# Regulation of the Distribution and Function of [$^{125}$I]Epibatidine Binding Sites by Chronic Nicotine in Mouse Embryonic Neuronal Cultures

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Received January 27, 2012; accepted April 23, 2012

## ABSTRACT

Chronic nicotine produces up-regulation of $\alpha4\beta2^*$ nicotinic acetylcholine receptors (nAChRs) (* denotes that an additional subunit may be part of the receptor). However, the extent of up-regulation to persistent ligand exposure varies across brain regions. The aim of this work was to study the cellular distribution and function of nAChRs after chronic nicotine treatment in primary cultures of mouse brain neurons. Initially, high-affinity [$^{125}$I]epibatidine binding to cell membrane homogenates from primary neuronal cultures obtained from diencephalon and hippocampus of C57BL/6J mouse embryos (embryonic days 16–18) was measured. An increase in $\alpha4\beta2^*$-nAChR binding sites was observed in hippocampus, but not in diencephalon, after 24 h of treatment with 1 $\mu$M nicotine. However, a nicotine dose-dependent up-regulation of approximately 3.5- and 0.4-fold in hippocampus and diencephalon, respectively, was found after 96 h of nicotine treatment. A significant fraction of total [$^{125}$I]epibatidine binding sites in both hippocampus (45%) and diencephalon (65%) was located on the cell surface. Chronic nicotine (96 h) up-regulated both intracellular and surface binding in both brain regions without changing the proportion of those binding sites compared with control neurons. The increase in surface binding was not accompanied by an increase in nicotine-stimulated $Ca^{2+}$ influx, suggesting persistent desensitization or inactivation of receptors at the plasma membrane occurred. Given the differences observed between hippocampus and diencephalon neurons exposed to nicotine, multiple mechanisms may play a role in the regulation of nAChR expression and function.

## Introduction

Nicotinic acetylcholine receptors (nAChRs) expressed in mammalian brain are ligand-gated ion channels assembled as pentamers composed of $\alpha$ ($\alpha2$-$\alpha7$) and $\beta$ ($\beta2$-$\beta4$) subunits. Major receptor subtypes in the central nervous system are homomeric $\alpha7^*$-nAChRs and heteromeric $\alpha4\beta2^*$-nAChRs (* denotes that an additional subunit may be part of the receptor) (Lukas et al., 1999). A lower density of $\alpha3\beta4^*$- and $\alpha6\beta2^*$- nAChR subtypes is also found in some brain regions (Baddick and Marks, 2011).

Chronic nicotine exposure induces up-regulation of nAChRs in mice (Marks et al., 1983), rats (Schwartz and Kellar, 1983), transfected oocytes (Fenster et al., 1999), stably transfected fibroblasts (Peng et al., 1994, 1997; Bencherif et al., 1995; Warpman et al., 1998; Whiteaker et al., 1998; Gentry et al., 2003), and embryonic neurons in culture (Bencherif et al., 1995; Dávila-García et al., 1999; Nashmi et al., 2003; Lomazzo et al., 2011; Govind et al., 2012). [$^3$H]nicotine binding sites are also increased in smokers. This increase is caused by an increase in $B_{max}$ with no change in $K_D$ (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). Some brain regions (e.g., thalamus) are less responsive to chronic nicotine-induced up-regulation than regions (e.g., hippocampus or cortex) where up-regulation occurs even after treatment with low doses of nicotine (Pauly et al., 1991; Flores et al., 1997; Sparks and Pauly, 1999; Nguyen et al., 2003; Marks et al., 2004).

Even though up-regulation of nAChRs occurs after chronic nicotine treatment of mice, function measured by $^{86}Rb^+$ ef-

flux or [³H]dopamine release from synaptosomal preparations remains at basal control levels or even decreases in a dose-dependent manner (Marks et al., 1993, 2004). The apparent decrease in receptor function produced by chronic ligand exposure may arise from ligand-induced persistent desensitization or inactivation of nAChRs (Peng et al., 1994; Gentry et al., 2003). However, it is also possible that the population of receptors that are increased by nicotine treatments remain in intracellular compartments such that receptors at the plasma membrane are unaltered or even reduced. This last hypothesis seems unlikely because of a recent report that demonstrates that receptors at the plasma membrane of neurons in primary culture are also up-regulated by chronic nicotine treatments (Lomazzo et al., 2011), similar to the observation with cells transfected with α4 and β2 nAChR subunits (Peng et al., 1994; Whiteaker et al., 1998). However, it has also been reported that chronic nicotine treatment increases total nAChR function as a consequence of the increased receptor expression in cell lines (Gopalakrishnan et al., 1996; Buisson et al., 2000), rat synaptosomes (Nguyen et al., 2004), and midbrain neurons (Nashmi et al., 2003, 2007).

Most of the studies on nAChR cellular distribution used cell lines expressing native or transfected receptors. An alternative approach, using embryonic neurons in culture that express native nAChRs, has the advantage of investigating cells that undergo differentiation and express several markers of mature neurons (Kaech and Banker, 2006). For example, primary cultures of hippocampal neurons express α7*- and α4β2*-nAChRs and show nicotinic-induced currents after 10 days in culture (Zarei et al., 1999). Furthermore, primary neurons in culture chronically treated with nicotine up-regulate nAChRs (Bencherif et al., 1995; Dávila-García et al., 1999; Nashmi et al., 2003; Lomazzo et al., 2011; Govind et al., 2012). Few studies have addressed the resulting functionality of the receptors expressed on neurons in primary culture after prolonged agonist exposure, although nAChR function has been measured after chronic nicotine exposure in cells expressing α4β2-nAChRs heterologously (Peng et al., 1994; Gopalakrishnan et al., 1996; Buisson et al., 2000; Gentry et al., 2003).

The current study investigates the regulation of the distribution and function of nAChRs in primary neuronal cultures isolated from hippocampus and diencephalon of mouse embryos. The effect of nicotine treatments on receptor density and distribution at surface and intracellular membranes was measured. We report here that cells prepared from hippocampus and diencephalon exhibit differences in nicotine-induced up-regulation measured by [¹²⁵I]epibatidine binding. The ratio between intracellular and surface receptors in both brain regions is unchanged by nicotine treatment. Moreover, receptor function assessed by fluorometric intracellular $Ca^{2+}$ detection is not increased by chronic nicotine treatment, indicating that the up-regulated receptors are less functional, consistent with the results obtained in mice chronically treated with nicotine (Marks et al., 1993, 2004). Differences in the regulation of ligand binding and receptor function obtained for hippocampus and diencephalon neurons after chronic nicotine treatment indicate that multiple mechanisms are responsible for the regulation of nAChRs.

## Materials and Methods

**Materials.** [¹²⁵I]epibatidine (2200 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). 2-(2-Bromoacetyloxy)-N,N,N-trimethylethanaminium bromide (BrACh), cytisine, (−)-nicotine hydrogen tartrate, polyethyleneimine, cytosine-β-D-arabino-furanoside, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 1,4-dithio-DL-threitol (DTT), methylcarbachol hydrochloride (MCC), and poly-L-lysine (>30,000 kDa) were purchased from Sigma (St. Louis, MO). HEPES half-sodium salt was from Roche Diagnostics (Indianapolis, IN). Neurobasal media, minimal essential medium, B27 supplement, Fluo4-NW calcium assay, Glutamax supplement, heat-inactivated horse serum, and TrypLE were purchased from Invitrogen (Carlsbad, CA).

**Primary Neuronal Cultures.** All experiments were approved by and carried out in accordance with the University of Colorado's *Guide for the Care and Use of Experimental Animals*. Primary cultures from embryonic mouse brains (embryonic days 16–18) were established as described previously (Kaech and Banker, 2006) with some modifications. The whole brain was placed in HBSS ($Ca^{2+}$ and $Mg^{2+}$ free) buffer (0.407 mM; 5.33 mM KCl, 0.441 mM $KH_2PO_4$, 4.17 mM $NaHCO_3$, 137.93 mM NaCl, 0.338 mM $Na_2HPO_4$, and 5.56 mM D-glucose) and separated from the meninges. Hippocampus and diencephalon (subcortical areas including thalamus, hypothalamus, and caudate nucleus) were dissected and minced into small pieces. Brain tissue was rinsed once with HBSS ($Ca^{2+}$ and $Mg^{2+}$ free) buffer and incubated with 0.5× TrypLE diluted in HBSS ($Ca^{2+}$ and $Mg^{2+}$ free) buffer for 15 min at 37°C. Tissue was mechanically dissociated through a heat-polished Pasteur pipette at room temperature. The cell suspension was centrifuged at 800g for 2 min and then resuspended in minimal essential medium supplemented with 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 50,000 to 75,000 cells/cm² over polystyrene plates coated with 0.1 mg/ml poly-L-lysine prepared in 0.1 M borate buffer, pH 8.5. After 24 h at 37°C, media were 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 mM L-glutamine). On the second day of culture, 10 μM cytosine-β-D-arabino-furanoside was added for 72 h at 37°C incubation to control the proliferation of glial cells. Cultures were kept in a humidified 5% $CO_2$-95% air incubator at 37°C. A nicotine stock (10 mM) was prepared fresh for every experiment by using maintenance medium and for dilutions. Chronic nicotine treatments were begun 12 to 14 days after plating. Depending on the experiment, cells received nicotine concentrations of 0.001 to 10 μM only once at the beginning of either a 24- or 96-h chronic treatment period at 37°C incubation.

**Preparation of Total Membranes.** After treatments were completed, neurons in culture were rinsed once with KRH buffer (144 mM NaCl, 2.2 KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM HEPES, pH 7.5) and then collected in 0.1× hypotonic KRH buffer by scraping the plate surface, triturated with an ultra turrax, and centrifuged at 25,000g for 15 min at 4°C. The pellets were washed four times by resuspension in ice-cold hypotonic KRH buffer followed by centrifugation. Cell membranes were resuspended in distilled-deionized water for the binding reaction (if carried out immediately) or hypotonic binding buffer and frozen at −70°C until assayed.

**[¹²⁵I]Epibatidine Binding to Cell Membrane Homogenates.** [¹²⁵I]epibatidine binding was measured as described previously (Whiteaker et al., 2000). Frozen washed pellets were resuspended in the overlying buffer and centrifuged at 25,000g for 15 min at 4°C. The supernatant was discarded, and the pellet was resuspended in ice-cold water. Resuspension volumes varied among samples cultured to adjust protein concentrations such that less than 10% of the [¹²⁵I]epibatidine was bound to the protein at the highest ligand concentration. Samples (5–20 μg of protein) were incubated in 96-well polystyrene plates for 2 h at 22°C in KRH buffer. Final incubation volume was 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.) at 4°C onto glass fiber filters that had been treated for 10 min with 0.5% polyethelenimine [top filter, MFS Type B (Advantec, Dublin, CA); bottom filter, Pall type A/E (Pall Life Sciences, Port Washington, NY)]. An Inotech Cell Harvester (Inotech Biosystems, 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 was counted at 80% efficiency by using a Packard Cobra Auto-Gamma Counter (PerkinElmer Life and Analytical Sciences). For all experiments, nonspecific binding was measured by including 100 μM cytisine in the incubation medium. All cultured brain regions from different treatment groups were assayed for binding by using 200 pM [$^{125}$I]epibatidine. Because [$^{125}$I]epibatidine binds with high affinity to several different nAChR subtypes (Whiteaker et al., 2000), differential inhibition by cytisine (50 and 150 nM) was used to distinguish two binding sites: cytisine-sensitive sites comprising α4β2* and cytisine-resistant sites representing a mixed population of receptors including α3β4* (for review see Marks et al., 2010).

**Alkylation of Cell Surface nAChR.** To evaluate the effect of chronic nicotine treatment on surface and intracellular binding sites, cells were treated as described above with 0 or 1 μM nicotine for 24 or 96 h, followed by alkylation of surface nAChR as described previously with some modifications (Free et al., 2005). After experimental treatment, primary neurons in culture were rinsed once with HBSS buffer, pH 7.4 (1.26 mM CaCl$_2$, 0.493 mM MgCl$_2$, 0.407 mM MgSO$_4$, 5.33 mM KCl, 0.441 mM KH$_2$PO$_4$, 4.17 mM NaHCO$_3$, 137.93 mM NaCl, 0.338 mM Na$_2$HPO$_4$, and 5.56 mM D-glucose), supplemented with 20 mM HEPES and 5 mM glucose, and then treated for 15 min at 37°C with 1 mM DTT prepared in the same buffer. Cultures were rinsed once with HBSS followed by 6-min incubation with 100 μM BrACh prepared in HBSS at 22°C. After rinsing with HBSS, the reaction was completed by adding 1 mM DTNB in HBSS for 15 min at 37°C. After the alkylation reaction, the neurons were rinsed once with HBSS, lysed with hypotonic ice-cold KRH buffer, and scraped from the plate. A set of cultures treated as described above, but omitting the BrACh incubation, was used to measure total [$^{125}$I]epibatidine binding. Whole particulate membranes were prepared as described above, and [$^{125}$I]epibatidine binding was subsequently measured by using the radioligand binding assay. Surface binding was calculated as the difference between total binding (no incubation with BrACh) and binding after receptor alkylation (intracellular).

**[$^{125}$I]Epibatidine Binding to Intact Neurons in Culture.** MCC, a quaternary amine that is poorly permeable across membranes, was used to quantify the density of receptors present on the surface of neurons in culture (Whiteaker et al., 1998). Neurons were assayed for total and MCC-resistant [$^{125}$I]epibatidine binding, which represents remaining binding at the internal pool of receptors. Surface binding was calculated by subtracting MCC-resistant from total specific [$^{125}$I]epibatidine binding. To establish optimal binding conditions several preliminary experiments were conducted. For the association kinetics, intact cells were incubated with 200 pM [$^{125}$I]epibatidine at 22°C for 0.5-, 1-, 2.5-, 5-, 10-, 20-, 40-, and 60-min reaction to determine time of attainment of equilibrium binding. MCC competition binding was done to establish a concentration of this ligand that produces maximal inhibition of [$^{125}$I]epibatidine binding. MCC concentrations used were 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM in a 20-min binding reaction. Counts were obtained by harvesting the cells and washing the filtered material with ice-cold KRH buffer. Nonspecific binding values were obtained by adding 100 μM nicotine to the binding reaction. Total binding was measured for samples containing no MCC. To evaluate the effect of chronic nicotine treatment on MCC-sensitive (surface) and MCC-resistant (intracellular) binding sites, cells were treated with 0, 0.001, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 30 μM nicotine for 96 h at 37°C. At the completion of the chronic treatment, cells were washed four times by incubation with mainte-

nance medium for 10 min at 37°C. Subsequently, cells were incubated for 20 min with 200 pM [$^{125}$I]epibatidine and either 0 or 1 μM MCC at room temperature. Cells were harvested and radioactivity was measured as described above.

**Ca$^{2+}$ Influx Determination.** Neurons prepared as described above were plated in 96-well plates coated with 1 mg/ml poly-L-lysine (optical bottom, black plates; Nalge Nunc International, Rochester, NY) and maintained in culture at 37°C for 12 to 14 days until drug treatments began. Cells were treated with 0, 0.001, 0.01, 0.1, 1, or 10 μM nicotine for 96 h at 37°C. After the nicotine treatment, cells were rinsed four times with maintenance media at 37°C for 10 min. Cells were then loaded with 80 μl of Fluo4-NW by incubation for 1 h at 37°C with 5% CO$_2$ (cell culture incubator) followed by a 15-min incubation in the dark at 22°C according to the manufacturer's instructions. HBSS buffer supplemented with 20 mM HEPES and 5.5 mM glucose was used to prepare Fluo4-NW and nicotine solutions. Fluorescence was measured in a plate reader model VICTOR (PerkinElmer Life and Analytical Sciences). Samples were excited at 485 nm, and emitted fluorescence was recorded at 535 nm every second after nicotine addition. Data are presented as area under the curve during 60-s readings and normalized by total Ca$^{2+}$ influx after cell lysis with 1% Triton X-100. Nicotine-stimulated fluorescence was determined by subtracting basal fluorescence values, obtained by adding 20 μl of HBSS-HEPES-glucose buffer, from the values obtained by adding 20 μl of a 5× nicotine stock. Dose-response curves were generated by using 0.001, 0.01, 0.1, 1, and 10 μM nicotine. A concentration of 10 μM nicotine achieved maximal response and was used to test the effect of chronic nicotine treatment.

**Data Calculations.** Cytisine-sensitive and cytisine-resistant [$^{125}$I]epibatidine binding sites were calculated by 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 cytisine concentration, $I$, $B_1$ and $B_2$ are the [$^{125}$I]epibatidine binding with IC$_{50}$ values IC$_{50-1}$ (cytisine-sensitive sites) and IC$_{50-2}$ (cytisine-resistant sites), respectively. IC$_{50-1}$ and IC$_{50-2}$ values used were 3.75 and 300 nM. Densities of cytisine-sensitive and cytisine-resistant sites were calculated from the [$^{125}$I]epibatidine binding measured with 0, 50, and 150 nM cytisine.

[$^{125}$I]Epibatidine displacement binding assays were performed in situ and in cell membrane preparations. Inhibition of binding to membranes was calculated by using a one-site inhibition model: $B_L = B/(1 + L/IC_{50})$. For MCC inhibition binding assays with intact cells, the following formula was used to calculate inhibitable and residual binding: $B_L = (B/(1 + L/IC_{50})) + B_R$, where $B_L$ is [$^{125}$I]epibatidine binding measured at any concentration of inhibitor, $L$, and $B$ represents binding sites sensitive to the inhibition with an apparent IC$_{50}$ value. $B_R$ corresponds to the uninhibited residual binding fraction. The inhibition constant $K_i$ was determined by using the Cheng and Prussoff equation (IC$_{50} = K_i^*(1 + L/K_d)$ (Cheng and Prusoff, 1973), where $L$ is the concentration of [$^{125}$I]epibatidine, and $K_d$ is the average high-affinity binding constant calculated from saturation binding experiments for the concentration of [$^{125}$I]epibatidine used in a specific experiment.

The association of [$^{125}$I]epibatidine binding in intact neurons was modeled by a double exponential increase as described previously (Lippiello et al., 1987) by using the following equation: $B_t = B_{fast}(1 - e^{-kfastt}) + B_{slow}(1 - e^{-kslowt})$ where $B_{fast}$ and $B_{slow}$ are the two states of the receptor with high and low rates of association, and $B_t$ is the total binding at time t.

The Hill equation was used to determine EC$_{50}$ and Hill coefficients for Ca$^{2+}$ influx experiments. SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA) was used for calculations and graphical presentation of the data. Statistical analyses were conducted by using Sigma Stat (Systat Software, Inc.). Specific analyses are described in the figure legends.

## Results

**Binding to Membrane Homogenates after Chronic Nicotine Treatment.** Neurons in culture were allowed to differentiate for at least 12 days before any drug treatment. This was done to assure that functional receptors were being expressed (Zarei et al., 1999). Binding of [$^{125}$I]epibatidine in untreated diencephalon cells (44.4 ± 1.7 fmol/mg protein) was significantly higher than in hippocampal cells (4.0 ± 0.3 fmol/mg protein). Chronic nicotine treatment for 96 h elicited a dose-dependent increase in total specific [$^{125}$I]epibatidine binding in both hippocampus neurons ($F_{4,34}$ = 45.5; $p$ < 0.001) and diencephalon neurons ($F_{4,33}$ = 95.8; $p$ < 0.001) (Fig. 1A; one-way ANOVA). $EC_{50}$ values for the nicotine-induced up-regulation of total specific [$^{125}$I]epibatidine binding in hippocampus and diencephalon were 180.7 ± 62.9 and 96.7 ± 39.8 nM, respectively. However, as illustrated in Fig. 1B, hippocampal cells exhibited significantly more up-regulation than did diencephalon cells (310.7 ± 42.1 and 77.2 ± 6.4% of control for 10 μM treatments in hippocampus and diencephalon, respectively).

Differential sensitivity to inhibition by cytisine can distinguish between the sites with relatively high affinity for cytisine (primarily α4β2*-nAChR sites) and those with relatively low affinity for cytisine (mixed nAChR sites) (Marks et al., 1998; Zoli et al., 1998; Whiteaker et al., 2000). The high-affinity [$^3$H]epibatidine binding sites in thalamus and hippocampus of mouse brain represent mostly α4β2*-nAChRs (Marks et al., 2010). To determine the effects of chronic treatment on these two components of [$^{125}$I]epibatidine binding sites, cultures of hippocampus and diencephalon cells were treated with 1 μM nicotine for 24 or 96 h.

After 24 h of 1 μM nicotine treatment, up-regulation of the cytisine-sensitive component (α4β2*-nAChR), but not the cytisine-resistant [$^{125}$I]epibatidine binding component (non-α4β2*, mixed receptor population including α3β4*-nAChR), in hippocampus was observed (Fig. 2A). In contrast, diencephalon cultures did not show a change in binding after a 24-h treatment (Fig. 2B). However, significant increases in cytisine-sensitive [$^{125}$I]epibatidine binding were observed for both hippocampus (Fig. 2A) and diencephalon (Fig. 2B) after a 96-h treatment with 1 μM nicotine. No change was observed for the cytisine-resistant component of [$^{125}$I]epiba-

tidine binding in hippocampus at any time point (Fig. 2A). However, the cytisine-resistant [$^{125}$I]epibatidine binding component showed a significant increase in diencephalon neurons after 96 h of treatment with nicotine (Fig. 2B).

**nAChR Alkylation.** One method to determine the distribution of the receptors in the plasma membrane versus intracellular membranes makes use of an alkylation technique reported previously for nicotinic receptors in chromaffin cells (Free et al., 2005). Neurons (12–14 days in culture) were treated with 1 μM nicotine for 24 and 96 h. After washing, samples were then treated with 1 mM DTT to reduce disulfide bonds and reacted with either 0 or 100 μM BrACh and 1 mM DTNB to reoxidize unreacted sulfhydryls. Neurons treated with DTT and DTNB, but not BrACh, provided a measurement of total [$^{125}$I]epibatidine binding. Samples treated with DTT, BrACh, and DTNB provided a measurement of the density of [$^{125}$I]epibatidine binding sites at both surface membranes (binding eliminated by BrACh treatment) and intracellular membranes (binding retained after BrACh treatment). Total specific [$^{125}$I]epibatidine binding in control hippocampus neurons (binding after DTT and DTNB treatment) was 6.3 ± 0.8 fmol/mg (Fig. 3A). After treatment with 1 μM nicotine for 24 and 96 h total specific [$^{125}$I]epibatidine binding in hippocampus was significantly higher than that of controls: 12.9* ± 1.6 and 27.7* ± 2.2 fmol/mg, respectively (Fig. 3A). The percentage of total [$^{125}$I]epibatidine binding sites expressed at the cell surface versus that in the intracellular pool was not significantly changed by treatment with nicotine in hippocampus [surface receptors: 57.8 ± 13.8% (0 h), 45.4 ± 9.0% (24 h), and 48.5 ± 11.2% (96 h)] (Fig. 3A). Total specific [$^{125}$I]epibatidine binding in control diencephalon neurons was 38.8 ± 2.8 fmol/mg protein (Fig. 3B). After treatment with 1 μM nicotine for 24 and 96 h [$^{125}$I]epibatidine binding site density was 37.1 ± 2.3 and 46.4* ± 3.3 fmol/mg, respectively (Fig. 3B). Statistically significant up-regulation was observed for diencephalon neurons treated for 96 h, but not 24 h. No differences in the percentage of surface receptors versus total receptors were noted on diencephalon cells after treatment with 1 μM nicotine for 96 h compared with untreated cells. However, there seemed to be a modest decrease in surface receptors as a percentage of total receptors after 24 h of nicotine treatment [surface receptors:
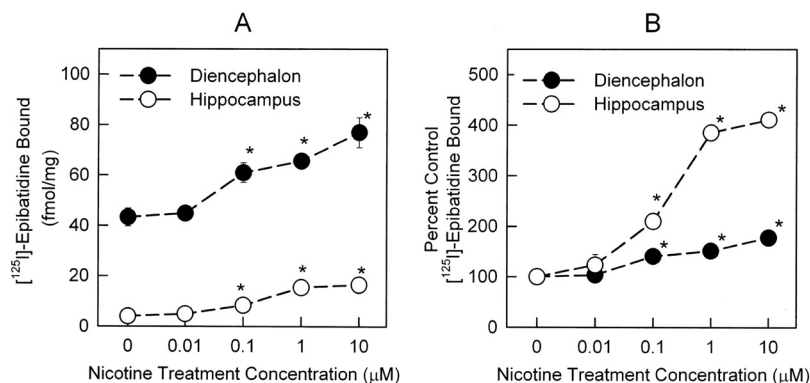


**Fig. 1.** Total specific [$^{125}$I]epibatidine binding in total membranes prepared from hippocampus and diencephalon cultures treated with nicotine for 96 h. Primary cultures of hippocampus (○) or diencephalon (●) were treated with nicotine concentrations of 0, 0.01, 0.1, 1, and 10 μM for 96 h. Binding was measured by using 200 pM [$^{125}$I]epibatidine in a 2-h binding reaction. Nonspecific binding was determined by including 100 μM cytisine in the binding reaction. A, total specific [$^{125}$I]epibatidine binding to the total membrane fractions prepared from the cultures. B, binding data transformed to percentage of control. Data represent mean ± S.E.M. of seven replicates from three independent experiments. Significant differences with respect to control (untreated) neurons were calculated by using one-way ANOVA, followed by Student-Newman-Keuls post hoc analysis. *, $p$ < 0.001.
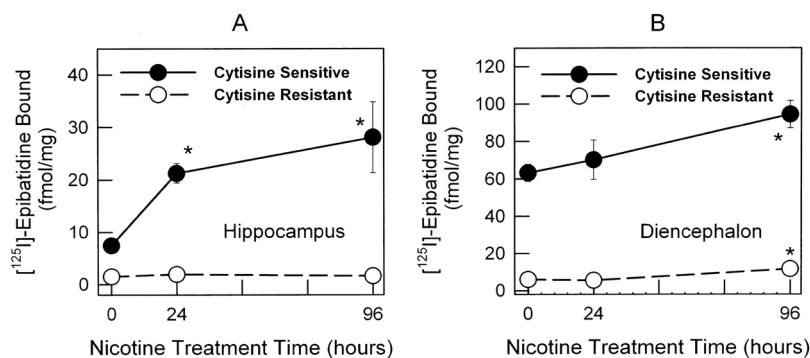
**Fig. 2.** Time course of the up-regulation of both cytisine-sensitive and cytisine-resistant components of $[^{125}I]$epibatidine binding in hippocampus and diencephalon cultures. Binding was measured in total membranes prepared from hippocampus and diencephalon cultures at 0, 24, and 96 h for untreated neurons and neurons treated with 1 $\mu$M nicotine. Nonspecific binding was determined by using 100 $\mu$M cytisine. Results are expressed as specific $[^{125}I]$epibatidine binding $\pm$ S.E.M. in fmol/mg protein. The effects of nicotine treatment on cytisine-sensitive (●) and cytisine-resistant (○) components in $[^{125}I]$epibatidine binding for hippocampus cultures (A) and diencephalon cultures (B) are shown. Significant differences from control untreated neurons were calculated by using one-way ANOVA, followed by a Student-Newman-Keuls post hoc analysis. *, $p < 0.05$.
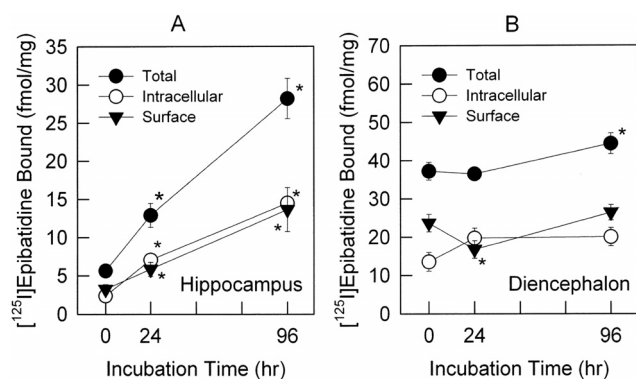


**Fig. 3.** Determination of $[^{125}I]$epibatidine binding to intracellular and surface membranes by alkylation of surface receptors. Primary neurons cultured from hippocampus and diencephalon were treated with 1 $\mu$M nicotine for 0, 24, or 96 h before the alkylation reaction as described under *Materials and Methods*. After alkylation, total membranes were prepared from the neurons, and total and intracellular $[^{125}I]$epibatidine binding was measured. Surface binding was obtained by subtraction of the intracellular binding from the average of total binding. Total (●), intracellular (○), and surface (▼) binding sites are shown for both hippocampus (A) and diencephalon (B) cultures. Data represent mean $\pm$ S.E.M. ($n = 13$, five independent experiments for 0 and 96 h; $n = 9$, four independent experiments for 24-h treatment). Statistical differences were determined by Kruskal-Wallis one-way ANOVA on ranks, post hoc Dunn's method. *, $p < 0.05$.

$63.6 \pm 6.0\%$ (0 h), $45.9 \pm 6.7\%$ (24 h) and $59.5 \pm 5.2\%$ (96 h)] (Fig. 3B).

**In Situ $[^{125}I]$Epibatidine Binding.** An additional method, with which to measure the cellular distribution of surface and intracellular $[^{125}I]$epibatidine binding sites, is the use of differential inhibition by MCC to inhibit surface binding. The principle behind the use of MCC as a selective inhibitor of surface receptors is its relative high hydrophilicity and resultant slow permeability across cell membranes as reflected in a lower partition coefficient compared with cytisine, nicotine, or epibatidine (logP = 0, 0.2, 1.2, and 2.2 for MCC, cytisine, nicotine, and epibatidine, respectively; PubChem project website, http://pubchem.ncbi.nlm.nih.gov). MCC displays relatively high affinity for $\alpha 4\beta 2^*$-nAChRs (Boksa and Quirion, 1987), allowing its use in relatively low concentrations. Preliminary studies were conducted to establish the appropriate conditions for selective inhibition of surface binding sites by MCC. Because MCC may slowly penetrate cell membranes and subsequently inhibit intracellular

sites, the time course for $[^{125}I]$epibatidine binding was determined to establish the minimum time required to reach equilibrium. For this purpose, an $[^{125}I]$epibatidine association binding was done in intact neurons in culture at 22°C. The association had two components. The fast component reached equilibrium in less than 1 min, making accurate calculation of rate constants unreliable. Estimate of the rate constant for the slow component of $[^{125}I]$epibatidine (200 pM) binding was possible. Values for $k_{slow} = 0.158 \pm 0.09$ and $0.147 \pm 0.018$ min$^{-1}$ with $t_{1/2}$ values of 4.4 and 4.7 min were determined for hippocampus (Fig. 4A) and diencephalon (Fig. 4D), respectively. Consequently, equilibrium binding was attained by using 20-min incubation. The 20-min incubation time was used for all subsequent experiments.

After establishing the appropriate incubation time, the pharmacology of MCC interaction with $[^{125}I]$epibatidine binding sites was investigated by using the 20-min incubation time. Inhibition of $[^{125}I]$epibatidine binding by MCC was measured in hippocampus and diencephalon cultures to obtain $K_i$ values (calculated by using a one-site inhibition curve fit with a residual population resistant to inhibition). The $K_i$ values calculated for inhibition of $[^{125}I]$epibatidine binding to intact cells were $83 \pm 38$ and $7.3 \pm 2.2$ nM for hippocampus (Fig. 4B) and diencephalon (Fig. 4E), respectively. High concentrations of MCC did not totally inhibit $[^{125}I]$epibatidine binding (Fig. 4, B and E). The residual binding calculated from the inhibition curves was $45.4 \pm 5.9$ and $33.2 \pm 3.6\%$ of the total for hippocampus and diencephalon, respectively. This component of specific $[^{125}I]$epibatidine binding resistant to inhibition by MCC represents intracellular receptors (Whiteaker et al., 1998). MCC inhibition curves were also determined for $[^{125}I]$epibatidine binding sites in cell membranes prepared from neurons in culture. $K_i$ values of $9.7 \pm 2.8$ and $10.5 \pm 3.6$ nM were measured for hippocampus (Fig. 4C) and diencephalon (Fig. 4F), respectively. Complete inhibition of $[^{125}I]$epibatidine binding to cell membranes by MCC was observed in both hippocampus (Fig. 4C) and diencephalon (Fig. 4F).

The preliminary characterization of the in situ binding establishes that using a 20-min incubation with $[^{125}I]$epibatidine and measuring inhibition of the binding by 1 $\mu$M MCC to inhibit surface binding sites is appropriate for examining the effects of chronic nicotine exposure on the distribution of $[^{125}I]$epibatidine binding sites. Primary cultures from hip-
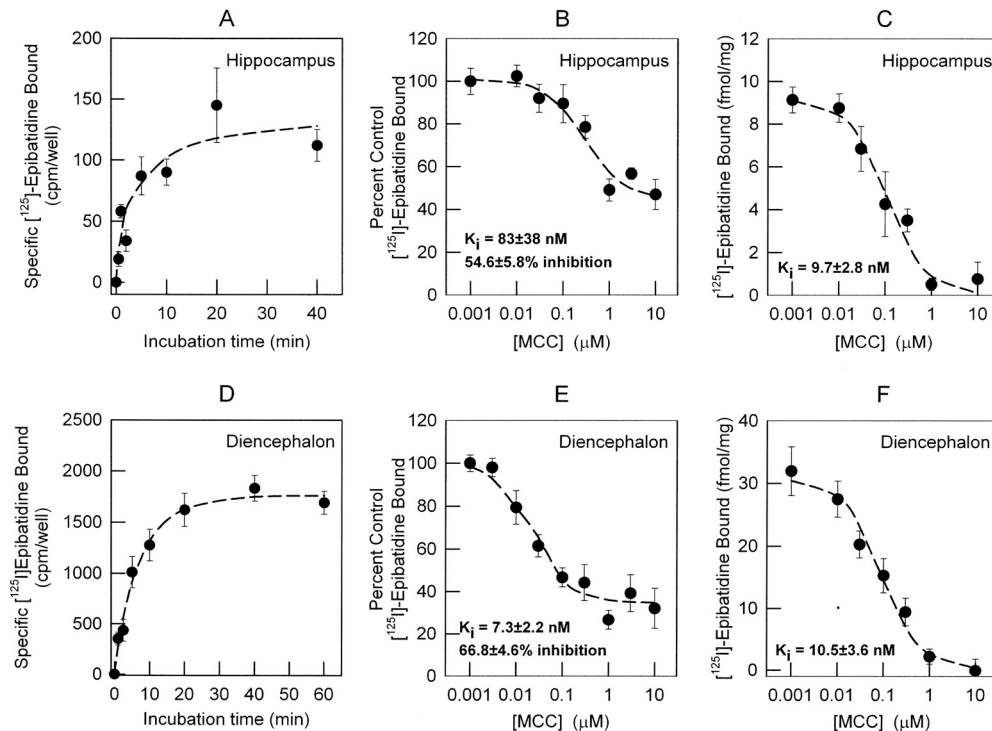
**Fig. 4.** Measurement of the time course for [$^{125}$I]epibatidine binding and comparison of MCC inhibition of [$^{125}$I]epibatidine binding in whole cells and cell membranes. A and D, the time courses for the binding of [$^{125}$I]epibatidine to intact neuronal cultures from hippocampus (A) and diencephalon (D) were measured by incubating the cells with 200 pM [$^{125}$I]epibatidine for the times indicated. Neurons (12–14 days) were seeded in 48-well plates and assayed for [$^{125}$I]epibatidine binding in the same culture dish at room temperature (22°C). Nonspecific binding was determined by including 100 $\mu$M nicotine in the binding reaction. The inhibition of [$^{125}$I]epibatidine binding by MCC in intact neurons was measured by adding the indicated concentrations of MCC (0.001–10 $\mu$M) to the incubation medium. B and E, IC$_{50}$ values and residual binding for both hippocampus (B) and diencephalon (E) neuronal cultures were calculated as described under *Materials and Methods*. C and F, IC$_{50}$ values for MCC in total membrane preparations from both hippocampus (C) and diencephalon (F) were calculated as described under *Materials and Methods*. Apparent $K_i$ values were calculated by using the Cheng-Prussof equation (apparent $K_D$ for [$^{125}$I]epibatidine binding in intact cells: 73 pM for hippocampus and 111 pM for diencephalon; apparent $K_D$ for membrane preparations: 25 pM). Data for the association kinetics represent the mean $\pm$ S.E.M. for four separate determinations. Data for binding in situ represent nine replicates from two independent experiments and are presented as percentage of specific binding in the absence of competitor. Binding in total membranes represents four replicates from two independent experiments and is presented as total specific binding normalized by protein concentration. All curves are least-squares fits of the data as described under *Materials and Methods*.

pocampus and diencephalon were exposed to one of eight concentrations of nicotine for 96 h. After washing to remove nicotine from the medium, cells were incubated with 200 pM [$^{125}$I]epibatidine for 20 min in the presence of 0 $\mu$M MCC (total binding sites) or 1 $\mu$M MCC (intracellular binding sites). Total [$^{125}$I]epibatidine binding was increased in a dose-dependent manner, with significant increases noted after treatment with 0.1 $\mu$M and higher concentrations of nicotine in both hippocampus (Fig. 5A) and diencephalon (Fig. 5B). Maximal binding after chronic treatment with 10 $\mu$M nicotine in hippocampus was 280 $\pm$ 40% of control (Fig. 5A) and in diencephalon was 175 $\pm$ 8% of control (Fig. 5B). The density of both surface and intracellular [$^{125}$I]epibatidine binding increased after chronic nicotine treatment as well. No differences in the percentage of surface receptors in either hippocampus (Fig. 5C) or diencephalon (Fig. 5D) were noted for any nicotine treatment dose. However, consistent with the results presented above, the percentage of surface receptors in diencephalon (80%) (Fig. 5D) is higher than the percentage of surface receptors in hippocampus (50%) (Fig. 5C).
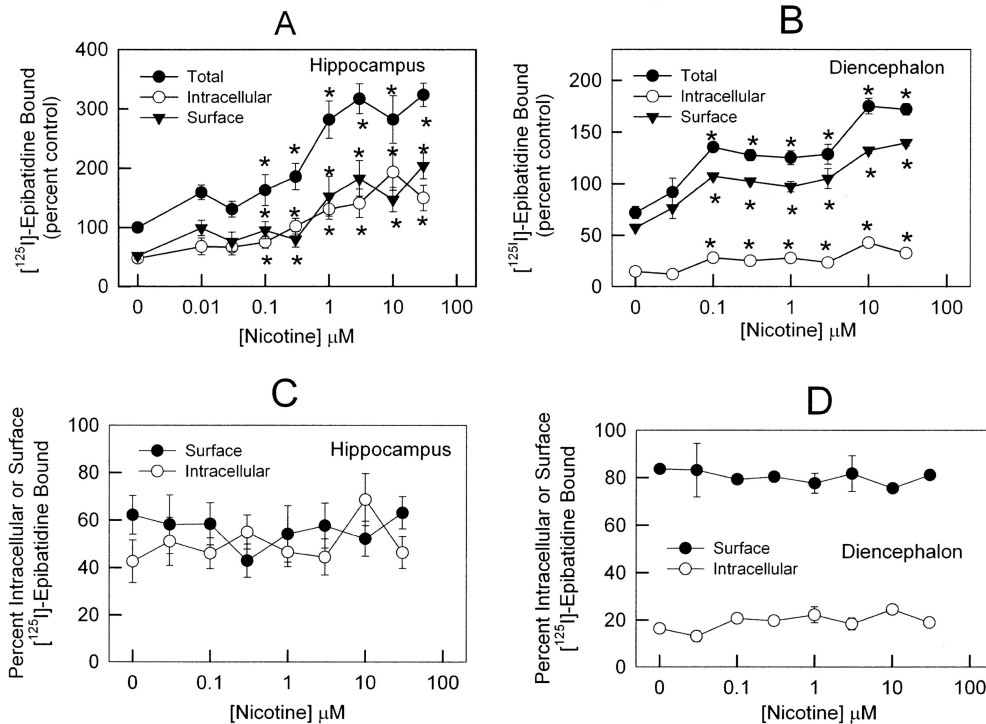
**nAChR Function after Chronic Nicotine Treatment.** The experiments described above demonstrate that chronic nicotine treatment increased the number of surface nAChRs measured with [$^{125}$I]epibatidine binding. To investigate the effects of chronic nicotine treatment on nAChR function,

nicotine-stimulated Ca$^{2+}$ influx was measured. Ca$^{2+}$ influx has been used to measure nAChR function in several systems, including primary neuronal cultures (for review see Shen and Yakel, 2009). Initially, nicotine dose-response curves were generated for control hippocampus and diencephalon neurons (Fig. 6, A–D). The effect of acute nicotine exposure on intracellular Ca$^{2+}$ in hippocampus is shown in Fig. 6A. An EC$_{50}$ value of 182.7 $\pm$ 1.7 was calculated (Fig. 6B). The effect of acute nicotine exposure on intracellular Ca$^{2+}$ in diencephalon is shown in Fig. 6C. An EC$_{50}$ value of 84.0 $\pm$ 5.1 nM was calculated (Fig. 6D). Hill coefficients were 1.02 $\pm$ 0.52 and 0.58 $\pm$ 0.66 in hippocampus and diencephalon, respectively. A 10 $\mu$M nicotine concentration was chosen to acutely stimulate control cells and cells that had been chronically treated with nicotine (0.01, 0.1, 1, and 10 $\mu$M nicotine for 96 h). Chronic nicotine treatment had no significant effect on the increase in intracellular Ca$^{2+}$ in hippocampal neurons elicited by acute nicotine stimulation (Fig. 6E). In contrast, the increase in intracellular Ca$^{2+}$ for diencephalon neurons elicited by acute nicotine stimulation was reduced approximately 60% after chronic treatment with 1 or 10 $\mu$M nicotine (Fig. 6E).

The functional activity of nAChR in response to chronic nicotine exposure in hippocampus and diencephalon was estimated by dividing the functional responses (Fig. 6E) by

**Fig. 5.** Distribution of nAChRs after chronic nicotine treatment by in situ MCC inhibition of [$^{125}$I]epibatidine binding. A and B, hippocampus (A) and diencephalon (B) neurons were treated for 4 days with 0.03, 0.1, 0.3, 1, 3, 10, and 30 μM nicotine for 96 h. Samples were incubated with 200 pM [$^{125}$I]epibatidine for 20 min at 22°C. Intracellular binding was determined by adding 1 μM MCC to inhibit [$^{125}$I]epibatidine binding to the surface receptors. Surface binding was calculated by subtracting intracellular binding from total binding in absence of 1 μM MCC. Total (●), intracellular (○) and surface (▼) [$^{125}$I]epibatidine binding are presented as the percentage of total binding to untreated samples for hippocampus (A) and diencephalon (B) cultures. C and D, surface (●) and intracellular (○) [$^{125}$I]epibatidine binding, as a percentage of the total binding after treatment with each of the indicated nicotine concentrations used for chronic treatment, for hippocampus (C) and diencephalon (D) is shown. Data represent specific cpm as percentage of control neurons. *, statistical differences ($p < 0.05$) from control cells for total, intracellular, and surface binding obtained by one-way ANOVA, post hoc Student-Newman-Keuls.

surface binding (Fig. 5, A and B). The estimated relative function per unit surface of the [$^{125}$I]epibatidine binding site decreased after chronic nicotine treatment by virtually the same extent for both hippocampus and diencephalon cells (Fig. 6F).

## Discussion

Chronic nicotine treatment increases the number of nAChR binding sites in rodent and human brain (Marks et al., 1983, 2011; Schwartz and Kellar, 1983; Benwell et al., 1988; Pauly et al., 1991; Breese et al., 1997; Perry et al., 1999; Sparks and Pauly, 1999; Nguyen et al., 2003), as well as in cells expressing nAChRs (Peng et al., 1994, 1997; Bencherif et al., 1995; Warpman et al., 1998; Whiteaker et al., 1998; Gentry et al., 2003), including primary neuronal cultures (Bencherif et al., 1995; Dávila-García et al., 1999; Nashmi et al., 2003; Lomazzo et al., 2011; Govind et al., 2012). Here, we demonstrate in primary neuronal cultures from hippocampus and diencephalon that chronic nicotine exposure elicits saturable, concentration-dependent up-regulation of high-affinity [$^{125}$I]epibatidine binding sites without changing the ratio of surface to intracellular receptors. However, the increase in plasma membrane receptors is not accompanied by an increase in function measured as nicotine-induced Ca$^{2+}$ influx, corroborating data obtained in chronically nicotine-treated mice (Marks et al., 1993, 2004) and cell lines expressing α4β2*-nAChRs (Peng et al., 1994; Gopalakrishnan et al., 1996; Fenster et al., 1999; Kuryatov et al., 2000), indicating that on average the surface receptors after chronic nicotine treatment are not fully functional.

**Neuronal Cultures Resemble Adult Brain.** The expression and regulation of nAChRs in primary neuronal cultures resemble that observed for adult brain.

Basal levels of high-affinity [$^{125}$I]epibatidine binding sites in hippocampus were relatively low respective to adult tissue, but diencephalon displayed a similar density as observed in the thalamus of adult mice (Marks et al., 2004). Likewise, reflecting the expression in adult rat brain, primary rat hippocampus and cortex neuronal cultures have a lower density of nAChR binding sites than subcortical tissue (Dávila-García et al., 1999).

Nicotine-induced nAChR up-regulation in rodents differs among brain regions: cortex or hippocampus show an approximately 2-fold increase in ligand binding, whereas thalamus has much less up-regulation (Marks et al., 1983, 2004, 2011; Flores et al., 1992; Sanderson et al., 1993; Sparks and Pauly, 1999; Nguyen et al., 2003). Likewise, less up-regulation was observed for primary striatal cultures than for primary cortical cultures (Lomazzo et al., 2011), a difference that is also observed after treatment in vivo. We also observed differences in the extent of nicotine-induced up-regulation for mouse neuronal cultures, finding 2.5- to 3.5-fold increases in total [$^{125}$I]epibatidine binding in hippocampus but a modest 0.4-fold increase in diencephalon cultures after chronic exposure to 1 μM nicotine. Part of this regional difference could arise from the different developmental stages of these two brain regions.

In addition to the increase in α4β2*-nAChRs (measured as cytisine-sensitive [$^{125}$I]epibatidine binding), a significant increase in cytisine-resistant [$^{125}$I]epibatidine binding sites was noted in diencephalon cells after 96 h of treatment with 1 μM nicotine. However, little change in cytisine-resistant sites (or the subset of nAChR measured under these conditions) after chronic nicotine treatment has been noted for animals (Flores et al., 1997; Dávila-García et al., 2003; Nguyen et al., 2003; Marks et al., 2004). However, small increases in cytisine-resistant [$^{125}$I]epibatidine binding sites, which failed to reach statistical significance, have been noted in several mouse brain regions after treatment with high doses of nicotine that resulted in plasma concentrations
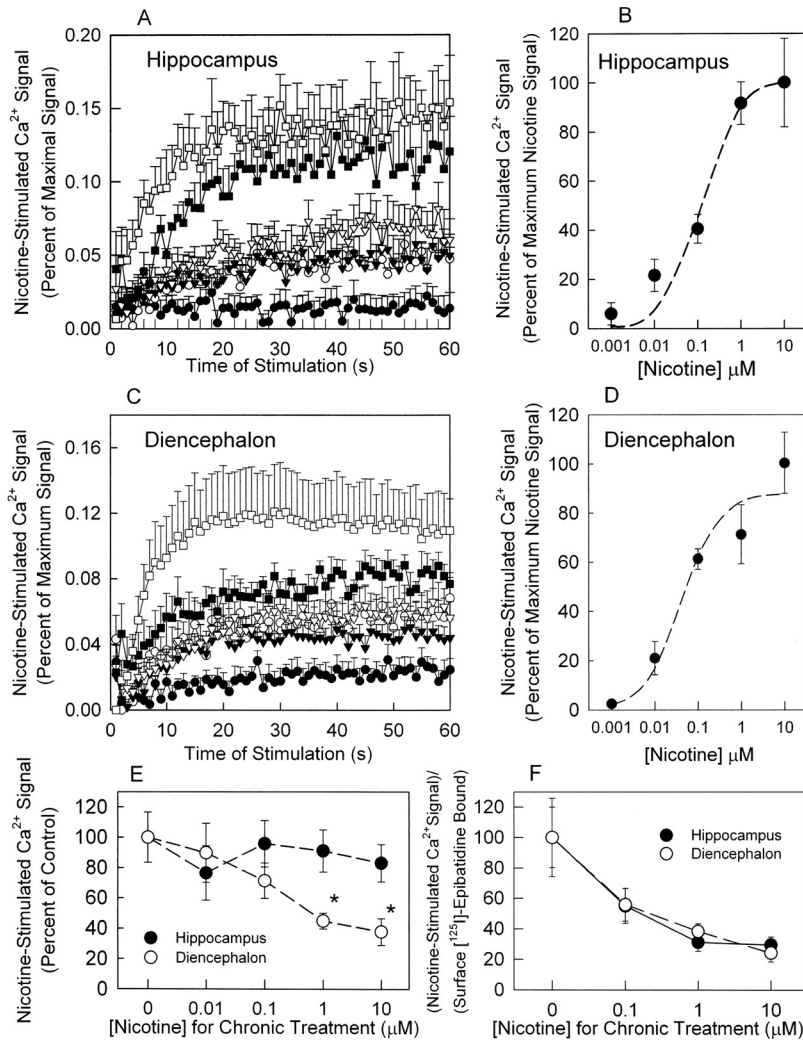
**Fig. 6.** Intracellular $Ca^{2+}$ determination in hippocampus and diencephalon neurons. A and C, time course of nicotine-induced $Ca^{2+}$ influx in untreated hippocampus (A) and diencephalon (C) neurons cultured for 12 days was measured by using these nicotine concentrations: 0 μM (●), 0.001 μM (○), 0.01 μM (▼), 0.1 μM (▽), 1 μM (■), and 10 μM (□). Each point represents the mean ± S.E.M. for four separate experiments. B and D, concentration-response curves were calculated by using the area under the curve for each nicotine concentration, as shown in A and C, for hippocampus (B) and diencephalon (D) and using nonlinear least-squares to fit the curves as described under *Materials and Methods*. E, subsequently, neurons were chronically treated with 0.01, 0.1, 1, and 10 μM nicotine for 4 days and then assayed for $Ca^{2+}$ influx stimulated with 10 μM nicotine. Results are presented as the percentage of the response measured for samples treated with 0 μM nicotine. F, the ratio of nicotine-stimulated increases in intracellular $Ca^{2+}$ normalized by the percentage of surface binding after chronic nicotine treatment (data from E) divided by the amount of surface [125I]epibatidine binding measured after the same nicotine treatment (shown in Fig. 5, A and B). Data represented as mean ± S.E.M. of three independent experiments (*n* = 3–4 each). Significant statistical differences were found by Kruskal-Wallis ANOVA test, post hoc Dunn's method. *, $p < 0.05$.

around 1 μM (Marks et al., 2004). Nicotine-induced up-regulation has been observed for α3β2- or α3β4-nAChRs after treatment with nicotine concentrations higher than those attainable in vivo (Peng et al., 1997; Meyer et al., 2001; Avila et al., 2003; Xiao and Kellar, 2004). The subunit composition of the nAChR expressed in primary mouse diencephalon neurons is currently unknown. The increase in cytisine-resistant [125I]epibatidine binding in diencephalon after chronic treatment with 1 μM nicotine could result from an up-regulation of α3β2*- or α3β4*-nAChRs.

**Plasma Membrane and Intracellular Distribution of [125I]Epibatidine Binding Sites and Effects of Chronic Nicotine Exposure.** nAChR up-regulation has been described in detail using animal and cellular models. However, few reports have focused on how receptors are distributed before and after chronic ligand exposure. Using two different approaches to determine surface and intracellular [125I]epibatidine binding, we demonstrate here that chronic nicotine treatment does not significantly change the percentage of high-affinity [125I]epibatidine binding sites located at the cell surface: surface receptors increase in parallel with the total receptors. Although the proportion of receptors on the cell surface differs between diencephalon (70–80%) and hippocampus (50%), the distribution of binding sites on surface and intracellular membranes remains constant after nicotine

treatment. Our results are completely consistent with those of a recent study demonstrating that the distribution of heteromeric nAChRs in rat primary cultures was unchanged by chronic nicotine treatment (Lomazzo et al., 2011). It should be noted that these results were found even though there were some technical differences between the studies, including methods for measuring the surface receptors and the age of the cultures when chronic nicotine treatment began. It should also be noted, in agreement with our findings, that the ratio of surface to intracellular epibatidine binding sites in M10 cells stably transfected with chicken α4β2-nAChRs, in which most of the receptors are intracellular (approximately 85%), was unchanged by chronic nicotine treatment as measured by MCC inhibition (Whiteaker et al., 1998), a result confirming increased receptor expression as measured by binding of mAb299, an α4 nAChR subunit-selective antibody (Peng et al., 1994).

**Receptor Function after Chronic Nicotine Treatment.** Despite the increase in receptors at the plasma membrane after chronic nicotine treatment, $Ca^{2+}$ influx elicited by acute nicotine exposure was decreased in diencephalon and virtually unchanged in hippocampus cultures. If the nicotine-stimulated increases in intracellular $Ca^{2+}$ are normalized to plasma membrane receptor densities, the effect of chronic nicotine treatment is similar for cells from both re-

gions: that is, chronic nicotine treatment decreases function per unit surface binding site (functionality ratio) (Fig. 6F). This result indicates that the up-regulated surface receptors are on average not fully functional. This finding is totally consistent with previous reports on chronically nicotine-treated mice where both $^{86}Rb^+$ efflux and [$^3$H]dopamine release were diminished compared with control saline-treated mice (Marks et al., 1993, 2004).

The functionality of nAChRs after chronic nicotine exposure remains controversial. Decreases in functionality ratio similar to those reported here have been observed previously in mice (Marks et al., 1993, 2004) or cell lines transfected with nAChRs (Peng et al., 1994, 1997; Gopalakrishnan et al., 1996; Fenster et al., 1999; Kuryatov et al., 2000; Avila et al., 2003). This decrease could result from functional inactivation of receptors after chronic nicotine exposure (Peng et al., 1994; Kuryatov et al., 2000; Gentry et al., 2003). However, it has also been reported that chronic nicotine treatment increases total nAChR function as a consequence of increased receptor expression in cell lines (Gopalakrishnan et al., 1996; Buisson and Bertrand, 2001), rat synaptosomes (Nguyen et al., 2004), and midbrain neurons (Nashmi et al., 2007). Differences in receptor composition, the functional response being measured, the specific cell type being investigated, or the species being examined no doubt contribute to the diversity of findings.

**Summary.** In conclusion, we observed significant differences between hippocampus and diencephalon neurons in culture with respect to density, distribution, and nicotine-induced up-regulation of high-affinity [$^{125}$I]epibatidine binding sites. The proportion of nAChR on the cell surface of cultures from both brain regions remained unchanged after chronic nicotine treatment, indicating that the number of receptors potentially available for function increased. However, this nicotine-induced increase in nAChR on the plasma membrane was not accompanied by an increase in function measured by agonist-stimulated increase in intracellular $Ca^{2+}$, suggesting that the up-regulated receptors are on average less functional than receptors that have not been chronically exposed to nicotine. The similarity in nAChR expression and the response of the nAChR to chronic nicotine treatment between mouse brain in vivo and mouse neurons in culture indicate that primary neuronal cultures are an experimental model suitable for studying the mechanism of nAChR expression and function.

#### Authorship Contributions

*Participated in research design:* Zambrano and Marks.
*Conducted experiments:* Zambrano and Salamander.
*Performed data analysis:* Zambrano and Marks.
*Wrote or contributed to the writing of the manuscript:* Zambrano, Salamander, Collins, Grady, and Marks.

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