and nBgt-binding sites [53,54]. High-affinity nicotine binding sites have been identified in the somatodendritic region of VTA DA neurons and on their terminals in the NAcc [55]. Using subunit-specific antibodies (Abs) to stain neurons at the light and electron microscope level, α4 subunits were found almost exclusively on dopaminergic neurons in the midbrain [56],

although α4 subunits have also been found on non-DA neurons in this site [57]. Most of the antibody staining was observed in the somatodendritic aspect of DA neurons. Similar results were obtained when fluorescently tagged α4 and β2 subunits were transfected into cultured rat VTA neurons [58]. This distribution is consistent with the above studies showing a greater contribution of nicotine receptors in the VTA to the DA releasing and behavioral effects of nicotine. The pharmacology of the response indicates at least two nicotinic subtypes are contributing, the α4β2 and α7 subunit subtypes [13,59,60]. However, binding data and studies using knockout mice [19,20,61] indicate that receptors containing α6 and/or β3 subunits also contribute.

In addition to those expressed in the somatodendritic region of VTA DA neurons, nAChRs are also expressed on the terminals of these neurons in the NAcc. Studies conducted in striatal synaptosomes have found that nicotine-stimulated DA release is modulated by pre-synaptic nicotinic receptors [62]. More recently, it was shown that nicotine acts at these receptors in the NAcc to amplify phasic relative to tonic firing induced DA release in this site [63,64]. Similar findings were obtained in electrophysiological studies of interpeduncular nucleus [65] and hippocampal neurons [66]. Because of the high permeability of nicotinic receptors to Ca^{2+} [4], it is believed that these receptors stimulate neurotransmitter release by increasing the pre-synaptic Ca^{2+} concentration in neuron terminals. Studies using immunoprecipitation with subunit-specific antibodies have shown that nicotinic receptors containing α4 and β2 subunits predominate in striatal preparations [67]. However, the nicotinic receptor antagonists nBgt [68,69] and α-Conotoxin MII [70,71], block ~50% of the nicotine-stimulated DA release in striatal slice and synaptosomal preparations, while αBgt fails to block release altogether. Autoradiography and binding experiments using ^{125}I-nBgt [53,54] or ^{125}I-α-Conotoxin MII [72] have shown labeling in these areas. Originally, α-Conotoxin MII was thought to be specific for α3β2 receptors [4]. More recent findings have shown that α-Conotoxin MII also binds α6-containing receptors [18,23,24,73].

1.3. Nicotine-induced conformational changes of nAChRs

Nicotine, like ACh, is a nicotinic receptor agonist. The binding of nicotine and ACh to nicotinic receptors cause a conformational change that either opens or closes the receptors' ion channels, thereby changing the receptors' functional state. Before binding agonist, the receptor is in the resting state and is nonfunctional. With agonist binding, receptors rapidly activate by opening the ion channel through the receptor. Activation is a metastable event. If the receptor remains bound by agonist, activation is quickly followed by a second conformational change into a nonfunctional state termed desensitization in which the channel is closed [74]. Normally, receptors rapidly recover from the desensitized state and enter the resting state when nicotine is removed. Several lines of evidence indicate that chronic exposure to nicotine causes some of the nicotinic receptors in the brain to undergo long-lasting state changes. These conformational changes are distinguished from activation and desensitization by much slower kinetics (on the order of hours to days). In one study, α4β2 receptors expressed in human embryonic kidney cells showed a dose-dependent loss of Ca^{2+} entry when exposed to 0.1–10 μM nicotine for an hour or longer [75]. Seven hours in the absence of nicotine was needed for full recovery. ^{3}H-nicotine binding studies in rat brains together with dopamine release and flux measurements also indicate that receptors are undergoing long-lasting changes. ^{3}H-nicotine binds to receptors from rat brain membranes at two very different rates. A rapid rate of binding is consistent with ^{3}H-nicotine binding to a high-affinity receptor (K_D =1 nM), and a much slower component is consistent with "isomerization" of low-affinity sites (K_D = 200 nM) into high-affinity sites [76]. The isomerization is very slow taking several hours to occur. Similarly, the nicotine-stimulated release of ^{3}H-dopamine from striatal synaptosomes, as is caused by activation of pre-synaptic nicotinic receptors, changes slowly after nicotine treatment. Nicotine-stimulated ^{3}H-dopamine release decreases after exposure to nicotine [77,78] as does ^{86}Rb efflux from the synaptosomes [79], presumably an assay of receptor activation. The loss of nicotine-stimulated ^{3}H-dopamine with nicotine treatment is both slow to occur and to reverse (≥5 h) and occurs in the nicotine concentration range of 0.1–1 μM nicotine (IC_{50} of 0.7 μM nicotine) [78]. These data are consistent with a long-lasting inactivation of nAChRs (see [80] for review). In contrast to desensitization, receptor function remains blocked for 60 min to several hours [78,81]. Similar long-lasting inhibition has been observed in dopaminergic cells of the ventral tegmental area: perfusion of 0.5 μM nicotine for longer than 5 min can cause a deeper state of desensitization [59]. Recovery of the ACh-induced current following the washout of nicotine requires nearly 30 min. During the continuous application of 100 μM nicotine, the number of open nAChR channels decreases exponentially because of receptor desensitization in acutely isolated habenula neurons.

It is possible upregulation is initiated by α4β2 activation. Alternatively, upregulation is initiated by desensitization as proposed by other groups [82]. While the data from synaptosome preparations is consistent with long-acting nAChR inactivation, there is little electrophysiological data in support of such inactivation. Almost all of the electrophysiological data was from studies using oocyte expression (e.g., [83]). However, a careful study indicates that oocytes take up and concentrate nicotine and subsequently secrete it, which causes desensitization of α4β2 receptors [84]. There is increasing evidence that a functional upregulation of nAChRs occurs on a longer time-scale. After nicotine removal, α4β2 nAChRs become "hyperfunctional" as revealed by increased evoked currents through the channel with higher affinity [85]. Functional upregulation has been demonstrated after 8-24 h of nicotine treatment in HEK cells [86–89] and in cultured VTA neurons [58]. *In vivo* nicotine injections enhance expression of functional (presumably α4β2) nAChRs in hippocampal slices [90]. Long-term (14 days) exposure to subcutaneous nicotine can cause upregulation of most likely α4β2-like nAChR number and increase receptor function as assessed by ^{86}Rb efflux in rat brain [91]. It should be noted that evidence for functional upregulation of nAChRs does not contradict evidence for long-lasting inactivation. Both may exist depending on nicotine concentration, exposure length and receptor subtype.

1.4. Nicotinic-induced upregulation of high-affinity nicotine binding

A slow acting change in nicotinic receptors caused by long-term exposure to nicotine is an increase or "upregulation" of high-affinity nicotine binding sites in the brain. By comparing the brains of smokers to those of non-smokers, chronic exposure to nicotine was shown to cause an increased number of ^{3}H-nicotine binding sites in postmortem human brains [92–94]. Similar increases in ^{3}H-nicotine binding were observed in mouse [95] and rat brains [96,97] in response to repeated administration of nicotine. This upregulation in response to nicotine is posttranscriptional because no change in receptor subunit mRNA levels is observed for upregulation of high-affinity nicotine binding sites *in vivo* caused by nicotine exposure as long as 10 days [51]. The nicotine-induced upregulation of binding sites has been interpreted as an increase in the number of nicotinic receptors and, consequently, an increased sensitivity to nicotine [98,99]. It is important to note that ^{3}H-agonist binding is not necessarily an exact measure of the number of nicotinic receptors. Because agonist binding alters receptor conformation and nAChR affinity for the agonist, as discussed

above, it is possible that nicotine exposure changes the receptor's affinity for nicotine. Equilibrium binding measurements were performed and there appeared to be no change in affinity for agonist after nicotine treatment [95]. However, equilibrium binding does not necessarily detect large changes in the receptors' affinity for agonist [76,100].

With nicotine levels achieved by smokers, the acute effect of nicotine following smoking is to activate and then desensitize receptors. Because nicotine is eliminated with a half-life of 2–3 h, blood nicotine levels accumulate to levels of 100–200 nM during the hours of repeated smoking, and even higher levels are achieved immediately following each cigarette [101–103]. Nicotine levels, thus, reach and are maintained at levels at which receptors should be upregulated. There is considerable evidence based on behavioral studies in rats that repeated exposure to nicotine causes sensitization of locomotor activity and DA overflow in the NAcc because the dopaminergic VTA neurons become more responsive to nicotine. A possible mechanism for the increased response to nicotine is the upregulation of the nicotinic receptors on the dopaminergic VTA neurons. This link between receptor upregulation and the sensitization has been interpreted as an increase in receptor numbers in response to the desensitization of the receptors by nicotine [99].

Nicotine-induced upregulation of high-affinity nicotine binding also occurs when α4β2 or α3β2 receptors are expressed in cells other than neurons. This indicates that the upregulation is a property intrinsic to the receptor as opposed to a regulatory property specific to the neurons in which the receptors are normally found. The nicotine-induced upregulation has been studied using chick α4β2 receptors stably expressed in mouse fibroblasts (M10 cells; [100,104–107]), human α4β2 receptors stably expressed in HEK cells [75,108] and rat α4β2 receptors transiently expressed in HEK cells [109]. The level of upregulation of α4β2 high-affinity ligand binding varied from a ~2.5-fold increase in the M10 cells up to a 15-fold increase in one of the HEK cell lines [108]. In most studies, the increase in binding was interpreted as an increase in the number of receptors. In two studies, an assay of receptor number was performed in parallel with the nicotine-induced increase in binding. In the first study [104], less than a 2-fold increase in surface receptors was measured using ^{125}I-mAb 299 (anti-α4) binding after 3 days of treatment. However, the nicotine-induced upregulation saturated much sooner, specifically 10–24 h after nicotine treatment [104,108]. In the second study [109], the binding of antibodies specific for α4 and β2 subunits increased, but less than the increase in upregulation assayed with agonist binding. When our laboratory repeated these experiments, we found that there was very little Ab binding because of cell fixation [88]. When repeated using living cells, there was no significant change in Ab binding in parallel with the 4–5-fold increase in agonist binding. A more recent study [110] using radio-immune assays with mAb 290 (anti-β2) showed increased antibody binding after nicotine exposure of HEK cells stably expressing human α4β2 receptors. Radio-ligand binding was not performed in parallel so that comparisons cannot be made.

2. Different assays of nicotine-induced upregulation

When comparisons are made between different studies of nicotine-induced upregulation, it should be considered how the upregulation was measured because this may vary from study to study. The original measurements were performed on rat and mouse brain preparations using radio-labeled agonist binding. First measurements of upregulation were made with 3H acetylcholine (ACh) [96] as well as with ^{3}H-nicotine [95] followed by ^{3}H-cytisine [111] and then with radio-labeled epibatidine [112] which can be tritiated [11] or ^{125}I-iodinated [113]. The most common assay used is to measure radio-labeled agonist binding to membrane preparations made from an aliquot of tissue from defined brain areas [91,94,114]. This assay can be refined by solubilizing with detergent and using subunit-specific antibodies to precipitate radio-ligand bound counts [115]. Another assay used to measure nicotine-induced upregulation in brain preparations is autoradiography on tissue sections [94,116,117]. While a number of studies have found a strong correlation between the findings using autoradiography and binding to membrane preparations, it should be kept in mind that the two techniques are assaying different pools of nAChRs under different conditions. Membrane preparations are composed mostly of plasma membrane and large intracellular membrane fragments. Internal membrane that fragment into microsomes, such as endoplasmic reticulum (ER) membranes, are mostly lost unless care is taken. Autoradiography, in contrast, assays receptors throughout the whole cell.

The assays used to measure nicotine-induced upregulation in cells transfected with nAChR subunits are more varied than for brain preparations. An advantage of using cultured cells transfected with nAChR subunits is that intact cells can be used for the radio-labeled agonist binding assays. When radio-labeled nicotine, cytosine or epibatidine are used, binding is to the whole cell population of the nAChR because these ligands are membrane permeant. While some studies have performed radio-ligand binding on intact cultured cells (e.g., [88,89,118]), many others (e.g., [108,109,119]) have used membrane preparations instead, which will assay a subpopulation of the nAChRs. Another assay used for nicotine-induced upregulation in transfected cells is binding of subunit-specific antibodies to the cell surface with ^{125}I-iodinated antibodies [104,120] or other assays to measure the amount of antibody binding (e.g., [109,88,110]). As described above, the results of these studies conflicted with respect to whether antibody binding correlated with radio-ligand binding.

Fluorescently tagged nAChR subunits have recently been applied to assay nicotine-induced upregulation in cells transfected with nAChR subunits, as well as in knock-in mouse models. Nashmi et al. [58] transfected α4 and β2 subunits, in which YFP and CFP were fused respectively within their cytoplasmic domains, into cultured HEK cells and neurons from ventral midbrain. Similar construct were used to engineer mice that express fluorescently tagged α4 subunits [57]. In both cases it was found that nicotine exposure increased α4-YFP subunit fluorescence consistent with nicotine-inducing increases in the number of α4 subunits. While the increase in α4-YFP subunit fluorescence correlated with functional upregulation of the α4β2 receptors, no measurements of radio-ligand were performed as an independent measure of the nicotine-induced upregulation.

3. Regulators of upregulation

A number of factors regulate different features of nicotine-induced upregulation. These include differences in nAChR subunit composition, changes in temperature and the release of pro-inflammatory cytokines all of which further complicate interpretation of different studies.

3.1. nAChR subunit composition

Several studies have determined that nAChR subunit composition can alter different aspects of nicotine-induced upregulation. If β4 replaces β2 subunits in either α3- or α4-containing nAChRs, the upregulation as assayed by the fold-increase of radio-labeled agonist binding in transfected cells is highly reduced [118,120,121]. The findings are somewhat ambiguous because replacement of β2 subunits by β4 causes an increase in total radio-

labeled agonist binding without nicotine treatment as if the β4-containing receptors are already upregulated without any nicotine treatment. There are also examples where addition of β4 to either α6 subunits [122] or α3 [123] subunits did result in significant degree of upregulation by nicotine.

The addition of two accessory subunits, α5 or β3 subunits to nAChRs appeared to block upregulation or downregulation by nicotine in several regions of the brain [124,125]. α5 subunits were found almost exclusively associated with α4β2 receptors in the several brain areas, and no nicotine-induced upregulation was observed [125]. A recent finding that α4β2 receptors in dopaminergic neurons in the VTA are not upregulated by nicotine [57], raises the question whether α4β2 receptors in these neurons are associated with α5. Since only a small percentage of receptors contain α5 subunits, the contribution from this upregulation-resistant receptor population may be too small to make an impact on a higher population of α4β2 receptors that can be upregulated. It is also possible that the addition of α5 subunits to α4β2 receptors has an effect similar to that observed with the addition of β4 subunits, where an increase in binding is observed without nicotine treatment as if the subunit addition causes upregulation without nicotine treatment. Interestingly, no nicotine-induced downregulation of α6 receptors in the striatum was observed if β3 subunits were present [124]. In mammalian cell lines co-expression of β3 subunits increased α6β2 and α6β4 receptor levels and the nicotine-induced upregulation of α6β2β3 receptors was enhanced compared to α6β2 receptors [122]. These effects of β3 subunits may help explain why in the striatum α6-containing receptors without β3 are downregulated by nicotine, while those containing β3 are unaffected [124].

We have compared the nicotine-induced upregulation of α3β2, α4β2 and α6β2 nAChRs in transfected cells [126]. Chronic nicotine exposure upregulated these receptors that showed differences in upregulation time course and concentration-dependence. The α6β2 receptor upregulation required higher nicotine concentrations than for α4β2 but lower than for α3β2 receptors. The α6β2 upregulation occurred 10-fold faster than for α4β2 and slightly faster than for α3β2. The data suggest that nicotinic receptor upregulation is subtype specific such that α6-containing receptors upregulate in response to transient, high nicotine exposures while sustained, low nicotine exposures upregulate α4β2 receptors.

3.2. Temperature

Lowering the temperature reduces the turnover of subunits [127–129], which in turn favors the assembly and folding of muscle nicotinic receptors [130]. For several nAChR subtypes transfected into mammalian cell lines, lowering the temperature to 29 °C or 30 °C greatly increases the number of high-affinity agonist binding sites on the nAChRs [122,131]. To some degree, these temperature-evoked increases correlate with an increase in subunit protein levels [126], but this question has not been carefully investigated. α4β2 receptors appear to be an exception as a 12-fold increase in ligand binding was observed with no change in total subunit protein [131]. These effects of temperature may combine with the effects of nicotine since cigarette smoking causes a drop in skin temperature of 1.5 °C [132].

3.3. Cytokines

Pro-inflammatory cytokines such as TNF-α and IL-1β can regulate nicotine-induced upregulation of nAChRs [121,133]. These cytokine effects are likely important in regulating nAChRs on blood cells in the periphery but also for nAChRs in brain (see [134,135], for a review). TNF-α release enhances the nicotine-induced upregulation of α4β2 and α3β2 receptors. This enhancement was attributed to an increase in receptor number as this enhancement was sensitive to transcriptional and translational inhibitors [121]. The response to pro-inflammatory cytokines was dependent on subunit composition with differences that depended on β subunit subtype. α7-type nAChRs antagonize pro-inflammatory cytokine release [136] that may act to counter balance TNF-α enhancement of α4β2 receptor upregulation. With the emerging concept of cholinergic modulation of inflammatory response, cross talk between inflammatory mediators and nicotinic receptors is becoming evident and should provide new insights for therapeutic interventions for inflammatory bowel diseases.

4. Mechanisms underlying nicotine-induced upregulation

The mechanisms causing the upregulation are the subject of significant debate. There is general agreement that the effect is posttranscriptional since nicotine treatment does not alter subunit mRNA levels in rat and mouse brains [51,100,104]. Furthermore, α4β2 receptors were upregulated by nicotine in M10 cells after protein synthesis was inhibited with cycloheximide indicating that the mechanisms involved in the upregulation are posttranslational. Six different posttranslational mechanisms have been proposed to underlie nicotine-induced upregulation: (1) cell surface turnover, (2) receptor trafficking to the surface, (3) subunit maturation and assembly in the ER, (4) changes in subunit stoichiometry, (5) block of subunit degradation in the ER and (6) nAChR conformational changes (see Table 1). We review each of theses mechanisms below.

4.1. Cell surface turnover

The first mechanism proposed to underlie nicotine-induced upregulation was the turnover of cell-surface receptors. Nicotine was found to slow the turnover of surface α4β2 receptors measured by cycloheximide inhibition of protein synthesis [104], which indicated that surface receptor number was increasing because of a nicotine inhibition of turnover. Other studies were unable to duplicate these results. Using other methods, one group found no change in turnover [100] while another group found that cycloheximide instead inhibited most of the upregulation [108]. Recently, using surface biotinylation of α4β2 receptors, nicotine was again found to slow surface turnover [137]. Two other studies [88,119] also using surface biotinylation of α4β2 receptors expressed in the same cells found no change in turnover. One of the groups [119] performed additional experiments co-transfecting with the α4β2 receptors a dominant-negative construct that blocked endocytosis. No effect was observed on the nicotine-induced upregulation further suggesting that surface α4β2 turnover is not affected during upregulation.

Table 1
Proposed mechanisms underlying nicotine-induced upregulation of nicotinic receptors.

	Mechanisms of upregulation	References
1	Cell surface turn over	Peng et al. [104]
2	Receptor trafficking	Harkness and Millar [109], Darsow et al. [119]
3	Subunit maturation and assembly	Nashmi et al. [58], Sallette et al. [89], Kuryatov et al. [137]
4	Changes in subunit stoichiometry	Nelson et al. [142], Moroni et al. [141]
5	Block of subunit degradation in the ER	Ficklin et al. [143], Rezvani et al. [144]
6	Conformational change	Vallejo et al. [88]

4.2. Receptor trafficking

Another mechanism suggested to be contributing to the nicotine-induced upregulation is an increase in receptor trafficking to the cell surface [109]. Our laboratory found that upregulation of high-affinity binding remains unchanged after treatment of the α4β2-expressing cells with Brefeldin A (BFA), which disperses the Golgi and blocks trafficking from the Golgi [88]. Also, formation of new high-affinity binding sites was blocked by BFA indicating that receptor maturation was blocked by BFA, which did not affect upregulation. Another group [119] found that upregulation of intracellular high-affinity binding remains unchanged after BFA treatment, but found that BFA blocked an increase in surface receptors suggesting an increase in exocytic trafficking to the surface. Contrary to this finding about BFA is our finding that there is no increase in anti-α4 and β2 Ab binding to the surface and that BFA blocks formation of new high-affinity binding sites [88].

4.3. Subunit maturation and assembly in the ER

A third mechanism that may contribute to the upregulation of α4β2 receptors is an increase in the intracellular receptor pool caused by increased receptor assembly and/or maturation of the subunits in the ER. There is precedence for such a mechanism causing increases in nAChR surface expression. Increased nAChR subunit assembly and maturation was determined to be the mechanism causing an upregulation of the muscle-type nicotinic receptor when intracellular cAMP levels increase [138,139]. Several studies have concluded that α4β2 receptor assembly and subunit maturation are increased during upregulation [58,89,109,137]. The studies proposed that nicotine may be acting as a molecular chaperone in the endoplasmic reticulum and promotes the assembly of subunits [137]. However, this study also found that in addition to assembly, the half-life of surface receptors are increased by nicotine exposure. Nashmi et al. [58] found that there is increased Forster resonance energy transfer (FRET) between YFP and CFP fused respectively to the cytoplasmic domains of α4 and β2 subunits. The increase in FRET was interpreted as increased assembly, but it also could be caused by a change in receptor conformation that brings the fused CFP and YFP closer together in the receptor.

Sallette et al. [89] directly assayed α4β2 receptor assembly using ^{35}S-methionine pulse-chase analysis during nicotine exposure. They observed a 2–3-fold increase in assembly that correlated well with a 2–3-fold increase in high-affinity binding. However, the measurements were performed without determining what percent of the synthesized subunits assemble into receptors and what percent of available subunits were precipitated with the Abs. Furthermore, the binding of the Abs used, anti-α4 mAb 299 and anti-β2 mAb 290, is conformation-dependent (e.g., [109]), and whether interactions with the mAbs changed with nicotine treatment were not tested. We found that 30–56% of synthesized α4 and β2 subunits assemble into receptors yet nicotine treatment caused a 4–7-fold increase in ligand binding [88]. This result indicates that even if the assembly efficiency were increased to 100%, assembly could not increase receptor numbers 4–7-fold. At most a 2-fold increase in the number of assembled receptors would be expected, and could only account for part of the measured 4–6-fold increase in ligand binding. Additional evidence contrary to subunit assembly mechanism being a factor is work from our laboratory. We used several independent measurements including nicotine treatment of surface receptors after biotinylation, Ab binding to surface α4β2 receptors, measurements of intracellular vs. surface pools of receptors and ^{35}S-methionine pulse-chase analysis to measure the efficiency of subunit assembly. All of these assays indicated that nicotine induces an increase in ^{125}I-epibatidine binding without significant changes in the number of surface or intracellular receptors. Furthermore, we found that receptors on the cell surface undergo upregulation of ^{125}I-epibatidine binding [88].

4.4. Changes in subunit stoichiometry

Measurements of the subunit stoichiometry of receptors *in vivo* have not been attempted because the subunit composition of the native nAChRs is not yet established. The issue of stoichiometry has been addressed for α4β2 receptors transfected into mammalian cell lines or xenopous oocytes. The possibility that α4β2 receptor stoichiometry actually varies was raised based on experiments in oocytes in which the ratio α4:β2 of injected subunit mRNA or cDNA was varied from 1:10 to 10:1 [140,141]. These studies found that changes in the ratio of the injected subunit mRNA or cDNA resulted in receptors with differences in the activation behavior of agonist-evoked currents, using mainly acetylcholine as the agonist [140]. The data was interpreted as a change in subunit stoichiometry with a α4:β2 ratio of 3:2 generating nAChRs with low ACh sensitivity and a ratio of 2:3 forming receptors with high ACh sensitivity. Expression of α4β2 receptors in mammalian cell lines suggested that nicotine-induced upregulation preferentially causes assembly of α4 and β2 subunits in a ratio of 2:3 [142]. This study attempted to directly measure α4β2 receptor stoichiometry by metabolically labeling the subunits with ^{35}S-labeled amino acids for 24 h and determined the ratio of ^{35}S-labeled α4 and β2 subunits found in fully assembled receptors for cells that were treated or untreated with nicotine. The ratio of ^{35}S-labeled α4:β2 subunits decreased with the nicotine treatment. The results are consistent with a change in stoichiometry but other explanations for these results are possible if nicotine is differentially affecting subunit assembly, maturation or turnover. While nicotine may be changing the subunit stoichiometry of the receptor achieved during its assembly, it is not clear how this would cause the changes in the number of high-affinity binding sites unless a conformational change also occurs that increases the number of high-affinity binding sites as suggested in the section above. The finding that α4β2 receptors on the cell surface undergo upregulation of ^{125}I-epibatidine binding [88,107] is inconsistent with a change in subunit stoichiometry because it is highly unlikely a change in subunit stoichiometry can occur for receptors once inserted onto the surface.

4.5. Block of subunit degradation in the ER

In the case of muscle nAChRs, assembly efficiency and cell surface receptor expression can be increased by blocking their proteosome-mediated ER-associated degradation (ERAD) without altering surface turnover [130]. Subsequent studies tested whether nicotine inhibits proteosomal degradation of neuronal nAChR subunits in a similar way. Over-expression of Ubiquilin-1 was found to promote ERAD of unassembled α3 and α4 subunits, thereby limiting the assembly of these subunits and also blocking an increase in surface receptors by nicotine treatment [143]. In a separate study, nicotine was observed to directly inhibit proteosome catalytic activity through a direct interaction of nicotine with the proteosome, independent of nicotinic receptor activation. Only α7-type nAChRs were shown to increase in receptor number because of this activity. Effects on α3 and α4 subunits were not tested [144].

4.6. nAChR conformational changes

The last mechanism proposed to underlie nicotine-induced upregulation is that the upregulated nAChRs can be in two separate

states and the transition between states is regulated by nicotine [88]. In one state, the resting state, the affinity for nicotine and other agonists is so low that no significant radio-labeled agonist binding can occur. However, agonists still bind to their site with the normal affinity that activates and desensitizes the receptors. In the other state, the upregulated state, the affinity for nicotine and other agonists is high such that radio-labeled agonist binding occurs. Exposure to nicotine-induces the transition from the resting to the upregulated state so that an increase in the number of high-affinity agonist binding sites is observed, but there is no increase in the number of receptors. The initial evidence in support of this mechanism was primarily that the number of receptors, the turnover, the assembly and the trafficking do not change [88]. As we have attempted to convey above, there is much published data contrary to this conclusion, and at this point, further experiments are needed in order resolve whether $\alpha4\beta2$ receptor numbers increase during nicotine exposure and whether the other receptor subtypes are affected.

Support for a conformational change comes from the findings that nicotine can induce functional upregulation of $\alpha4\beta2$ nAChRs [87]. Work from the Bertrand lab [86,87] indicates that this $\alpha4\beta2$ nAChR functional upregulation is largely caused by $\alpha4\beta2$ nAChRs entering a different functional state as evidence by a change in the desensitization rate, a change in single-channel conductance and a change in the ACh concentration dependence of activation. The functional state change is consistent with a conformational change, but does not indicate how nicotine causes the conformational change. As discussed above, it could be induced by a change in receptor stoichiometry during assembly in the ER. Alternatively, nicotine may act as a "chemical chaperone" during assembly in the ER and favor a conformation change independent of a change in subunit stoichiometry [89]. Finally, as we have proposed [88], nicotine may interact with nAChRs at the cell surface, as well as with intracellular nAChRs, and induce the conformational change that alters function and affinity for agonist. Our findings that pre-existing, cell surface receptors can be upregulated by nicotine [88] supports this latter mechanism.

5. Does nAChR upregulation contribute to nicotine-induced sensitization?

Upregulation of nicotinic receptors was initially hypothesized to underlie both tolerance to nicotine's locomotor depressant effect [145] as well as sensitization to its locomotor activating effect [39,146]. However, it was eventually determined that a correlation in time does not always exist between upregulation of nicotine receptors and these behaviors, ruling out receptor upregulation as the primary cause of nicotine-induced locomotor sensitization. It is still conceivable that nicotine-induced upregulation of nAChRs, which precedes sensitization, represents an early step in a sequence of neuronal events that ultimately leads to sensitized responding [26]. Activation of nicotine receptors is necessary for the induction of locomotor and NAcc DA sensitization by nicotine and other stimulants [43]. While nAChR upregulation is observed widely in brain following exposure to nicotine, there are several reasons to suspect that nAChRs in the VTA play a critical role in the induction of sensitization by nicotine. As outlined above, these receptors have been implicated in the ability of nicotine to increase locomotion and NAcc DA overflow as well as to support self-administration. Also, nicotine exposure upregulates these receptors [18,58,147], enhances the synaptic strength and produces LTP of excitatory synapses onto VTA DA neurons [148,149]. Interestingly, blocking nicotine receptors in the VTA, but not in the NAcc, during exposure to nicotine blocks the induction of long-term locomotor sensitization observed 3 weeks later. In addition, the same moderate intermittent nicotine exposure regimen used to produce locomotor sensitization also produced a transient upregulation of nicotine receptors in the VTA but not in the NAcc [150]. This upregulation was observed 3 h but not 3 days or 3 weeks after nicotine exposure. Thus, transient upregulation was observed selectively in VTA nicotine receptors that are critical for the induction of sensitization by nicotine.

Exposure to nicotine → nAChR upregulation → LTP → DA sensitization → behavioral sensitization (drug addiction).

Functional upregulation of nAChRs in VTA DA neurons should increase excitability and would favor induction of drug-induced LTP of the excitatory inputs they receive. Ultimately, these neuronal events would promote the induction of long-term sensitization in the reactivity of these cells and the expression of behavioral manifestations of this sensitization. Sensitization of midbrain dopamine neuron reactivity is known to enhance drug self-administration [151,152]. Recently, chronic continuous nicotine exposure was shown to upregulate $\alpha4$ containing nicotine receptors specifically in midbrain GABA neurons and increase inhibition of mesoaccumbens DA activity in the period immediately following exposure [57]. Given that withdrawal symptoms are prevalent during this period and the ability of nicotine to activate NAcc DA release is decreased [153], such findings are not entirely surprising. It remains to be determined whether this type of intense chronic regimen ultimately leads to sensitized responding to nicotine and whether more moderate intermittent regimens that are known to produce sensitization show different cell specificity in the receptor upregulation they induce.

Acknowledgements

This work was supported by NIH grants: NS043782 (WNG), DA019695 (WNG & PV) and the Peter F. McManus Foundation (WNG).

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