

ORIGINAL ARTICLE

Density of $\alpha 4\beta 2^*$ nAChR on the surface of neurons is modulated by chronic antagonist exposure

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[¹²⁵I]epibatidine, dihydro- β -erythroidine, mecamylamine, neuronal culture, nicotine, nicotinic receptor

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Abstract

The expression of high-affinity $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors (nAChR) increases following chronic exposure to nicotinic agonists. While, nAChR antagonists can also produce upregulation, these changes are often less pronounced than achieved with agonists. It is unknown if nAChR agonists and antagonists induce receptor upregulation by the same mechanisms. In this study, primary neuronal cultures prepared from cerebral cortex, hippocampus, diencephalon, and midbrain/hindbrain of C57BL/6J mouse embryos were treated chronically with nicotine (agonist), mecamylamine (noncompetitive antagonist) or dihydro- β -erythroidine (competitive antagonist) or the combination of nicotine with each antagonist. The distribution of intracellular and surface [¹²⁵I]epibatidine-binding sites were subsequently measured. Treatment with 1 μ mol/L nicotine upregulated intracellular and cell surface [¹²⁵I]epibatidine binding after 96 h. Chronic dihydro- β -erythroidine (10 μ mol/L) treatment also increased [¹²⁵I]epibatidine binding on the cell surface; however, mecamylamine was ineffective in upregulating receptors by itself. The combination of 1 μ mol/L nicotine plus 10 μ mol/L mecamylamine elicited a significantly higher upregulation than that achieved by treatment with nicotine alone due to an increase of [¹²⁵I]epibatidine binding on the cell surface. This synergistic effect of mecamylamine and nicotine was found in neuronal cultures from all four brain regions. Chronic treatment with nicotine concentrations as low as 10 nmol/L produced upregulation of [¹²⁵I]epibatidine binding. However, the effect of mecamylamine was observed only after coincubation with nicotine concentrations equal to or greater than 100 nmol/L. Vesicular trafficking was required for both nicotine and nicotine plus mecamylamine-induced upregulation. Results presented here support the idea of multiple mechanisms for nAChR upregulation.

Abbreviations

ANOVA, analysis of variance; ARA C, cytosine β -D-arabinofuranoside; BrACh, 2-(2-bromoacetyloxy)-N,N,N-trimethylethanaminium bromide; DMSO, dimethyl sulfide; DTT, 1,4-dithio-DL-threitol; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); HBSS, Hank's balanced salt solution; HEPES, 4-(2-hydroxyethyl)-piperazine ethanesulfonic acid; KRH, Krebs-Ringer-HEPES; MEM, minimal essential medium; nAChR, nicotinic acetylcholine receptors; PEI, polyethylenimine.

Introduction

Chronic exposure to nicotine elicits a reversible increase (termed upregulation) of the nicotinic acetylcholine

receptors (nAChR) principally for the high-affinity $\alpha 4\beta 2^*$ nAChR subtype (Marks et al. 1983; Schwartz and Kellar 1983; Whiteaker et al. 1998; Davila-Garcia et al. 1999; Fenster et al. 1999; Lomazzo et al. 2011; Zambrano et al.

2012). Upregulation of $\alpha 4\beta 2^*$ nAChR is also found in postmortem brains of smokers compared with nonsmoker human subjects (Benwell *et al.* 1988; Breese *et al.* 1997; Perry *et al.* 1999). In vivo PET scan studies confirm that smokers have a higher density of nAChR than nonsmokers and the regional differences in the extent of upregulation similar to those seen with animal studies (Staley *et al.* 2006; Mukhin *et al.* 2008). Although upregulation of $\alpha 4\beta 2^*$ nAChRs occur in human smokers, the role of upregulation in nicotine dependence is not clear. Our early studies suggested a relationship between the rate of $\alpha 4\beta 2^*$ nAChR upregulation during nicotine treatment and return to baseline following termination of treatment and the rate of gain and loss of tolerance to the acute effects of nicotine (Marks *et al.* 1985). Recently, it has been shown that upregulation of $\alpha 4\beta 2^*$ nAChRs is correlated with nicotine withdrawal-induced learning deficits in mice (Wilkinson *et al.* 2013). Since cognitive impairment caused by nicotine withdrawal is a common consequence of smoking cessation and is a strong predictor of relapse (Rukstalis *et al.* 2005). The proposed role of $\alpha 4\beta 2^*$ upregulation in withdrawal-induced learning deficits is highly relevant to nicotine dependence.

The mechanisms that modulate nAChR expression after chronic nicotine exposure have not been fully elucidated. However, it is well established that this increase is not due to changes in mRNA production (Marks *et al.* 1992; Peng *et al.* 1994). Studies in systems expressing $\alpha 4\beta 2^*$ nAChR heterologously suggest that chronic nicotine treatments increase the assembly of new receptors at the endoplasmic reticulum by a chaperone effect of nicotine (Kuryatov *et al.* 2005; Lester *et al.* 2009). The chaperone hypothesis postulates that the interaction of nicotine with surface receptors is not necessary for nAChR upregulation, but a concentration of the drug enough to bind and stabilize the specific nAChR subtype in the endoplasmic reticulum is required (Lester *et al.* 2009).

Studies of the effects of chronic exposure to nicotinic antagonists on nAChR expression have yielded inconsistent results. nAChR upregulation has been observed in mice chronically infused with the open channel blocker mecamylamine (Collins *et al.* 1994; Pauly *et al.* 1996). In addition, mecamylamine produced a synergistic effect when it was coadministered with nicotine (Collins *et al.* 1994; Peng *et al.* 1994; Pauly *et al.* 1996; Davila-Garcia *et al.* 1999). However, other studies have reported that daily injection of rats with mecamylamine had no effect on nAChR expression when used alone or when coadministered with nicotine (Schwartz and Kellar 1985). Similarly mecamylamine treatment of non-neuronal cells transfected with $\alpha 4$ and $\beta 2^*$ subunits did not alter receptor expression (Gopalakrishnan *et al.* 1997; Darsow *et al.*

2005; Kuryatov *et al.* 2005). Competitive nAChR antagonists, such as dihydro- β -erythroidine, produced upregulation in N2A cells transfected with $\alpha 4\beta 2^*$ nAChR although this effect was modest compared to the magnitude of upregulation produced by the treatment with nicotine or cytosine (Srinivasan *et al.* 2012). Other studies confirm a low efficacy of dihydro- β -erythroidine to produce nAChR upregulation (Yang and Buccafusco 1994; Gopalakrishnan *et al.* 1997).

In this study, we compared the effects of chronic treatment of primary mouse neuronal cell culture with nicotine and the nAChR inhibitors mecamylamine (a noncompetitive antagonist which binds in the channel) and dihydro- β -erythroidine (a competitive antagonist) on the expression at both intracellular and surface nAChR. While the effect of treatment with nAChR antagonists on receptor distribution using primary neuronal cultures has not yet been described, nAChR in both cell surface and intracellular compartment are upregulated by chronic nicotine treatments (Lomazzo *et al.* 2011; Zambrano *et al.* 2012). We describe here that nAChR antagonists have an effect on upregulation only in [125 I]epibatidine sites located at the cell surface. These results support the idea of multiple mechanisms for nAChR up-regulation.

Materials and Methods

Materials

Brefeldin A and mecamylamine hydrochloride was purchased from Tocris Bioscience (Bristol, United Kingdom). [125 I]Epibatidine (2200 Ci/mmol) was purchased from Perkin-Elmer Life Science (Waltham, MA). 2-(2-bromoacetyloxy)-*N,N,N*-trimethylethanaminium bromide (BrACh), cytosine, cytosine β -D-arabino-furanoside (ARA C), 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB), dihydro- β -erythroidine, 1,4-dithio-DL-trethol (DTT), (-)-nicotine hydrogen tartrate, polyethyleneimine (PEI), and poly-L-lysine (>30,000 kDa) were purchased from Sigma Aldrich Chemical Company (St. Louis, MO). The 4-(2-Hydroxyethyl)-piperazineethanesulfonic acid (HEPES) half-sodium salt was from Roche Diagnostics Corporation (Indianapolis, IN). Neurobasal media, Minimal essential media (MEM), B27 supplement, GlutamaxTM, inactivated horse serum, and TrypLE express were purchased from Invitrogen (Carlsbad, CA).

Methods

Primary neuronal cultures

All experiments were approved by the Animal Care and Utilization Committee of the University of Colorado,

Boulder, and carried out in accordance with procedures approved by the National Institutes for Health. Primary cultures from embryonic mouse brains (embryonic day E16 to E18) were prepared as previously described (Zambrano *et al.* 2012). Briefly, embryo brains were placed in Hank's balanced salt solution (HBSS, Ca²⁺ and Mg²⁺-free) buffer and separated from the meninges. The forebrain was initially separated from the brain stem, the hippocampus was dissected from the cortex. The remaining brain stem was separated into diencephalon and mid-brain/hindbrain regions. Brain tissues were minced into small pieces, rinsed once with HBSS, and then incubated for 15 min at 37°C with 0.5X TrypLE express diluted with HBSS. TrypLE solution was replaced by MEM supplemented with 10% Horse serum and the minced tissue were mechanically disaggregated using a heat polished Pasteur pipette. The resulting cell suspension was centrifuged at 800g for 2 min and then suspended in MEM containing 10% Horse Serum, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.25 mg/mL amphotericin B. Isolated neurons were seeded at a density of 100,000 cells/cm² over polystyrene plates coated with 0.1 mg/mL poly-L-lysine prepared in 0.1 mol/L borate buffer pH 8.5. After 24 h, media was changed to maintenance media (neurobasal media supplemented with B27, 100 U/mL penicillin, 100 U/mL streptomycin, 0.25 mg/mL amphotericin B and 2 mmol/L L-glutamine. On the second day of culture, 10 µmol/L ARA C was added for 48 h in order to control proliferation of glial cells. Cultures were kept in a humidified 5% CO₂-95% air incubator at 37°C.

Stock solutions (10 mmol/L) of nicotine, dihydro-β-erythroidine and mecamylamine were prepared in maintenance media and kept frozen at -20°C until used. Chronic drug treatments with nicotine and/or the antagonists (mecamylamine and dihydro-β-erythroidine) were performed up to 96 h in neurons that have been cultured for 12–14 days. Brefeldin A (1 µmol/L) was prepared in maintenance media from a 10-mmol/L stock dissolved in dimethyl sulfoxide (DMSO) and used alone or in combination with 1 µmol/L nicotine and/or 10 µmol/L mecamylamine. DMSO controls were also performed. Treatment time for these studies was 24 h.

Preparation of total membranes

At the end of the experimental treatment, the medium was removed from each well which was then rinsed once with Krebs-Ringer-HEPES (KRH) buffer (NaCl, 144 mmol/L; KCl, 2.2 mmol/L; CaCl₂, 2 mmol/L; MgSO₄, 1 mmol/L; HEPES, 25 mmol/L; pH = 7.5). Cells were lysed by scrapping the plate surface in 0.1X hypotonic

KRH buffer. Hypotonic buffer containing the cell lysates was collected and centrifuged at 25,000g for 10 min at 4°C to obtain total membrane pellets. The pellets were washed 3 times by resuspension in ice-cold hypotonic KRH buffer followed by centrifugation. Cell membrane pellets were resuspended in distilled-deionized water for the binding reaction (if carried out immediately) or in the hypotonic 0.1X KRH-binding buffer and frozen at -20°C until assayed.

[¹²⁵I]epibatidine binding to cell membrane homogenates

[¹²⁵I]Epibatidine binding was measured as described previously (Whiteaker *et al.* 2000). Frozen cell membrane pellets were thawed and centrifuged at 25,000g for 10 min. The supernatant was discarded and the pellet was resuspended in distilled-deionized water. Re-suspension volumes varied among samples to adjust protein concentrations such that less than 10% of the [¹²⁵I]epibatidine was bound to the protein at a concentration of 200 pmol/L of the radioligand. Samples (5–20 µg protein) were incubated in 96-well polystyrene plates for 2 h at room temperature in KRH buffer with a final incubation volume of 30 µL. At the completion of the binding reaction, samples were diluted with 200 µL of ice-cold KRH buffer and filtered under vacuum (0.2 atm.) onto glass fiber filters that had been treated with 0.5% polyetheleneimine (top filter, MFS Type B; bottom filter, Pall Type A/E). An Inotech Cell Harvester (Inotech Biosystems International, Rockville, MD) was used to collect the samples, which were subsequently washed five times with ice-cold buffer. Filters containing the washed samples were transferred to glass culture tubes and radioactivity counted at 80% efficiency using a Packard Cobra Auto-Gamma Counter (Packard Instruments, Downers Grove, IL). For all the experiments, nonspecific binding was measured by including 100 µmol/L cytosine in the incubation medium.

Alkylation of cell surface associated nAChR

Alkylation of surface nAChR was carried out as described previously (Free *et al.* 2005; Zambrano *et al.* 2012). Briefly, primary neurons in culture were rinsed once with HBSS buffer pH 7.4 supplemented with 20 mmol/L HEPES and then treated for 15 min at 37°C with 1 mmol/L DTT prepared in HBSS to reduce disulfide bonds. Cultures were rinsed once with HBSS followed by 6 min incubation at room temperature with 0 or 100 µmol/L BrACh prepared in HBSS. After removing the BrACh solutions, samples were rinsed once with HBSS. Reduced disulfide groups that had not been alkyl-

ated were reoxidized by adding 1 mmol/L DTNB in HBSS and incubating for 15 min at 37°C. After the alkylation/reoxidation reactions, the neurons were rinsed once with HBSS, lysed with hypotonic ice-cold KRH buffer, and scraped from the plate. The set of cultures that had been treated with 0 $\mu\text{mol/L}$ BrACh was used to measure total [^{125}I]epibatidine binding. To evaluate the effect of chronic nicotine treatment on BrACh alkylated (cell surface) and residual unalkylated (intracellular) binding, cells were treated with nicotine (0, 1, 10, 100 and 1000 nmol/L) for 24, 48, 72 and 96 h. Whole particulate membranes were prepared as described above (preparation of total membranes) and [^{125}I]epibatidine binding was subsequently measured using the radioligand-binding assay also described above ([^{125}I]epibatidine binding to cell membrane homogenates). Surface binding was calculated as the difference between total binding (no incubation with BrACh) and binding after alkylation (intracellular).

Data calculations

Total specific [^{125}I]epibatidine binding was determined for neuronal cultures from all brain regions. Previous experiments showed that cytosine-resistant [^{125}I]epibatidine-binding range from 3% to 10% of in these primary neuronal cultures. Consequently, cytosine-sensitive $\alpha 4\beta 2^*$ nAChR is the predominant subtype measured by 200 pmol/L [^{125}I]epibatidine binding (unpublished data). Cytosine-sensitive and cytosine-resistant [^{125}I]epibatidine binding sites were calculated for MB/HB cultures using a two site inhibition model: $B_I = B_1/(1 + I/IC_{50-1}) + B_2/(1 + I/IC_{50-2})$, where B_I is the [^{125}I]epibatidine binding at either cytosine concentration, I , B_1 and B_2 are the [^{125}I]epibatidine binding with IC_{50} values IC_{50-1} (cytosine-sensitive sites) and IC_{50-2} (cytosine-resistant sites), respectively. IC_{50-1} and IC_{50-2} values used were 3.75 and 300 nmol/L. Densities of cytosine-sensitive and cytosine-resistant sites were calculated from the [^{125}I]epibatidine binding measured with 0, 50 and 150 nmol/L cytosine. SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA, USA) was used for calculations and graphical presentation of the data. Statistical analyses were conducted using SPSS software (IBM Corp., Somers, NY, USA). Two-way analysis of variance (ANOVA) was performed to calculate nicotine-mecamylamine interaction or time-treatment interaction. One-way ANOVA analysis was performed for total, intracellular and surface binding components to detect differences among treatments. A Student's t -test was performed in the dose-response study for each nicotine concentration to compare the effect of mecamylamine. Tukey's post-hoc test was run for multiple comparison analysis after one way ANOVA.

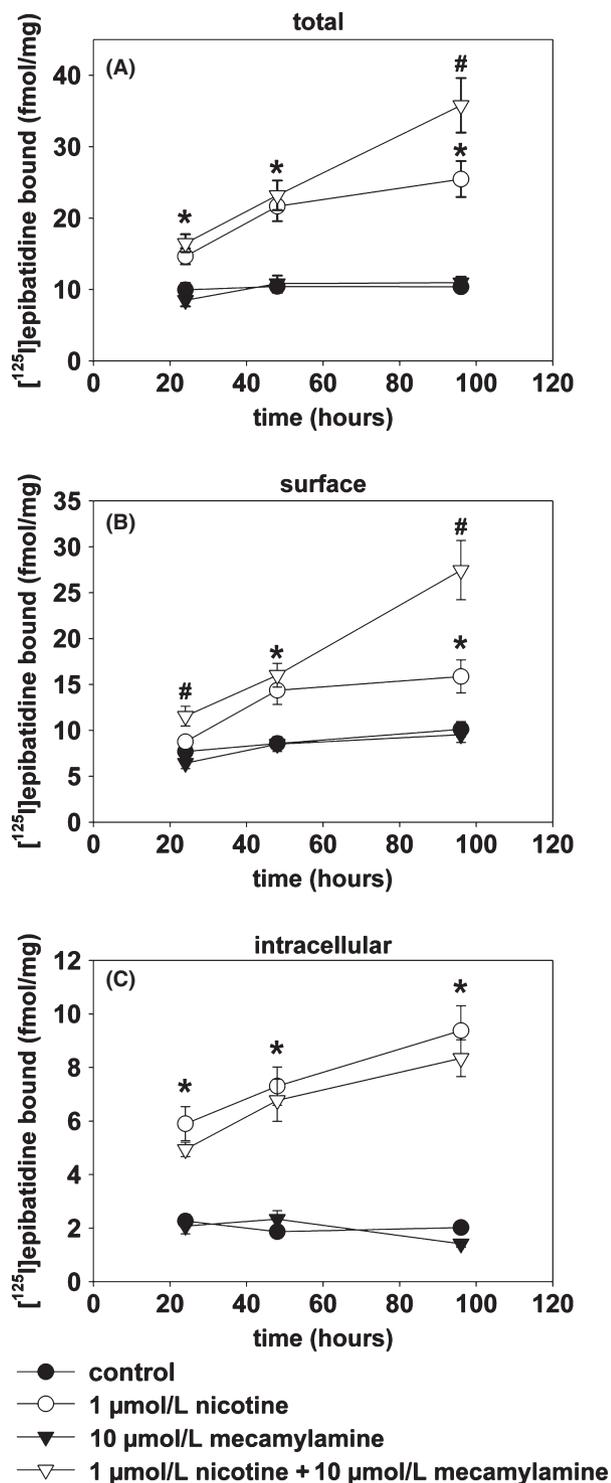
Results

[^{125}I]Epibatidine binding sites in primary cortical neurons upregulate as a function of time of treatment with nicotine alone and in coinubation with mecamylamine

Cortical neurons prepared from E16 to E18 mouse embryos were used to investigate the effects of treatment with 1 $\mu\text{mol/L}$ nicotine and/or 10 $\mu\text{mol/L}$ mecamylamine on [^{125}I]epibatidine-binding sites over three different time points. Cultured neurons were maintained for 12–14 days before drug treatments were begun. Functional $\alpha 4\beta 2^*$ nAChR in hippocampal neurons are detectable after 10 days of culture (Zarei et al., 1999). Following 24, 48 or 96 h of drug treatment, samples were analyzed for total [^{125}I]epibatidine binding as well as binding specifically localized to the surface and intracellular membranes (Free et al. 2005; Zambrano et al. 2012). Briefly, surface nAChR-binding sites are covalently bound with BrACh while intracellular-binding sites are left intact. Total binding is measured in neurons that followed same reaction in absence of BrACh, and then surface binding sites are obtained by subtracting total minus intracellular sites. The amount of total [^{125}I]epibatidine binding in control samples as well as those treated with 10 $\mu\text{mol/L}$ mecamylamine was unchanged throughout the treatment period (Fig. 1A). In contrast, a time-dependent increase in total [^{125}I]epibatidine binding was observed following treatment with 1 $\mu\text{mol/L}$ nicotine compared with control neurons (a $47 \pm 18\%$ increase after 24 h, a $108 \pm 27\%$ after 48 h and a $146 \pm 29\%$ increase after 96 h, Fig. 1A). Increases in total [^{125}I]epibatidine binding were also observed following treatment with 1 $\mu\text{mol/L}$ nicotine plus 10 $\mu\text{mol/L}$ mecamylamine ($65 \pm 20\%$, $122 \pm 28\%$ and $245 \pm 43\%$ increases over the control at 24, 48, and 96 h, respectively, Fig. 1A). Furthermore, total [^{125}I]epibatidine binding for samples treated for 96 h with both drugs was significantly greater than that observed for samples treated with nicotine alone ($P < 0.05$, Tukey post hoc test).

The pattern of upregulation for [^{125}I]epibatidine binding sites localized on the cell surface was generally similar to that for total binding. Increases were noted after 48 and 96 h of treatment but not after 24 h. A striking increase was noted for samples treated with both nicotine and mecamylamine for 96 h and resulted from a selective increase of binding sites only in the neuronal surface (Fig. 1B). Increases in [^{125}I]epibatidine binding on the cell surface appear to be slower than those measured for intracellular binding.

Receptors localized to the intracellular membranes were upregulated after 24, 48, and 96 h of nicotine treatment.



The pattern of intracellular change did not differ between samples treated with nicotine alone or nicotine plus mecamylamine. Total, intracellular and cell surface binding [^{125}I]epibatidine binding for control and mecamylamine-

Figure 1. Time-course study of total, intracellular, and surface [^{125}I]epibatidine binding. Primary neuronal cultures (12–14 days in culture) prepared from cortex (E16–E18) were challenged with 1 μmol/L nicotine, 10 μmol/L mecamylamine or the combination of both drugs. Specific total (A), surface (B) and intracellular (C) binding was determined after 24, 48 and 96 h of treatment. Data represent mean \pm SEM of specific [^{125}I]epibatidine binding in membrane preparations normalized by protein content for 12 to 18 replicates obtained from 3 to 4 independent experiments. Asterisks indicate that nicotine and nicotine plus mecamylamine treatments differ from control, but not each other. Pound symbols indicate significant differences between nicotine plus mecamylamine cotreatment from control and nicotine alone.

treated samples (without nicotine) did not differ at any time point for (Fig. 1A, B, and C).

Chronic nicotine and nicotine cotreatment with mecamylamine differentially up-regulate [^{125}I]epibatidine binding in primary cultures prepared from four brain areas

The results described above for the effect on nicotine and/or mecamylamine treatment on the expression and distribution of [^{125}I]epibatidine-binding sites confirmed that chronic nicotine treatment increases the density of these sites and that the increased expression resulting from an interaction between nicotine and mecamylamine is localized to receptors in the surface. In order to evaluate whether these changes also occur in regions in addition to cortex, primary cell cultures prepared from hippocampus, diencephalon (including thalamus and striatum) and midbrain/hindbrain in addition to cortex were treated with nicotine and/or mecamylamine for 96 h.

The density of total [^{125}I]epibatidine-binding sites for untreated samples varied substantially among the regions (as fmol/mg protein, cortex: 13.0 ± 0.9 ; hippocampus: 8.6 ± 0.7 ; diencephalon: 31.1 ± 2.4 and midbrain/hindbrain: 97.4 ± 11.5). The percentage of binding sites on the cell surface was higher than that at the intracellular membranes for all the brain regions (87.7% of the total [^{125}I]epibatidine binding for cortex, 77.3% for hippocampus, 74.8% for diencephalon and 84.1% for midbrain/hindbrain, Fig. 2).

Treatment with 1 μmol/L nicotine elicited significant increases in total [^{125}I]epibatidine binding in cortex ($114 \pm 26\%$), hippocampus ($204 \pm 42\%$), diencephalon ($58 \pm 16\%$) and midbrain/hindbrain ($42 \pm 10\%$) (Fig. 2A–D). Treatment with mecamylamine alone did not affect binding. Cotreatment with nicotine plus mecamylamine elicited greater increase in total [^{125}I]epibati-

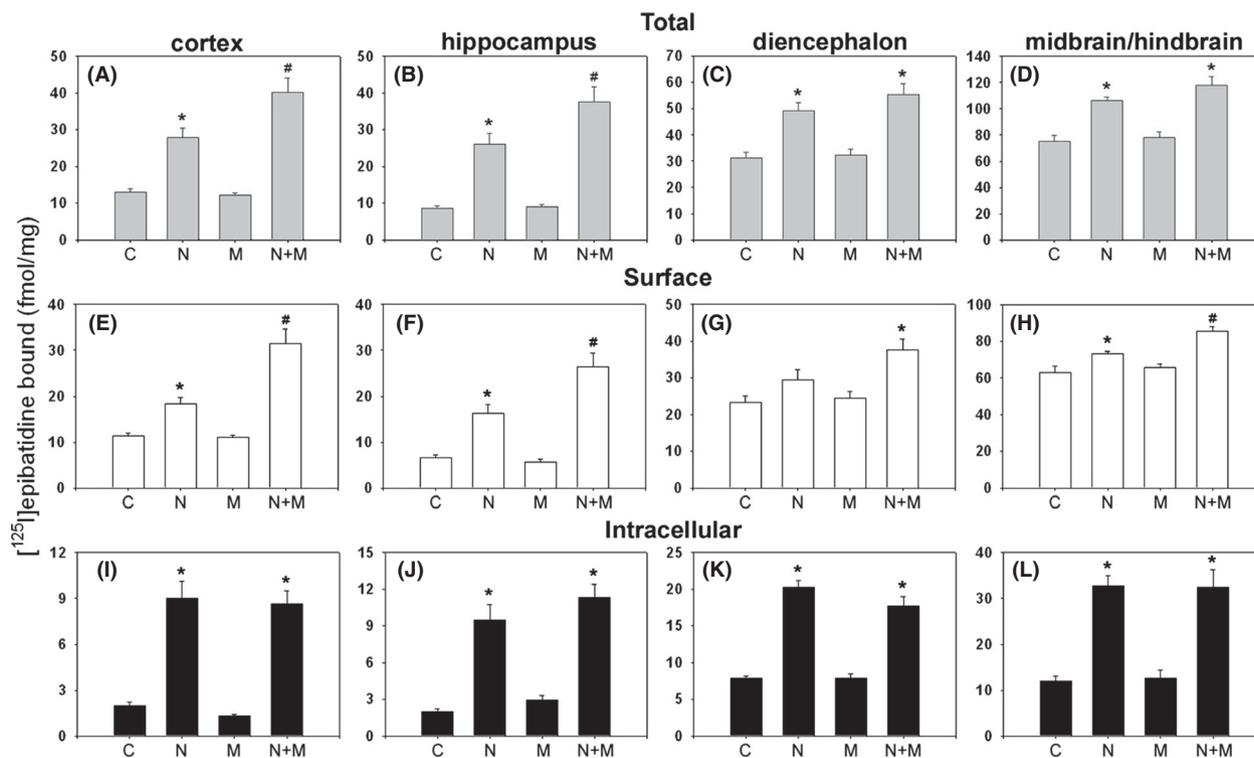


Figure 2. Comparison of mecamylamine and nicotine-induced [125 I]epibatidine-binding upregulation across brain regions. Primary neuronal cultures (12–14 days in culture) prepared from cortex, hippocampus, diencephalon, and midbrain/hindbrain regions were treated with 1 μ mol/L nicotine, 10 μ mol/L mecamylamine or the combination of both for 96 h. Specific total (A–D), surface (E–H) and intracellular (I–L) [125 I]epibatidine binding was determined. C, N, M, and N+M in the graphs represent control, 1 μ mol/L nicotine, 1 μ mol/L mecamylamine, and 1 μ mol/L nicotine plus 1 μ mol/L mecamylamine, respectively. Data are presented as mean \pm SEM of 11–12 replicates obtained from 4 independent experiments. Asterisks indicate significant differences between 1 μ mol/L nicotine and control. Pound symbols indicate significant differences between 1 μ mol/L nicotine and 1 μ mol/L nicotine plus 10 μ mol/L mecamylamine cotreatment.

dine binding in both cortex ($209 \pm 37\%$, consistent for results reported above, Fig. 1) and hippocampus ($338 \pm 60\%$) than treatment with nicotine alone (Fig. 2A and B). In contrast, diencephalon and midbrain/hindbrain cultures did not show significantly increased binding induced by nicotine and mecamylamine cotreatment over that observed following treatment with nicotine alone (Fig. 2C and D, nicotine by mecamylamine interaction $F_{1,32} = 0.64$ and $F_{1,32} = 0.21$, respectively).

As was the case for total [125 I]epibatidine binding, treatment with nicotine alone significantly increased cell surface [125 I]epibatidine binding in cortex ($61 \pm 15\%$), hippocampus ($146 \pm 35\%$), diencephalon ($27 \pm 16\%$), and midbrain/hindbrain ($17 \pm 7\%$) (Fig. 2E–H, main effect of nicotine: $F_{1,44} = 69.59$, $F_{1,54} = 68.87$, $F_{1,31} = 17.06$, and $F_{1,32} = 38.13$, respectively, $P < 0.001$ for all regions). Furthermore, cotreatment with nicotine and mecamylamine resulted in a further increase in [125 I]epibatidine binding in both cortex ($177 \pm 30\%$, consistent with results in Section 2.1 above), and hippocampus ($299 \pm 56\%$) (nicotine by mecamylamine interaction

$F_{1,42} = 16.96$ $P < 0.001$, $F_{1,53} = 9.10$ $P < 0.005$, respectively). As was observed for total binding, upregulation from nicotine plus mecamylamine treatment in diencephalon ($62 \pm 18\%$) and midbrain/hindbrain ($36 \pm 8\%$) was not significantly different from nicotine alone.

The upregulation pattern of intracellular [125 I]epibatidine binding was similar for all four brain regions. Intracellular binding in cells chronically treated with mecamylamine did not differ from that of controls. Chronic treatment with nicotine significantly increased intracellular binding in all four brain regions (main effect of nicotine: cortex, $F_{1,44} = 96.50$, $361 \pm 80\%$ increase; hippocampus, $F_{1,54} = 87.40$, $382 \pm 90\%$ increase; diencephalon $F_{1,31} = 185.15$, $158 \pm 16\%$ increase; midbrain/hindbrain, $F_{1,32} = 69.88$, $173 \pm 30\%$ increase, $P < 0.001$ for all regions). In contrast to the cell surface binding, the density of intracellular binding in cells treated with nicotine plus mecamylamine did not differ from that of cells treated with nicotine alone (Fig. 2I–L). All the difference in upregulation produced by mecamylamine was observed in the cell surface and only in the presence of nicotine.

Nicotine-mecamylamine interactions in the presence of nanomolar nicotine concentrations: Mecamylamine requires a minimum nicotine "threshold" to produce synergistic upregulation of receptors

The results described above demonstrate that mecamylamine increased [¹²⁵I]epibatidine binding in primary neuronal cultures only if nicotine was also present. The 1 μmol/L concentration of nicotine used in those studies is known to activate α4β2*-nAChR. However, the plasma level of nicotine in human cigarette smokers varies from 6 nmol/L after overnight abstinence to 250 nmol/L shortly after smoking (Jarvik *et al.* 2000; Brody *et al.* 2006). In order to evaluate whether the concentrations of nicotine observed in tobacco users also result in a nicotine-dependent effect of mecamylamine on the expression of [¹²⁵I]epibatidine-binding sites, primary neuronal cultures prepared from cortex and midbrain/hindbrain were treated with 10 μmol/L mecamylamine in the presence of 1, 10, and 100 nmol/L nicotine. Primary neurons from these cortex and midbrain/hindbrain were selected because they represent low and high α4β2* nAChR density regions, respectively, and also differ in their response to chronic nicotine exposure displaying relatively high and low upregulation as percentage from control (Compare Fig. 2A and B for total [¹²⁵I]epibatidine binding in cortex and midbrain/hindbrain respectively).

A significant nicotine concentration-dependent increase in total [¹²⁵I]epibatidine binding was observed for both brain regions (Fig. 3A cortex: $F_{3,17} = 8.27$, $P < 0.001$; Fig. 3B midbrain/hindbrain: $F_{3,20} = 8.35$, $P < 0.001$). Samples treated with either 10 or 100 nmol/L nicotine had significantly more [¹²⁵I]epibatidine binding than control samples (Fig. 3A cortex: $33 \pm 9\%$ and $55 \pm 18\%$; Fig. 3B midbrain/hindbrain: $28 \pm 9\%$ and $32 \pm 10\%$). Mecamylamine significantly increased [¹²⁵I]epibatidine binding in the presence of 100 nmol/L nicotine in midbrain/hindbrain neurons (Fig. 3B, $53 \pm 13\%$ increase respect to control, $t_9 = 3.26$, $P < 0.01$) compared to 100 nmol/L nicotine alone ($32.2 \pm 10.0\%$ increase respect to control).

The effect of nicotine and mecamylamine on cell surface [¹²⁵I]epibatidine binding was similar to the effect on total binding for cortical cultures (Fig. 3C, $F_{3,17} = 4.68$, $P < 0.05$) and midbrain/hindbrain cultures (Fig. 3D, $F_{3,20} = 8.58$, $P < 0.001$). Cortical samples treated with 100 nmol/L nicotine ($33 \pm 4\%$ increase from control) and midbrain/hindbrain cultures treated with 10 nmol/L nicotine ($27 \pm 6\%$) or 100 nmol/L nicotine ($24 \pm 6\%$) had significantly higher binding than control samples. No upregulation of [¹²⁵I]epibatidine binding at the cell surfaces was observed following treatment with mecamylamine

alone or mecamylamine in the presence of 1 or 10 nmol/L nicotine (Fig. 3C and D). However, cell surface binding following treatment with mecamylamine plus 100 nmol/L nicotine was significantly higher than that observed following treatment with nicotine alone in cortex ($91 \pm 14\%$ increase, $t_{10} = 4.31$, $P < 0.01$, comparing nicotine to nicotine plus mecamylamine) and midbrain/hindbrain ($48 \pm 4\%$, $t_9 = 6.47$, $P < 0.001$).

The pattern of increase in intracellular [¹²⁵I]epibatidine binding following treatment with 100 nmol/L nicotine was similar for both brain regions (Fig. 3E–F). Addition of mecamylamine did not change intracellular binding from that of nicotine alone. Although chronic nicotine treatment tended to increase intracellular [¹²⁵I]epibatidine binding in cortex, this apparent increase was not statistically significant for 10 nmol/L nicotine ($F_{3,17} = 2.39$, $P > 0.05$). In contrast, the increase in intracellular binding sites in midbrain/hindbrain cells treated with 100 nmol/L nicotine was statistically significant ($F_{3,20} = 10.66$, $P < 0.001$).

Chronic nicotine alone and in combination with dihydro-β-erythroidine produce differential effects on [¹²⁵I]epibatidine binding

The experiments described above evaluated the interaction between nicotine and the noncompetitive antagonist, mecamylamine. In order to evaluate the interaction between a competitive antagonist and nicotine, primary neuronal cultures prepared from cortex, hippocampus, diencephalon, and midbrain/hindbrain were chronically treated for 96 h with nicotine (1 μmol/L) and/or dihydro-β-erythroidine (10 μmol/L).

The effects of chronic nicotine and/or dihydro-β-erythroidine treatment on total [¹²⁵I]epibatidine binding are illustrated in Figure 4A–D. The response of total [¹²⁵I]epibatidine binding to chronic treatment with nicotine and/or dihydro-β-erythroidine was similar in three of the brain regions tested. Treatment with nicotine significantly increased total binding in cortex ($153 \pm 32\%$ increase from control; main effect of nicotine: $F_{1,30} = 39.58$, $P < 0.001$), diencephalon ($58 \pm 16\%$; $F_{1,26} = 31.09$, $P < 0.001$) and midbrain/hindbrain ($46 \pm 9\%$; $F_{1,29} = 18.83$, $P < 0.001$). Although there was a tendency for [¹²⁵I]epibatidine binding to increase following treatment with dihydro-β-erythroidine ($31 \pm 8\%$ increase for cortex, $26 \pm 15\%$ for diencephalon, $30 \pm 9\%$ for midbrain/hindbrain), these changes were not significant. Similarly, while there was a tendency for the effects of cotreatment with nicotine plus dihydro-β-erythroidine to elicit an increase in [¹²⁵I]epibatidine binding, this increase was not significantly different from that measured following

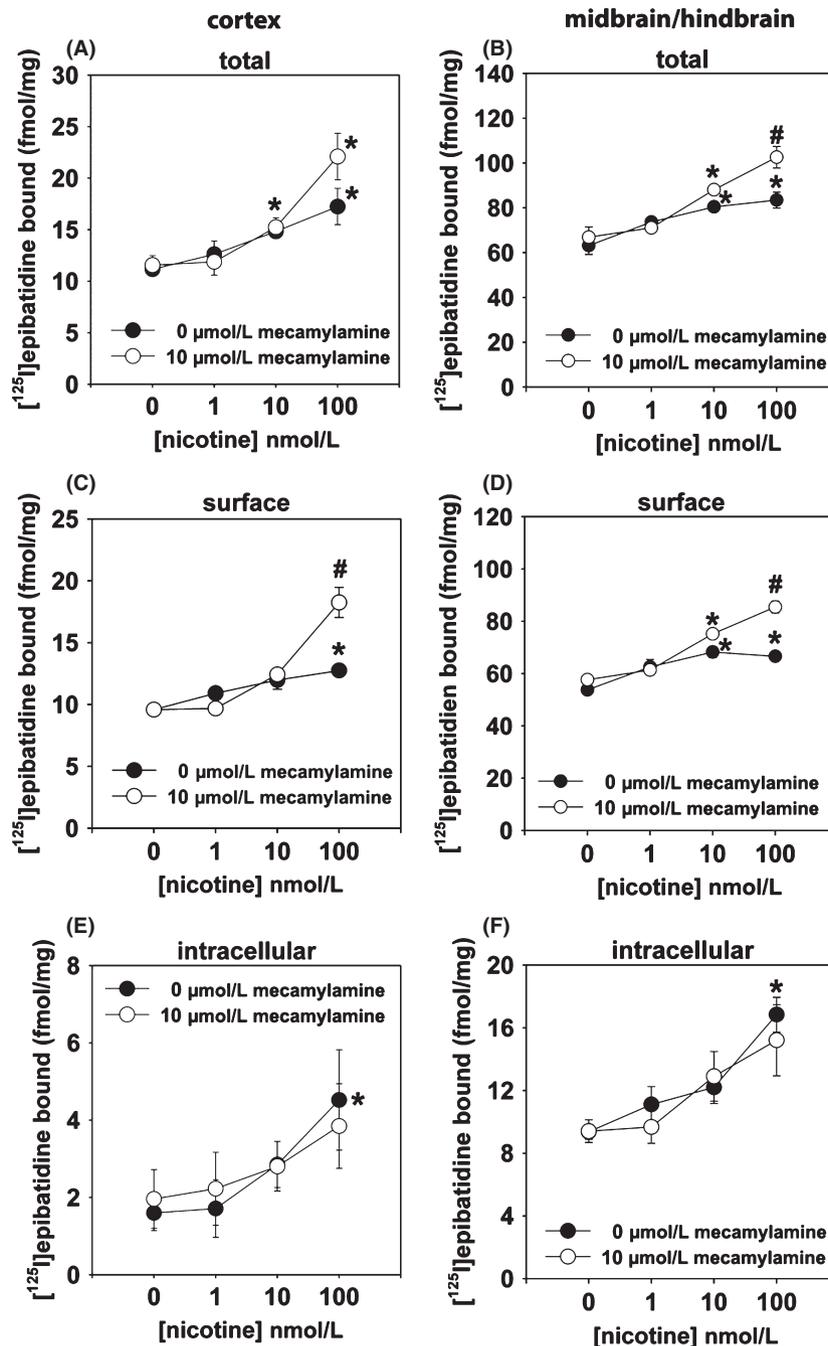


Figure 3. Effect of mecamlamine on a low-nicotine concentration response. Neuronal cultures (12–14 days in culture) prepared from cortex and midbrain/hindbrain were treated with 1, 10, and 100 nmol/L nicotine for 96 h in the presence or absence of 10 μmol/L mecamlamine. Specific total (A–B), surface (C–D) and intracellular (E–F) [125I]epibatidine binding was determined. Data are presented as mean ± SEM of 5–6 replicates obtained from 2 independent experiments. Asterisks indicate significant difference between 100 nmol/L nicotine and 100 nmol/L nicotine plus 10 μmol/L mecamlamine treatments.

nicotine treatment alone. The pattern for response was somewhat different in hippocampus in which chronic treatment with either nicotine or dihydro-β-erythroidine significantly increased [125I]epibatidine binding (2-way ANOVAs, main effect of nicotine, $F_{1,18} = 59.24$,

$P < 0.001$; main effect of dihydro-β-erythroidine, $F_{1,18} = 14.35$, $P < 0.001$). The increase measured following treatment with 1 μmol/L nicotine was greater than that observed for other regions ($230 \pm 76\%$ increase from control), while the binding following treatment with

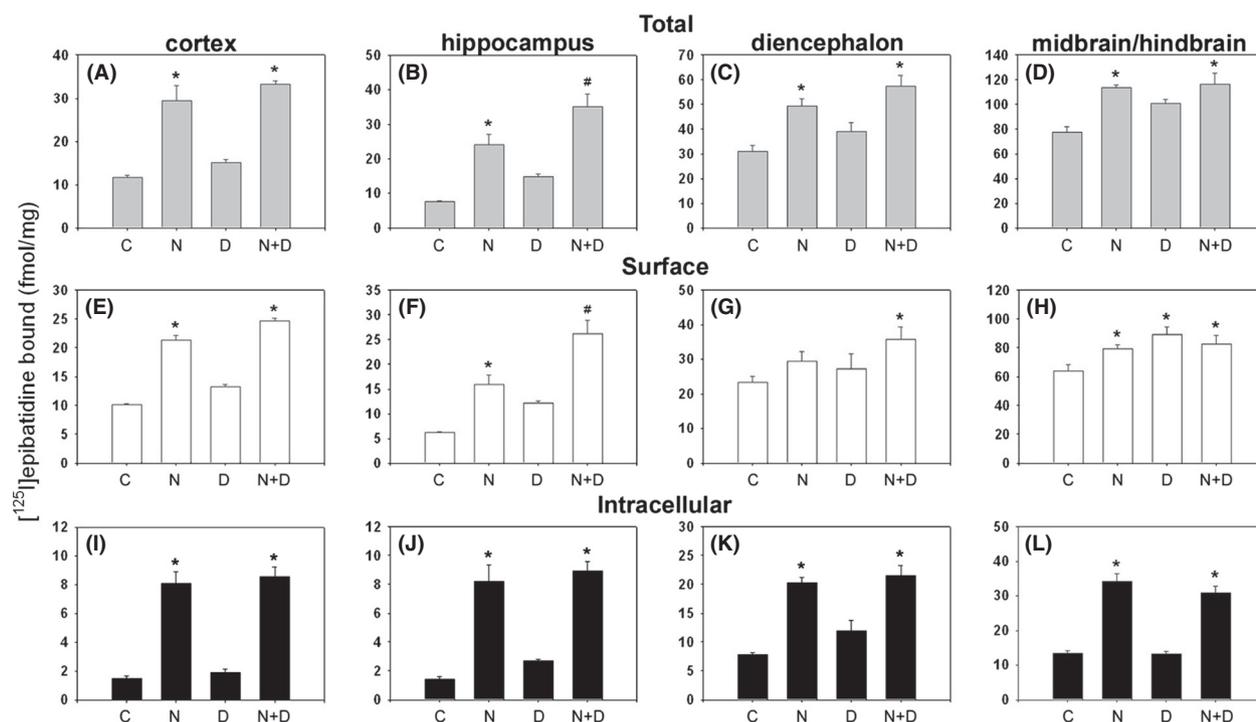


Figure 4. Comparison of chronic nicotine and dihydro- β -erythroidine on [125 I]epibatidine-binding sites. Primary neuronal cultures (12–14 days in culture) prepared from cortex, hippocampus, diencephalon and midbrain/hindbrain regions were treated with 1 μ mol/L nicotine, 10 μ mol/L dihydro- β -erythroidine or the combination of both drugs for 96 h. Specific total (A–D), surface (E–H) and intracellular (I–L) [125 I]epibatidine binding was determined. C, N, D, and N+D in the graphs represent control, 1 μ mol/L nicotine, 10 μ mol/L dihydro- β -erythroidine, and 1 μ mol/L nicotine plus 10 μ mol/L dihydro- β -erythroidine, respectively. Data are presented as mean \pm SEM of 8–9 replicates obtained from 3 independent experiments. Asterisks indicate significant differences from control. Pound symbols indicate significant differences between 1 μ mol/L nicotine and 1 μ mol/L nicotine plus 10 μ mol/L dihydro- β -erythroidine cotreatment.

dihydro- β -erythroidine alone tended to increase ($103 \pm 34\%$), but was not significantly different from control (Tukey test, $P > 0.05$). However, the increase in total [125 I]epibatidine after treatment with nicotine plus dihydro- β -erythroidine of $391 \pm 105\%$ was significantly greater than that observed following treatment with nicotine alone (Tukey test, $P < 0.05$).

Except for some relatively subtle quantitative differences, the patterns for the effects of chronic nicotine and/or dihydro- β -erythroidine on [125 I]epibatidine-binding sites on the cell surface of each of the four regions are similar to those reported above for total [125 I]epibatidine binding (Fig. 4E–H). Chronic nicotine treatment increases [125 I]epibatidine binding, while in general chronic dihydro- β -erythroidine treatment has less effect. With the exception of hippocampus, [125 I]epibatidine binding following cotreatment with nicotine and dihydro- β -erythroidine is not significantly higher than binding following treatment with nicotine alone (in hippocampus, $168 \pm 60\%$ increase following nicotine alone and $354 \pm 97\%$ increase following nicotine plus dihydro- β -erythroidine).

The pattern of increase in intracellular [125 I]epibatidine binding was similar for all four brain regions (Fig. 4I–L).

Chronic dihydro- β -erythroidine treatment did not change intracellular binding from that of controls. Nicotine treatment significantly increased binding in all four brain regions (main effect of nicotine: cortex, $F_{1,30} = 84.02$, $448 \pm 91\%$ increase; hippocampus, $F_{1,18} = 83.50$, $498 \pm 172\%$ increase; diencephalon $F_{1,25} = 91.38$, $158 \pm 16\%$ increase; midbrain/hindbrain, $F_{1,28} = 147.93$, $155 \pm 22\%$ increase, $P < 0.001$ for all brain regions). Cells treated with nicotine plus dihydro- β -erythroidine did not differ from cells treated with nicotine alone.

Brefeldin A treatment blocks upregulation of [125 I]epibatidine binding in cultured cortical neurons

Vesicular trafficking between ER and Golgi organelles has previously been shown to be necessary for $\alpha 4\beta 2$ -nAChR upregulation at the cell surface but not at the ER of HEK293 cells transfected with $\alpha 4$ and $\beta 2$ nAChR subunits (Darsow et al. 2005). Brefeldin A is a fungal antibiotic that blocks the transport of proteins from the endoplasmic reticulum to the Golgi system (Lippincott-Schwartz et al. 1989). In our studies, 1 μ mol/L brefeldin A was

used for 24 h to observe the role of the protein secretory pathway on nicotine and mecamylamine-induced $\alpha 4\beta 2^*$ nAChR upregulation.

Consistent with the results reported in Figure 1 for cultured cortical neurons, chronic treatment with nicotine significantly increased total binding of [125 I]epibatidine (Fig. 5A, $F_{6,81} = 15.32$, $P < 0.001$), and intracellular binding (Fig. 5C, $F_{6,81} = 15.83$, $P < 0.001$) but not in the neuronal surface compared to control DMSO-treated neurons. In contrast, the increases following treatment with nicotine plus mecamylamine were observed not only in total [125 I]epibatidine binding (Fig. 5A, $F_{6,81} = 15.32$, $P < 0.001$) and intracellular binding (Fig. 5C, $F_{6,81} = 15.83$, $P < 0.001$), but also for binding on the neuronal surface (Fig. 5B, $F_{6,81} = 9.88$, $P < 0.001$) compared to control DMSO-treated neurons. Chronic treatment with mecamylamine alone did not significantly alter either total, cell surface or intracellular binding compared to control DMSO-treated neurons.

Brefeldin A treatment decreased upregulation induced by nicotine treatment. A significant effect of brefeldin A on nicotine-induced upregulation of total [125 I]epibatidine binding (Fig. 5A, $F_{6,81} = 15.32$, $P < 0.001$) appeared to be primarily a consequence of its blockade of the increase in intracellular [125 I]epibatidine binding (5C, $F_{6,81} = 15.83$, $P < 0.001$) since no significant effect on [125 I]epibatidine binding on the surface was observed at 24 h of nicotine treatment. Brefeldin A treatment also blocked upregulation of total [125 I]epibatidine binding induced by nicotine plus mecamylamine (Fig. 5A, $F_{6,81} = 15.32$, $P < 0.001$) in both surface (Fig. 5B, $F_{6,81} = 9.88$, $P < 0.001$) and intracellular [125 I]epibatidine binding (Fig. 5C, $F_{6,81} = 15.83$, $P < 0.001$). In summary, the synergistic effect of mecamylamine and nicotine on surface [125 I]epibatidine binding requires endoplasmic reticulum to Golgi vesicle trafficking.

Discussion

The results presented here provide new information about the effects of antagonist exposure on nAChR expression and confirm previous observations of nAChR regulation by chronic nicotine in primary neuronal cultures.

We and others have observed that a larger proportion of nAChR are expressed at the cell surface of primary neuronal cultures than are expressed in cell lines transfected with nAChR (Whiteaker et al. 1998; Davila-Garcia et al. 1999; Lomazzo et al. 2011; Zambrano et al. 2012). More than 70% of the total binding sites are located on the cell surface of each of the four brain areas examined. This high expression of surface receptors no doubt reflects the fact that primary neuronal cultures develop an extended network of neurites in culture that greatly

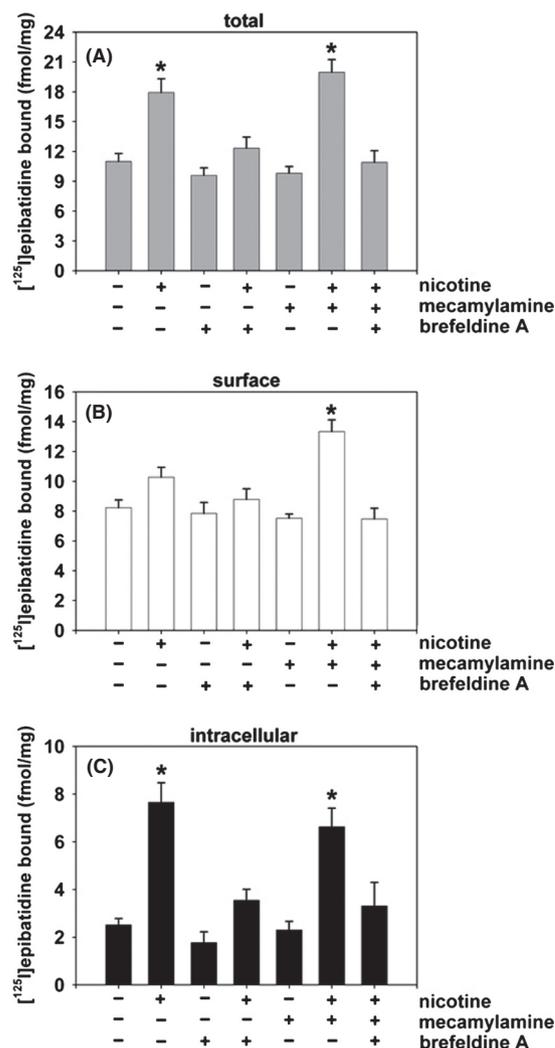


Figure 5. Effect of vesicular trafficking on mecamylamine and nicotine-induced [125 I]epibatidine-binding upregulation. Primary neuronal cultures (12–14 days in culture) prepared from cortex were treated for 24 h with 1 μ mol/L nicotine, 10 μ mol/L mecamylamine, 1 μ mol/L brefeldin A, or combination of those drugs treatments. Specific total (A), surface (B) and intracellular (C) [125 I]epibatidine binding was determined. Data are presented as mean \pm SEM of 9–18 replicates obtained from 3–4 independent experiments. (B) Brefeldin A treatments blocked the upregulation of surface [125 I]epibatidine sites up-regulated by nicotine and mecamylamine cotreatment. (C) Brefeldin A also blocked the upregulation of intracellular [125 I]epibatidine sites elicited by nicotine alone or in combination with mecamylamine. (A) As a result, upregulation of total [125 I]epibatidine sites elicited by nicotine alone or in combination with mecamylamine are blocked by Brefeldin A. Asterisks in A and C indicate significant differences between nicotine group or nicotine mecamylamine cotreatment group compared to control, mecamylamine and all brefeldin A groups determined by one way ANOVA followed by Tukey post hoc test. Asterisk in B denotes a significant difference of nicotine mecamylamine cotreatment compared to all other group treatments determined by one way ANOVA followed by Tukey post hoc test.

expands the surface area of each cell. The response of cultured neurons to chronic nicotine treatment is consistent with responses observed *in vivo*. Particularly noteworthy, is the observation that the extent of upregulation of nAChR observed for cells derived from different brain regions varies significantly, a result that is in agreement with what has previously been reported for primary rat neurons in culture (Davila-Garcia *et al.* 1999). Neurons prepared from diencephalon and midbrain/hindbrain displayed a modest upregulation after nicotine chronic treatments, consistent with analogous brain regions in rodents chronically treated with nicotine (Marks *et al.* 1983, 2004, 2011; Flores *et al.* 1992; Sanderson *et al.* 1993; Sparks and Pauly 1999; Nguyen *et al.* 2003). The parallels between nAChR regulation *in vivo* and in primary cell culture suggest that similar mechanisms may be regulating nicotine-induced upregulation *in vivo* and *in vitro*. Chronic nicotine treatment tended to elicit an increase in the density of receptors expressed on the cell surface. In addition, all regions displayed a substantially greater increase in the intracellular pool of [¹²⁵I]epibatidine-binding sites.

Although there is agreement that chronic agonist treatment elicits an upregulation of $\alpha 4\beta 2^*$ -nAChR, receptor upregulation has not been uniformly observed following chronic antagonist treatment. For example, a significant increase in total specific [³H]nicotine binding was observed following chronic mecamlamine treatment by constant infusion for 10 days in mice; cotreatment with nicotine and mecamlamine elicited an additive extent of upregulation (Collins *et al.* 1994; Pauly *et al.* 1996). Two studies using rats investigated the effect of mecamlamine on nAChR upregulation. Twice daily coadministration of nicotine and mecamlamine elicited a larger upregulation in nAChR-binding sites than was observed in rats injected twice daily with nicotine alone, a result similar to that for mice (McCallum *et al.* 2000). In contrast, no effect of chronic mecamlamine treatment was observed with once per day injections (Schwartz and Kellar 1985). Differences in attaining a threshold concentration of the drug necessary to produce nAChR upregulation or the necessity for coadministration of nicotine and mecamlamine when given by injection could account for these disparities.

The effect of mecamlamine treatment has also been studied in cell lines. Three independent studies in cell lines transfected with $\alpha 4\beta 2$ nAChR and exposed to nicotine concentrations ranging from 500 to 1 mmol/L found no effect of mecamlamine cotreatment, even though relatively high mecamlamine concentrations were used (Gopalakrishnan *et al.* 1997; Darsow *et al.* 2005; Kuryatov *et al.* 2005). These differential responses *in vivo* and *in vitro* suggest that endogenous nAChR stimulation by ACh or exogenous stimulation by nicotine may be necessary to elicit receptor regulation following mecamlamine

treatment. Our results support this suggestion. While we observed that chronic treatment with mecamlamine alone had no measurable effect on [¹²⁵I]epibatidine binding, [¹²⁵I]epibatidine binding following cotreatment with nicotine and mecamlamine was significantly higher than that following treatment with nicotine alone. Furthermore, the elevated nAChR expression observed following cotreatment was confined to the cell surface reinforcing the proposal that different mechanisms are responsible for the upregulation elicited by nicotine and mecamlamine treatment. A synergistic interaction between nicotine and mecamlamine has also been reported for transfected M10 cells expressing $\alpha 4\beta 2$ nAChR; the addition of nicotine to the transfected cells chronically treated with mecamlamine increased [³H]-nicotine binding (Peng *et al.* 1994). It should also be noted that treatment of these cells with high concentrations of mecamlamine alone increased receptor expression. It is not obvious why different responses to the combination of nicotine and mecamlamine are reported for cultured rat neurons (no effect observed in Davila-Garcia *et al.* 1999) and cultured mouse neurons (increased response with nicotine and mecamlamine, current study). In addition to the different species used to obtain the embryonic primary neuronal cultures both studies (Davila-Garcia *et al.* 1999 and present study) differ in the extent (4 days versus 7 days of chronic nicotine treatment, respectively) and nicotine concentration (1 μ mol/L vs. 10 μ mol/L, respectively) but used the same 10 μ mol/L smecamlamine concentration. Perhaps by using higher nicotine concentration for an extended period of time neurons may have attained a maximal nAChR upregulation. Consequently, additional upregulation by the antagonist may not be possible.

We observed that chronic treatment with the competitive antagonist, dihydro- β -erythroidine, elicited an upregulation of cell surface [¹²⁵I]epibatidine binding in midbrain/hindbrain cultures and did not block the response to nicotine. Total and surface [¹²⁵I]epibatidine binding in hippocampus following cotreatment with nicotine and dihydro- β -erythroidine was significantly higher than that observed with either drug alone. This increased binding following cotreatment likely occurs because the upregulation following treatment with 1 μ mol/L nicotine is not maximal. As was the case with chronic mecamlamine plus nicotine treatment, the increase in [¹²⁵I]epibatidine binding following dihydro- β -erythroidine treatment was confined to the cell surface. In agreement to our results, no effect of dihydro- β -erythroidine was observed in rat primary cortical neuronal cultures treated chronically (8 days) with 10 μ mol/L nicotine (Davila-Garcia *et al.* 1999). The authors postulated that the lower potency of the competitive inhibitor relative to nicotine

resulted in insufficient occupancy necessary to produce nAChR upregulation. The lower potency of a nAChR antagonist to produce nAChR upregulation was also documented for HEK cells transfected with the human $\alpha 4\beta 2$ nAChR. In this system, the EC_{50} value for upregulation of [3H]cytisine-binding sites was 107 $\mu\text{mol/L}$ for dihydro-Q-erythroidine compared to 0.49 $\mu\text{mol/L}$ for nicotine (Gopalakrishnan *et al.* 1997). In contrast, it was reported that intracerebral injection with a high concentration of dihydro- β -erythroidine did produce upregulation of specific [3H]cytisine binding, although the increase was smaller than that elicited by methylcarbachol. [3H]Cytisine-binding following coadministration of dihydro- β -erythroidine and methylcarbachol was reduced to the level found after administration of dihydro- β -erythroidine alone, a result consistent with the competitive effect of these drugs (Yang and Buccafusco 1994).

The effect of both inhibitors in this study was consistently found for [^{125}I]epibatidine-binding sites preferentially located in the cell surface. Altered receptor trafficking is one possible mechanism or increased stability of receptors at the surface is a second possibility. The role of vesicle trafficking has previously been studied in HEK293T cells transiently transfected with $\alpha 4\beta 2$ nAChR (Darsow *et al.* 2005). Upregulation of surface binding by nicotine was prevented when the exocytic traffic at the endoplasmic reticulum was blocked by brefeldin A. However, the total binding was unaffected, prompting the conclusion that all the upregulated receptors remained at the endoplasmic reticulum. We examined the effects of brefeldin A on receptor expression to study the role of trafficking in our system. Our data show that exposure of cultured cortical neurons to 1 $\mu\text{mol/L}$ nicotine for 24 h produces a modest upregulation of [^{125}I]epibatidine binding in the surface that was not significantly changed by brefeldin A treatment. However, brefeldin A treatment reduced the upregulation of intracellular [^{125}I]epibatidine binding elicited by nicotine treatment. Furthermore, the addition of brefeldin A prevents the increase of both the surface and intracellular [^{125}I]epibatidine binding elicited by nicotine and mecamylamine cotreatment for 24 h. The decrease in surface binding by brefeldin A may be due to a decreased trafficking of receptors from the endoplasmic reticulum. This potential change is consistent with the results with HEK cells transfected with $\alpha 4\beta 2$ -nAChR (Darsow *et al.* 2005). However, in contrast to the accumulation of receptors at the endoplasmic reticulum reported for the transfected cells following brefeldin A treatment, the intracellular binding in primary cultures decreased. This somewhat unexpected decrease of intracellular [^{125}I]epibatidine binding, may arise by degradation of newly synthesized nAChR through the ubiquitin-proteasome system that has previously been reported to

metabolize $\alpha 3$, $\beta 2$ and $\beta 4$ nAChR subunits (Rezvani *et al.* 2010) rather than retention in the endoplasmic reticulum. Brefeldin A treatment (1 micro mol/L) for 96 h was also tested and was toxic to neurons. This toxicity prevented longer term analysis of brefeldin A effects.

In conclusion, the results reported here indicate that both mecamylamine and dihydro- β -erythroidine modulate upregulation of nAChR-binding sites only on the surface of neurons. Upregulation by mecamylamine requires interaction of receptors with nicotine while dihydro- β -erythroidine was able to produce upregulation in the absence of nicotine. Additionally, our results suggest that exocytic trafficking is required for both agonist and antagonist upregulation of nAChR. If the effect of mecamylamine on surface $\alpha 4\beta 2^*$ nAChR is blocked by brefeldin A by decreasing the trafficking of receptors to the cell surface or alternatively by blocking the trafficking of a different protein that stabilizes receptors at the plasma membrane, as reported before for the scaffold protein 14-3-3 η (Jeanclos *et al.* 2001), remains to be established.

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Authors Contribution

Zambrano, Marks and Grady participated in the research design. Zambrano, Short and Salamander conducted the experiments. Zambrano and Marks performed the data analysis. Zambrano, Marks, Short, and Grady wrote or contributed to the writing of the manuscript.

Disclosures

None declared.

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