Molecular Pharmacology Fast Forward. Published on March 16, 2005 as doi:10.1124/mol.104.009282
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MOLPHARM/2004/009282

Expression of Native α3β4* Neuronal Nicotinic Receptors: Binding and Functional Studies Investigating Turnover of Surface and Intracellular Receptor Populations

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Molecular Pharmacology Fast Forward. Published on March 16, 2005 as DOI: 10.1124/mol.104.009282
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MOLPHARM/2004/009282

Running Title: α3β4* Nicotinic Receptor Expression and Turnover

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Number of Text Pages: 38
Number of Tables: 0
Number of Figures: 8
Number of References: 40

Abstract Words: 215
Introduction Words: 752
Discussion Words: 1739

Abbreviations: nicotinic acetylcholine receptor (nAChR), norepinephrine (NE) Dulbecco's Modified Eagle Medium (DMEM), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB).

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Abstract

Several pathological conditions involve alterations in expression of neuronal nicotinic acetylcholine receptors (nAChRs). While some studies have addressed processes involved with muscle nAChR expression, knowledge of the regulation of neuronal nAChRs is particularly sparse. The following studies were designed to investigate cellular mechanisms involved with expression of neuronal $\alpha3\beta4^*$ nAChRs. Catecholamine secretion assays and receptor binding studies coupled with receptor alkylation were used to study the nAChR regulation and turnover. Alkylation of adrenal nAChRs results in a rapid and complete loss of receptor-mediated neurosecretion and surface [^{3}H]epibatidine binding sites. Following alkylation both neurosecretory function and nAChR binding slowly (24-48 hrs) return to pre-alkylation levels. When cells are treated with the protein synthesis inhibitor puromycin, after alkylation, receptor-mediated neurosecretion does not recover. Chronic (24-48 hrs) puromycin treatment, in the absence of alkylation, results in a slow, time-dependent shift to the right, followed by a downward shift, in the nicotine concentration-response curve, documenting a disappearance of surface nAChRs. Puromycin treatment alone also results in a loss to both surface and intracellular [^{3}H]epibatidine binding sites. nAChR $\beta4$ subunit levels are significantly decreased following treatment with puromycin. These data support a constitutive turnover of adrenal $\alpha3\beta4^*$ nAChRs, requiring continual *de novo* synthesis of new receptor protein.

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Introduction

Nicotinic acetylcholine receptors (nAChRs) are ligand gated ion channels that have essential physiological roles in the central and peripheral nervous systems and are thought to be the primary mediators of nicotine addiction. Neuronal nAChRs have also been associated with a variety of other neurological disease states, all of which involve changes in expression or distribution of the nAChRs (Lindstrom, 1997). Several processes have been described that regulate neuronal nAChR expression and directly influence their functional activity. These processes have a temporal component. Loss of receptor function via desensitization occurs rapidly, usually within seconds or minutes. Receptor downregulation develops over several hours. Receptor up-regulation occurs with some neuronal nAChR subtypes and also develops over several hours. Finally, tolerance and dependence generally take days to weeks to develop. Several mechanisms are likely involved with these processes including alterations in receptor internalization, receptor recycling, receptor degradation and/or receptor synthesis.

At least three separate steps in the receptor expression/turnover process may regulate the number of nAChRs expressed on the cell surface. These include alterations in nAChR formation and assembly (Mitra *et al.*, 2001), nAChR transport to the cell surface (Rothhut *et al.*, 1996;Keller *et al.*, 2001), or nAChR stabilization in the cellular membrane (Peng *et al.*, 1994). The rate-limiting processes involved in nAChR turnover remain to be determined and may vary with different disease conditions. For example, stabilization within cellular membranes is thought to occur during prolonged agonist exposure resulting in nAChR up-regulation (Peng *et al.*, 1997;Peng *et al.*, 1994), a condition seen in tobacco addiction. However, a decrease in nAChR expression in

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specific brain regions is associated with Alzheimer's disease (Warpman and Nordberg, 1995 Hellstrom-Lindahl et al., 1999). Understanding how neurons regulate the expression of nAChRs may lead to methods for manipulating nAChR levels that could be used in the treatment of several conditions including Alzheimer's disease.

Few studies have addressed the question of surface trafficking or turnover of neuronal nAChRs. Several studies have investigated the surface trafficking of muscle nAChRs (e.g., (Marchand *et al.*, 2002). Neuronal nAChRs may traffic in a manner similar to their muscle counterpart. However, since multiple heteromeric ($\alpha3\beta4^*$,$\alpha3\alpha5\beta4$, $\alpha3\alpha5\beta2\beta4$, $\alpha4\beta2$, $\alpha4\alpha5\beta2$) and homomeric ($\alpha7$, $\alpha8$, $\alpha9$) subtypes (Lukas *et al.*, 1999) of neuronal nAChRs are expressed, the mechanisms for regulation and trafficking could vary between subtypes. The "rules" which govern the trafficking and surface expression of any nAChR subtype have not yet been defined. Most of what is known about nAChR trafficking and turnover comes from studies using transfected cells or oocytes. The interpretation of results using cell lines is complicated by the finding that receptor expression and assembly are both host cell and receptor subtype dependent (Sweileh *et al.*, 2000). Furthermore, marked differences in surface trafficking have been seen when comparing native to recombinant systems expressing $\alpha7$ nAChRs (Kassner and Berg, 1997). These findings highlight gaps in the understanding of neuronal nAChR regulation and indicate the importance of using native systems to examine questions relating to receptor regulation.

The ability to study neuronal nAChRs in their native environment and directly correlate cellular and molecular changes in nAChRs with changes in neurosecretion is a

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distinct advantage of using primary cultured bovine adrenal chromaffin cells. Evidence exists that these cells contain multiple populations of neuronal nAChRs. α7 containing nAChRs are thought to be expressed (Wilson and Kirshner, 1977;Garcia-Guzman *et al.*, 1995) and functional (Lopez *et al.*, 1998) on adrenal chromaffin cells, but the primary nAChR subtype responsible for catecholamine release in these cells is the mAb35 nAChR (Gu *et al.*, 1996). Binding studies support the characterization of these nAChRs as α3β4* nAChRs (Free *et al.*, 2002). The demonstration that bovine chromaffin cells contain mRNA for α3, α5, β4 nAChRs further supports this subunit composition (Criado *et al.*, 1992;Campos-Caro *et al.*, 1997;Wenger *et al.*, 1997;Garcia-Guzman *et al.*, 1995;Campos-Caro *et al.*, 1997;Wenger *et al.*, 1997;Free *et al.*, 2002). Therefore, cultured bovine chromaffin cells present an ideal model to investigate regulation of native neuronal nAChRs.

Our laboratory has recently demonstrated that cultured adrenal chromaffin cells contain a substantial population of fully assembled intracellular nAChRs (Free and McKay, 2003). The studies presented here seek to determine the contribution of pre-assembled intracellular pools of nAChRs to the complement of functional surface receptors and to deduce the mechanisms by which chromaffin cells regulate α3β4* nAChR surface expression. Our data support the necessity of constitutive *de novo* protein synthesis for the regulation of neuronal nAChR expression, and suggest that the pre-assembled intracellular pool may not play a primary role in the normal maintenance of α3β4* nAChR surface expression.

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Materials and Methods

Materials. Nicotine hydrogen tartrate, puromycin, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and components of N2+ media were obtained from the Sigma Chemical Company (St. Louis, MO). Dithiothrietol was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Dulbecco's Modified Eagle Medium (DMEM) and DMEM/F12 were obtained from GIBCO/BRL (Grand Island, NY). Bromoacetylcholine bromide was purchased from Research Biochemicals Incorporated (Natick, MA). [^{3}H]Norepinephrine ([^{3}H]NE), specific activity, 12.0-15.0 Ci/mmol), and (±)[5,6-bicycloheptyl-^{3}H]-epibatidine (specific activity, 66.6 Ci/mmol) were purchased from Dupont-New England Nuclear Corporation (Boston, MA). mAb35 (anti-acetylcholine receptor monoclonal antibody) was obtained from a hybridoma cell line purchased from American Type Culture Collection (Rockville, MD). The cells were cultured and the antibody was concentrated and purified using techniques described previously (Gu *et al.*, 1996).

Isolation and Primary Culture of Bovine Adrenal Chromaffin Cells. Adrenal chromaffin cells were dissociated from intact glands and plated in supplemented DMEM, as previously described (Maurer and McKay, 1994). Cells were plated on 24 well plates at a density of 1-2x10^5 cells per well for functional studies and 5X10^5 cells per well for intact cell binding studies. Two days after plating, media were replaced with a modified, serum-free, N2+ medium previously described by our laboratory (Maurer and McKay, 1994). DMEM and N2+ media were supplemented with 250 ng/ml amphotericin B, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10 µM 5-fluoro-2'-deoxyuridine. One day prior to experimentation, the culture medium was

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removed and replaced with medium free of amphotericin B and 5-fluoro-2'-deoxyuridine. Cells were used 4-7 days after isolation.

Catecholamine Secretion Studies. A [^{3}H]NE assay was used to monitor catecholamine release from cultured cells (McKay and Schneider, 1984). The amount of radioactivity released following a 5 min incubation with nicotine (stimulated release) or without nicotine (basal release) was determined using liquid scintillation spectroscopy. The radioactivity remaining in the cells was extracted with 8% trichloroacetic acid (TCA) and also quantified. Results are expressed as either a percentage of total (% total) incorporated [^{3}H]NE released under the treatment conditions, or as a percentage of net release (% net release) which is equal to % total minus % basal (nonstimulated) release. When KCl (56 mM) was used to stimulate catecholamine release, the sodium concentration of the buffer was reduced to maintain isotonicity.

Turnover Studies. For nAChR turnover studies, two methods were used to examine nAChR turnover. First, adrenal nAChRs were irreversibly inactivated via alkylation with bromoacetylcholine using techniques previously published by our laboratory (Gu *et al.*, 1996;Wenger *et al.*, 1997;Free and McKay, 2001). Briefly, cells were treated with 1mM dithiothreitol for 15 min at 37º C in order to reduce receptor disulfide bonds. Cells were then washed with a physiological salt solution for 15 min and then treated with 100 µM bromoacetylcholine for 6 min at room temperature. After washing (15 min), the disulfide bonds were reoxidized via treatment with 1 mM DTNB [5,5'-dithio-bis(2-nitrobenzoic acid)] for 15 min at 37º C. Following a 5 min wash, cells were placed back into N2+ media for chronic studies. Second, adrenal nAChRs were

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treated with the monoclonal antibody, mAb35 (50 nM), by adding it to the culture media for 24 hrs. Cells were washed and placed back in N2+ media to allow for recovery.

[^{3}H]Epibatidine Binding to Intact Bovine Adrenal Chromaffin Cells in Culture. Binding to adrenal nicotinic receptors in intact cells was performed using techniques previously described by our laboratory (Free and McKay, 2003). Briefly, cells were incubated for 60 min at room temperature in binding buffer containing 2 nM [^{3}H]epibatidine and 1µM αBGT to eliminate binding to αBGT binding sites. After the 60 min incubation, the binding buffer was aspirated and the cells were rapidly washed. Cells were extracted in 1M NaOH and the cells scraped from the plates. The cellular extracts were neutralized and counted using liquid scintillation spectroscopy. Non-specific binding was determined in the presence of 300 µM nicotine and typically represented 50 - 55% of the total binding. When binding to only surface nAChRs was investigated, nonspecific binding was determined in the presence of 5 mM carbachol, an impermeant cholinergic receptor agonist. Under these conditions nonspecific binding usually represented 65 -75% of total binding. This higher value likely represents a combination of nonspecific binding and specific intracellular binding (Free and McKay, 2003).

Production of peptide conjugates and an anti-bovine β4 polyclonal antibody. Amino acid sequences of the intracellular loops of bovine α3, α5, α7 and β4 receptors were aligned for homology, and sequences unique to β4 were identified by inspection. Peptides were synthesized with a carboxyterminal cysteine for conjugation via the thiol group, and purity was assessed using mass spectroscopy and capillary

electrophoresis. The 22-mer β4 peptide (RPRQQPSRAPQSSLARLTKSEC) was conjugated with keyhole limpet hemocyanin (Calbiochem) using sulfo-succinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC, Pierce) as previously described (Hermanson, 1996). The conjugates were dialyzed against phosphate buffer (pH 7.3) and frozen prior to use at a concentration of 1.4 - 2.25 mg/ml. Peptide synthesis, analysis and conjugation were done in the Protein Core facility, Institute for Cell and Molecular Biology, University of Texas at Austin. Immunizations were performed by Cocalico Biologicals, Inc. (Reamstown, PA). Each conjugate was thawed, agitated to resuspend any precipitated protein, emulsified with complete Freund's adjuvant, and injected into multiple sites on the backs of two rabbits. Subsequent injections were given as emulsions with incomplete Freund's adjuvant at approximately two week intervals, and bleeds were tested for activity and specificity by western blot against endogenous and recombinant receptor proteins.

Western Blot Analyses. Equivalent amounts of protein were separated in 10% SDS-polacrylamide gels and then electroblotted onto Hybond membranes (Amersham Pharmacia Biotech, UK). The blots were probed overnight at 4°C with a 1:2500 dilution of polyclonal antibody to the bovine β4 peptide described above. Bound antibodies were detected using a 1:2500 dilution of an anti-rabbit IgG linked to horseradish peroxidase (AmershamPharmaciaBiotech, Piscataway, NJ). Western blots were probed and detected according to the manufacturers instructions, using enhanced chemilumniscence (ECL, AmershamPharmaciaBiotech, Little Chalfont, UK). Blots were

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visualized using a Biochemi™ Imaging System (UVP, Inc. Upland, CA). Molecular weights were determined using LabWorks™ imaging software (UVP, Inc. Upland, CA).

Northern Blot Analyses. Chromaffin cells grown on 60 mm dishes either were not treated, alkylated with bromoacetylcholine, or were alkylated and allowed to recover for 24 or 48 hr. RNA was isolated by Trizol® (GIBCO/BRL, Grand Island, NY). Northern blot analysis was performed using 1% (w/v) agarose gels containing 7.4% (v/v) formaldehyde in 20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate at pH 7.0. Equal amounts of RNA were run on each lane of the gel. After electrophoresis, the RNA was transferred to GeneScreen Plus (Dupont-New England Nuclear Corporation, Boston, MA) in 10 X SSC according to the manufacturer's instructions. Bovine α3, α5, and β4 and mouse GAPDH (Ambion, Austin, TX) cDNAs were labeled with α-^{32}P-dCTP using the Prime-It Rm T® random primer labeling kit (Stratagene, La Jolla, CA). The ^{32}P-labeled probes were hybridized to the GeneScreen Plus membrane in 5 X SSPE, 50% deionized formamide, 5 X Denhardt's Solution, 1% SDS, 10% Dextran Sulfate, and 100 mg/ml of salmon sperm DNA at 42°C. The filters were washed in 2 X SSPE at room temperature, 2 X SSPE, 2% SDS at 65°C, 0.1 X SSPE for 45 mins, and 0.1% SDS at room temperature for 15 mins. The blots were exposed to X-ray (Kodak XAR-5) film at -70°C with an intensifying screen. A Molecular Dynamics phosphoimager was used to quantify the signal intensities in each lane. The nAChR signals were normalized to the GAPDH signals to measure relative changes in mRNA levels.

Data analyses. Results were calculated from the number of observations (n) performed in duplicate (intact cell binding studies) or triplicate (catecholamine secretion

studies). All experiments were performed on 2-4 different cell isolations. Experimental values were compared using the t-test ($p<0.05$) or Dunnett's multiple comparison test ($p<0.05$), as indicated.

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Results

Paradigms to investigate nAChR turnover: Two distinct paradigms were employed in order to investigate nAChR turnover processes. The first paradigm involves irreversible alkylation of surface nAChRs via treatment with the cell impermeant alkylating agent, bromoacetylcholine. Previous studies from our laboratory have demonstrated that nAChR alkylation with bromoacetylcholine results in a rapid and complete loss of nAChR mediated catecholamine release from bovine adrenal chromaffin cells (Gu *et al.*, 1996;Wenger *et al.*, 1997). The second paradigm involves antigenic modulation via treatment with the anti-nAChR antibody mAb35 and results in a partial loss of nAChR function (Gu *et al.*, 1996).

[^{3}H]Epibatidine binding experiments (using 2 nM epibatidine, 80% receptor occupancy) on intact adrenal chromaffin cells were designed to directly investigate the ability of alkylation to eliminate surface nAChRs. Intact cell binding experiments allow for the examination of changes to 1) total nAChRs (Rt, surface plus intracellular nAChRs), 2) surface nAChRs (Rs), and 3) intracellular nAChRs (Ri). Previous studies from our laboratory document the feasibility of this approach (Free and McKay, 2003). As seen in Figure 1, alkylation produced a 40% loss of binding to Rt and a nearly complete loss of binding to Rs. Alkylation had no effect on binding to Ri (data not shown). When the cells were allowed to recover for 48 hrs following alkylation, binding to both Rt and Rs significantly increased to near prealkylation levels (Fig. 1). These binding studies document the nearly complete loss of surface adrenal nAChRs after alkylation and their recovery from alkylation within 48 hrs. Treatment with mAb35

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resulted in a partial loss of surface receptor expression (data not shown) consistent with functional data (Gu *et al.*, 1996;Wenger *et al.*, 1997).

Transcriptional consequences of the loss of functional surface nAChRs. We investigated the transcriptional consequences of the loss of functional surface nAChRs via alkylation on nAChR mRNA levels. mRNA was isolated from cultured adrenal chromaffin cells immediately following nAChR alkylation (recovery = 0 hr) and 24 hr and 48hr after alkylation (i.e., recovery from alkylation). As demonstrated in Figure 2, no significant changes were observed in α3, α5, and β4 nAChR subunit mRNA levels either immediately following nAChR alkylation and during the recovery period (24 and 48hr). Untreated cells maintained an unchanging level of nAChR subunit mRNAs expression during this time period (data not shown). These data demonstrate that mRNA levels are not likely regulated after the loss of functional nAChRs via alkylation, consistent with a constant rate of $\alpha 3\beta 4^*$ nAChR subunit protein synthesis.

Effects of puromycin on recovery of nAChR mediated functional responses: Translational effects. To investigate the importance of protein synthesis in the recovery of nAChRs after the loss of functional nAChRs via alkylation, the protein synthesis inhibitor puromycin (10µg/ml) was included in the media during recovery from nAChR alkylation. As seen in Figure 3A, alkylation of cultured chromaffin cells resulted in a complete loss of functional nAChRs, as documented by a decrease in nAChR-mediated catecholamine release. Significant recovery of the functional response is observed after allowing cells to incubate in media alone for 24 hrs following receptor alkylation. This functional recovery is prevented by inclusion of the protein synthesis

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inhibitor puromycin in the recovery media (Fig. 3A) implicating the need for protein synthesis.

To investigate whether puromycin's effects were specific for alkylation-induced functional recovery, similar experiments were conducted after functional downregulation via antigenic modulation using the anti-nAChR antibody, mAb35. As seen in Figure 3B, treatment of cultured chromaffin cells with mAb35 resulted in a partial loss of secretory function that significantly recovered 24 hrs after removal of the antibody. With puromycin present during the recovery period, the return of function was completely inhibited (Fig. 3B). It is important to note that catecholamine release stimulated by depolarizing concentrations of KCl was not affected by treatment (24hr) with puromycin (15.2% ± 1.7% and 15.5% ± 2.3%, respectively). These data provide evidence for the effects of puromycin on nAChR recovery and not on a distal step in the stimulus-secretion pathway. These studies support the need for *de novo* protein synthesis in recovery of nAChR-mediated catecholamine release following two mechanistically distinct paradigms that eliminate functional surface nAChRs.

Involvement of constitutive nAChR turnover in recovery processes. In addition to their sensitivity to protein synthesis blockade, the two paradigms show similar recovery rates (Gu *et al.*, 1996;Wenger *et al.*, 1997). The above findings indicate that recovery of nAChR-mediated neurosecretion may be due to constitutive nAChR turnover, rather than stimulated nAChR synthesis. Thus, inhibition of new protein synthesis should decrease the number of functional surface nAChRs. To address this hypothesis, the effects of puromycin treatment on nicotine-stimulated catecholamine release were investigated. As seen in Figure 4A, when cells were treated with 10 µg/ml

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puromycin for 24 and 48 hrs, there was a time-dependent, rightward-shift, followed by a downward shift of the nicotine concentration-response curves. These shifts of agonist concentration-response curves are typically seen when receptors are lost in cells expressing spare receptors, as we have previously demonstrated in adrenal chromaffin cells (Wenger *et al.*, 1997), and do not represent actual changes in receptor affinity (for review, see Ruffolo, 1982). These effects of puromycin were also reversible. Figure 4B demonstrates a leftward and upward shift of the nicotine concentration-response curves after puromycin was removed, until full recovery of secretory function returned after 48 hrs. These data are consistent with a loss of surface receptors followed by a return of surface receptors. These data demonstrate the necessity of *de novo* protein synthesis for the maintenance of functional $\alpha3\beta4^*$ nAChRs and support constitutive nAChR turnover.

Effects of puromycin treatment on [^{3}H]epibatidine binding sites. To directly address the importance of *de novo* protein synthesis in maintaining both the surface and intracellular pools of nAChRs, [^{3}H]epibatidine binding experiments (2 nM, 80% receptor occupancy) were performed on intact cells. Puromycin treatment for 24 hrs significantly decreased the number of [^{3}H]epibatidine binding to both Rt and Rs (Fig. 5A). Binding to Ri was also significantly decreased with puromycin treatment (Fig. 5B). These effects of puromycin were reversible. Following a 48 hrs recovery period, both Rs and Ri sites significantly recovered (Fig. 5), albeit to apparently slightly different degrees. These data indicate that *de novo* protein synthesis is necessary for the maintenance of both surface and intracellular nAChRs.

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Effects of puromycin treatment on β4 nAChR protein levels. Binding and functional studies confirm that puromycin treatment decreases the expression of Rs. However, these studies do not rule out the potential that puromycin treatment is affecting a protein independent of the nAChR protein, such as a protein needed for nAChR assembly or trafficking. To address this issue, the ability of puromycin to inhibit the synthesis of nAChR protein was investigated.

To examine changes in nAChR protein a novel anti-bovine β4 nAChR polyclonal antibody was created for use in western blots to measure bovine β4 nAChR subunit levels. To ensure that specificity of this polyclonal antibody, it was tested using HEK293 cells heterologously expressing individual, (α3, α5, and β4) bovine nAChR subunits. As demonstrated in Figure 6, the antibody bound to a single band in the HEK 293 cell line transfected with the bovine β4 nAChR subunit protein. No proteins were detected in either untransfected, control HEK 293 cell lines, or those expressing either the bovine α5 or α3 nAChR subunit protein.

In native bovine adrenal chromaffin cells the anti-bovine β4 polyclonal antibody detected 3 proteins (52.5Kd, 51Kd, 48Kd) in the range of predicted molecular weight for the β4 subunit (Fig. 7, left-most lane). The observation of differences in apparent stability of the 3 proteins is unknown but may be due to differences in specific degradation rates of the proteins or differences in susceptibility to proteases. Differences in size of the band seen when the β4 subunit was expressed in HEK cells compared to the multiple bands observed in chromaffin cells may be due to differences in cell-specific subunit protein modifications. To determine the ability of puromycin

treatment to inhibit the synthesis β4 protein, western analysis was conducted on protein isolated from cells treated with puromycin for either 24 or 48 hrs. Figure 7 shows a representative blot of cultured cell extract, demonstrating significantly decreased β4 nAChR subunit levels following treatment of the cells with puromycin for 24 and 48 hrs. These data are consistent with puromycin acting to reduce the synthesis of adrenal nAChRs.

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Discussion

Despite the importance of nAChR turnover and regulation in a number of pathological conditions, very few studies have examined such mechanisms in native systems. We hypothesized that perturbation of surface nAChRs results in a compensatory increase in the surface expression of nAChRs. Contrary to our hypothesis, we found that nAChR antigenic nAChR modulation or loss of functional nAChRs do not produce compensatory increases in transcriptional or translational processes and that constitutive turnover accounts for replacement of surface nAChRs. We also found that de novo protein synthesis is necessary for the replacement of surface nAChRs and have no evidence that intracellular pools of nAChRs play a role in surface expression under our conditions of nAChR downregulation.

These studies are important since very little is know about processes mediating surface expression of functional neuronal nAChRs. In addition, gaining a better understanding of mechanisms that regulate expression of functional nAChRs receptors may be relevant to ameliorating the loss of nAChRs that is seen with Alzheimer's disease, where nAChR receptor loss or downregulation is occurring. The majority of neuronal nAChR regulation studies investigated nAChR up-regulation in response to chronic agonist treatment. These studies have resulted in a better understanding of nAChR subtype-specific sensitivity to up-regulation (Olale *et al.*, 1997;Flores *et al.*, 1997) and up-regulatory mechanisms (Peng *et al.*, 1994;Fenster *et al.*, 1999). However, they have not clearly addressed the underlying mechanisms of nAChR turnover and how cells respond to receptor downregulation. One important observation from chronic agonist treatment studies is that different nAChR subtypes may be differentially

regulated both in terms of response to chronic agonist treatment (Olale *et al.*, 1997;Wang *et al.*, 1998) and native versus recombinant systems (Sweileh *et al.*, 2000). Paradoxical results for up-regulation studies using $\alpha3\beta4$ nAChRs have been reported in recombinant systems (Meyer *et al.*, 2001;Wang *et al.*, 1998). These findings highlight the difficulties of studying $\alpha3\beta4$ nAChR turnover using up-regulation paradigms, and indicate the necessity of additional methods for investigating $\alpha3\beta4$ nAChR turnover. The current studies seek to define approaches for studying nAChR regulation in native systems and to identify regulatory events for $\alpha3\beta4$* nAChRs.

To address questions regarding turnover of native $\alpha3\beta4$* nAChRs, two paradigms were used: nAChR alkylation and nAChR antigenic modulation. Our laboratory has previously characterized these paradigms using functional (neurosecretion) assays (Gu *et al.*, 1996;Wenger *et al.*, 1997) We have now coupled these paradigms to direct radioligand binding assays to define changes in both surface and intracellular nAChRs for the study of nAChR turnover (Free and McKay, 2003). In the studies presented here, both alkylation and mAb35 treatment produced decreases in surface nAChR binding, which recovered with time to control levels. These results parallel previously published functional data (Gu *et al.*, 1996;Wenger *et al.*, 1997). Receptor alkylation is a useful paradigm for studies involving expression of $\alpha3\beta4$* nAChR subtypes since alkylation results in a complete loss of nAChR-mediated function and [^{3}H]epibatidine binding. This contrasts with the antigenic modulation paradigm that results in an incomplete reduction in nAChR-mediated neurosecretion (Gu *et al.*, 1996;Wenger *et al.*, 1997) and a correspondingly smaller reduction in [^{3}H]epibatidine

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binding to surface nAChRs. These findings support the use of these mechanistically distinct paradigms to investigate α3β4*nAChR turnover and indicate that they may be useful to differentiate populations of nAChRs.

As receptor alkylation caused a loss of surface receptors, we hypothesized that chromaffin cells might respond by increasing transcription of neuronal nAChR subunit genes. When adult muscle is denervated, increased synthesis of extrajunctional nAChRs occurs with an increase in subunit RNAs (Linden and Fambrough, 1979;Goldman *et al.*, 1988). During myogenesis, increased expression of surface nAChR s coincides with increases in nAChR subunit mRNAs (Evans *et al.*, 1987). In contrast to what is observed for muscle nAChRs, no increases in transcript levels for α3, β4, or α5 subunits were observed over the 48 hr period. While it appears that transcriptional regulation does play a vital role in both developmental and basal levels of nAChR expression, very little evidence supports transcriptional regulation playing a role in changes in receptor expression during disease. Most nAChR mRNA levels are not altered in Alzheimer's disease or by nicotine exposure (Nordberg, 2001;Marks *et al.*, 1992). These findings suggest that the primary mechanism of change in nAChR expression is likely via post-transcriptional or trafficking modifications. Our findings are consistent with this in that we see no changes in mRNA levels after loss of functional nAChRs supporting a lack of transcriptional regulation.

We found that inhibition of protein synthesis was able to prevent recovery of nAChR-mediated catecholamine secretion. When protein synthesis was inhibited in these cells, a reduction in surface nAChR binding sites was observed. In addition,

puromycin treatment caused loss of nAChR-mediate function and a time-dependent shift in the nicotine concentration-response curve. These effects were not due to a loss of cells or a generalized toxicity since, when the inhibitor was removed both nAChR function and binding returned to pretreatment levels**.** Previous studies have also shown that in the continued presence (48 hrs) of the protein synthesis inhibitors, actinomycin D or cyclohexamide, cell survival for cultured chromaffin cells is at least 70-80% (Cardenas *et al.*, 1995). In addition, puromycin treatment did not affect secretion stimulated by depolarizing concentrations of KCl. These findings support the necessity for *de novo* protein synthesis in the return of functional receptors to the surface after downregulation, implying a lack of receptor recycling and the need for continual replacement due to nAChR turnover on the cell surface.

In addition, our results using puromycin treatment alone indicate that constitutive de novo protein synthesis is also required to maintain normal levels of nAChRs. This is interesting in that adrenal chromaffin cells have a large pool of intracellular nAChRs, 2-3 times the number of surface nAChRs (Free and McKay, 2003) which might be expected to move to the surface in response to downregulation or as part of the normal turnover/cycling process. The data presented here suggest that the intracellular pool of receptors does not play a primary role in recovery after alkylation or in normal turnover since new receptor synthesis is required in both cases. These findings are however consistent with previous studies that suggest only a small percentage of the intracellular binding sites in chick ciliary ganglion neuron (Stollberg and Berg, 1987) or muscle cells (Pestronk, 1985) are destined for surface expression. The precise physiological implications of intracellular nAChRs remain unclear. These intracellular nAChRs may

consist of two distinct pools: nAChRs destined for immediate surface expression and nAChRs stored as an independent pool. Intracellular nAChR pools in other tissues (Stollberg and Berg, 1987) and recombinant cell lines (Whiteaker *et al.*, 1998) can be modulated (Rothhut *et al.*, 1996) and may be involved with the receptor turnover process. It is also possible that these intracellular nAChRs may represent internalized receptors that were once on the receptor surface. This however, is unlikely since they maintain a high affinity for [^{3}H]epibatidine (Free and McKay, 2003). Internalized receptors would be expected to degrade more rapidly when they are internalized (Xu and Salpeter, 1999) and therefore would not likely represent a significant amount of high affinity binding. Furthermore, it is not likely that newly internalized old receptors would be sensitive to puromycin, as is the α3β4* intracellular nAChR pool. Another intriguing possibility is that the intracellular receptors lack the necessary signals to be targeted for surface expression and are therefore, shunted away from the trafficking pathway. This contention is supported by studies showing muscle nAChRs must interact with certain regulatory elements in the endoplasmic reticulum to be directed to the cell surface (Keller *et al.*, 2001). Regardless, our data suggest that intracellular α3β4* nAChRs do not contribute to normal surface nAChR turnover and likely are an independent pool.

Constitutive nAChR turnover is supported by puromycin's effects on both surface and intracellular nAChRs and on secretion. However, in addition to its effects on the synthesis of nAChR protein, puromycin treatment may also affect the synthesis of other proteins possibly involved with trafficking, insertion, or internalization of adrenal α3β4*

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nAChRs. Our data do not indicate that puromycin significantly affects proteins involved with receptor internalization as we have previously documented an internalization rate for mAb35 induced turnover (Wenger *et al.*, 1997) which followed a similar time course as loss of surface receptors following puromycin treatment. These studies do not, however, rule out puromycin having effects on synthesis of a trafficking/chaperone or assembly proteins, as several studies have demonstrated that nAChRs interact with chaperone proteins (Keller *et al.*, 1996;Jeanclos *et al.*, 2001). We would hypothesize that since the cell makes many more fully assembled nAChRs than are inserted on the cell surface (Free and McKay, 2003), a chaperone/trafficking protein likely exists, and may represent the limiting regulatory factor for shunting nAChRs to the cell surface. Regardless of any effects on other proteins, our Western analyses document that puromycin treatment lowers the levels of β4 subunit protein under our treatment conditions.

Based on our findings, we propose a model for surface expression of α3β4* nAChRs (Fig. 8). Surface expression of nAChRs is dependent on constitutive turnover. Adrenal cells contain a large intracellular population of nAChRs that bind epibatidine with high affinity, whose function is not understood (Free and McKay, 2003) (Fig. 8, dotted box). This intracellular nAChR pool, as well as the nAChRs expressed on the cell's surface, depends on protein synthesis (step 2). The predominant route of nAChR surface expression is via step 3 (Fig. 8) and is dependent on *de novo* protein synthesis. We found no evidence that preformed, intracellular pools of nAChRs are involved with surface expression (step 5); however, we can not definitively rule this possibility out.

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Based on data in muscle nAChRs, it is not likely that endocytosis of surface nAChRs (step 6) contributes significantly to the intracellular pool of nAChRs.

In summary, our findings highlight the importance of continual turnover of $\alpha 3\beta 4^*$ nAChRs and demonstrates that lack of receptor recycling and the necessity of *de novo* protein synthesis in the maintenance on surface nAChRs. Because of the involvement of nAChRs in nicotine addiction and their possible roles in a number of disease states, a more thorough understanding of cellular and molecular mechanisms involved with nAChR expression, downregulation and turnover are of critical importance. These studies significantly add to the current understanding of how neuronal cells regulate nAChR number and turnover. Further investigation of this pathway will likely provide novel targets for the manipulation of receptor number such as chaperone proteins, or other post-translational modulators that underlie receptor trafficking.

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MOLPHARM/2004/009282

Footnotes

This project was supported by NIH Grant DA10569 (DBM).

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[2] Professor Paul D. Gottlieb died during the preparation of this manuscript after a brief illness. He was a valued friend and mentor and will be sorely missed.

Molecular Pharmacology Fast Forward. Published on March 16, 2005 as DOI: 10.1124/mol.104.009282
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Legends for Figures

Figure 1. Effects of alkylation and recovery from alkylation on [^{3}H]epibatidine binding to cultured adrenal chromaffin cells. [^{3}H]Epibatidine binding experiments (2 nM, ~80% receptor occupancy) were performed on the cells using either the permeable, competitive ligand, 300μM nicotine (Surface and Intracellular Binding) to measure specific binding of the radioligand to both surface and intracellular nAChRs, or the impermeable, competitive ligand, 5mM carbachol (Surface Binding) to measure specific binding of the radioligand to surface nAChRs. Some cells were alkylated (ALK) prior to the binding reaction. In addition, some cells were alkylated and then allowed to recover by incubating in culture media for an additional 48 hrs (ALK + 48 Hr Rec) prior to the binding reaction. Data are represented as means ± SEM (n=4).

Figure 2. Effects of alkylation-induced loss of functional surface nAChRs on nAChR subunit mRNA levels. Cells were alkylated and RNA was isolated immediately (control), 24, and 48 hr after alkylation. Northern blots were probed with bovine β4, α5, and α3 cDNAs then reprobed with GAPDH. The blots were exposed to film with an intensifying screen and then analysed using a phosphoimager to quantify the signal intensities in each lane. The nAChR subunit RNA signals were normalized to the GAPDH signals to measure relative changes in mRNA levels. The control signals were set at 100%. Slight changes in relative mRNA levels were seen which were not statistically significant (α3 24 hr=98%, 48 hr=127%; α5 24 hr=86%, 48 hr=86%; β4 24 hr=96%, 48 hr=87%).

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Figure 3. Effects of puromycin on recovery of nAChRs after nAChR downregulation. Cultured chromaffin cells were first loaded with [^{3}H]NE, as described in the Methods section. **A**. Effects of puromycin on recovery from alkylation-induced loss of functional surface nAChRs. Cells were either not treated (Control) or alkylated using 100µM bromoacetylcholine (Alkylated). Some groups of alkylated cells were not allowed to recover and immediately stimulated (No Rec). Other groups were washed and allowed to recover by incubation for 24 hrs either in culture media (24 hr Rec) or culture media containing 10 µg/ml puromycin (24 hr Rec + PUR). **B**. Effects of puromycin on recovery from mAb35 induced nAChR downregulation. Cultured adrenal chromaffin cells were either not treated (Control) or treated with 10 nM mAb35 for 24 hr (24 hr mAb35 Treated). Some groups of mAb35 treated cells were not allowed to recover and stimulated immediately (No Rec). Other groups were washed and allowed to recover by incubation for 24 hrs either in culture media (24 hr Rec) or culture media containing 10 µg/ml puromycin (24 hr Rec + PUR). Following all of these treatments, cells were washed (5 min) and then simulated with 10µM nicotine. Data are expressed as a percent of total cellular [^{3}H]NE content released during the 5 min stimulation period. Values are means ± SEM (n = 4-6). Dashed line represents basal, nonstimulated, catecholamine release.

Figure 4. **A**. Time-dependent effects of puromycin on nicotine-stimulated adrenal secretion. Cultured adrenal chromaffin cells were first loaded with [^{3}H]NE, as described in the Methods section. Cells were then incubated either 1) in culture media (Control), 2) in 10 µg/ml puromycin for 24 hrs (24 hr Puromycin Treated), or 3) in 10 µg/ml puromycin

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for 48 hrs (48 hr Puromycin Treated). Cells were then washed and stimulated with the indicated concentrations of nicotine. Data are expressed as percentage of net stimulated release, *i.e.,* total release minus basal release (mean ± SEM) for each concentration (n = 4 - 6). **B**. Time-dependent recovery of nicotine-stimulated secretion after puromycin treatment. Cells were first loaded with [^{3}H]NE, as described in the Methods section. Cells were then incubated either 1) in culture media (Control), 2) in 10 µg/ml puromycin for 48 hrs (48 hr Puromycin Treated), 3) in 10 µg/ml puromycin for 48 hrs, followed by culture media for 24 hrs (24 hr Recovery), or 4) in 10 µg/ml puromycin for 48 hrs, followed by culture media for 48 hrs (48 hr Recovery). Cells were then washed and stimulated with the indicated concentrations of nicotine. Data are expressed as a percentage of stimulated release, *i.e.,* total release minus basal release (mean ± SEM) for each concentration (n = 3).

Figure 5. A. Effects of puromycin treatment on total and surface nAChRs. Cultured chromaffin cells were incubated either 1) with culture media for 24 hrs (-, control), 2) with culture media containing 10 µg/ml puromycin for 24 hrs (PUR), or 3) with culture media containing 10 µg/ml puromycin for 24 hrs, followed by a 48 hr recovery period in media (PUR + 48 hr Rec). Following the treatment period, [^{3}H]epibatidine binding experiments (2 nM, ~80% receptor occupancy) were performed. To measure specific binding to both surface and intracellular nAChRs, the permeable, competitive ligand, 300µM nicotine was used (Surface and Intracellular Binding). To measure specific binding to surface nAChRs, alone, the impermeable, competitive ligand, 5mM carbachol was used (Surface Binding). Data are represented as a percentage of control specific

binding (1777 ± 185 cpm/culture). Values represent means ± SEM (n = 4). **B**. Effects of puromycin treatment on intracellular nAChRs. Cells were incubated either 1) with culture media for 24 hrs (-, control), 2) with culture media containing 10 µg/ml puromycin for 24 hrs (PUR), or 3) with culture media containing 10 µg/ml puromycin for 24 hrs, followed by a 48 hr recovery period in media (PUR + 48 hr Rec). To investigate the effects of puromycin treatment on intracellular binding, binding to surface nAChRs was eliminated by alkylation, as described in the Methods. Following puromycin treatment and alkylation, [^{3}H]epibatidine binding experiments (2 nM, ~80% receptor occupancy) were performed. Specific binding was measured using the permeable, competitive ligand, 300µM nicotine. Data are represented as a percentage of control specific binding (1463 ± 157 cpm/culture). Values represent means ± SEM (n = 4).

Figure 6. Western Blot of transfected HEK cell extracts. 20 ug of extracts from HEK control, HEK alpha 5-transfected, HEK alpha 3-transfected, or HEK beta4-transfected cells were separated by 10% PAGE and analyzed by Western blotting, as described in methods. The blot was probed with a 1:1000 dilution of the anti-β4 nAChR polyclonal antibody, PC 218, at 4°C overnight. Following hybridization with anti-rabbit HRP- linked 2°Ab for 1 hr, the blot was visualized by autoradiography using Amersham ECL.

Figure 7. Effects of puromycin treatment on nAChR β4 protein levels. Cultured chromaffin cells were either not treated (control 24hrs, control 48hrs) or treated for 24 (puro 24hrs) or 48 (puro 48hrs) hrs in the presence of 10 µg/ml puromycin. Following treatment, cells were washed once and extracted by scraping in extraction buffer.

Samples were then analyzed via western blot as described in Methods, probed with the anti-β4 nAChR polyclonal antibody (described in Figure 6) and visualization using ECL after incubation with an anti-rabbit HRP-conjugated antibody. Band size was determined by comparison with prestained protein standards. Data are shown as a representative blot. The four bands in the first land starting with the top are 56 kD, 52.2 kD, 51kD, and 48 kD.

Figure 8. Proposed model for surface expression of adrenal α3β4* nAChRs. Several steps may be involved with the expression of pentameric nAChRs (solid cylinders): 1, transcription; 2, translation; 3, trafficking of newly synthesized nAChRs to surface; 4, trafficking of newly synthesized nAChRs to intracellular pool (dotted box); 5, trafficking of nAChRs from intracellular pool to surface; 6, endocytosis of nAChRs from surface to intracellular pool; 7, degradation of intracellular nAChRs (mottled cylinder). Additional description of the model is found in the text.

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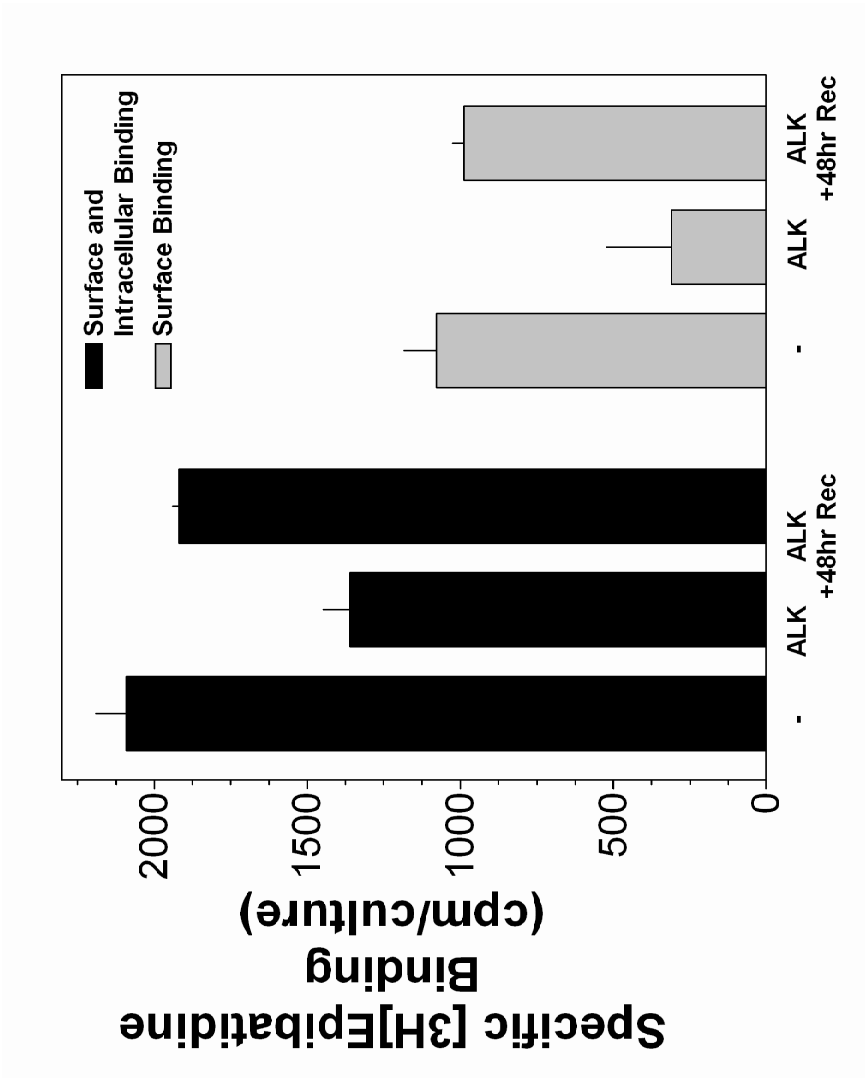

Figure 1

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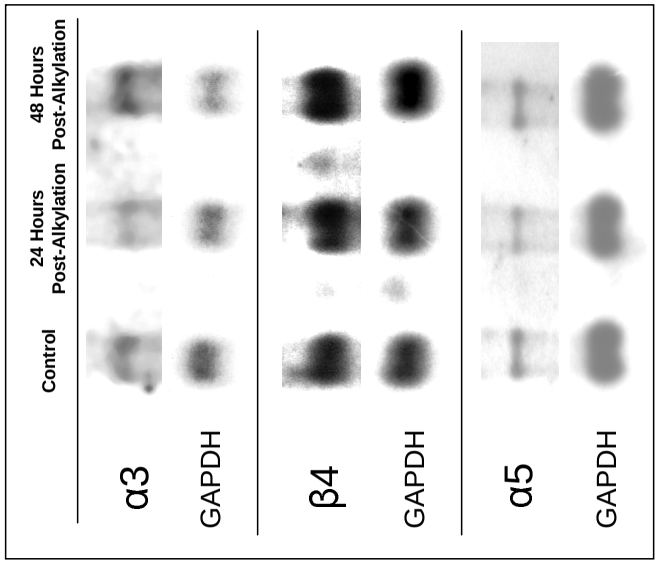

Figure 2

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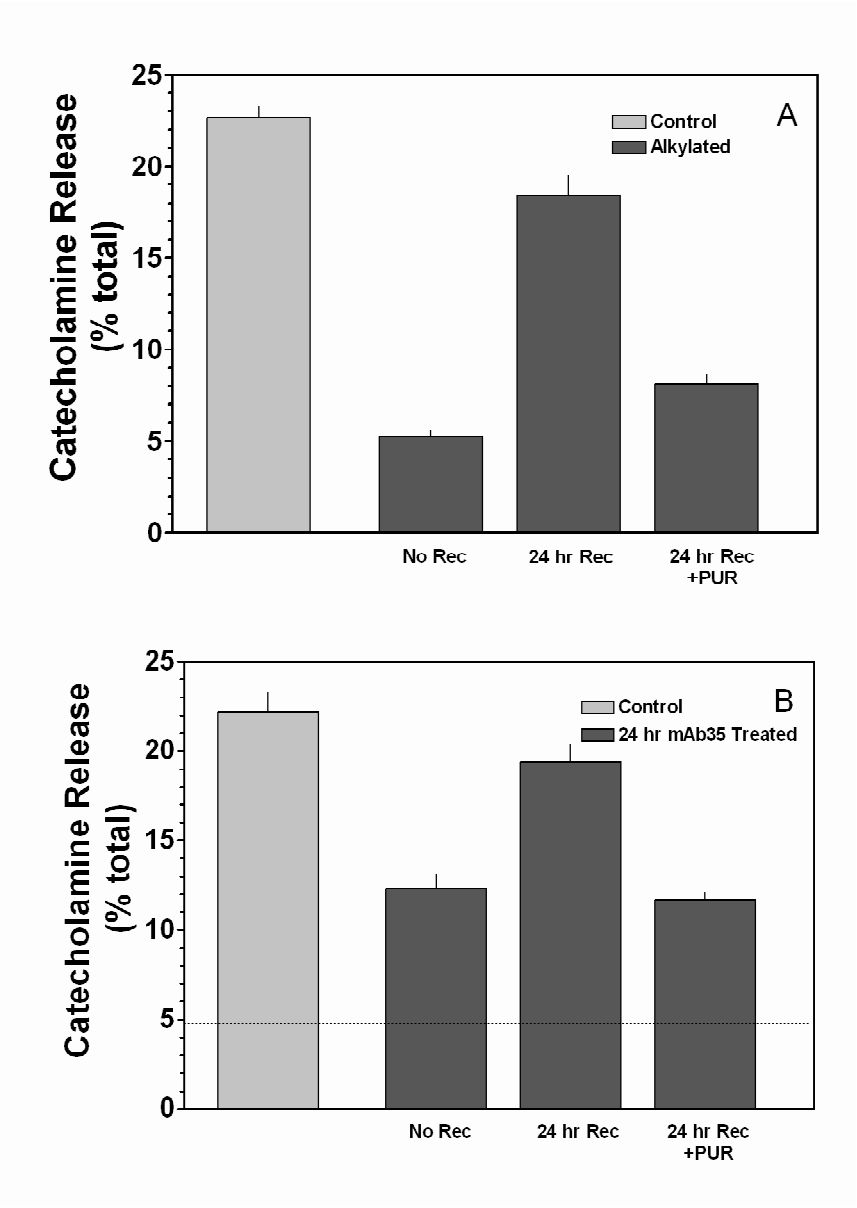

Figure 3

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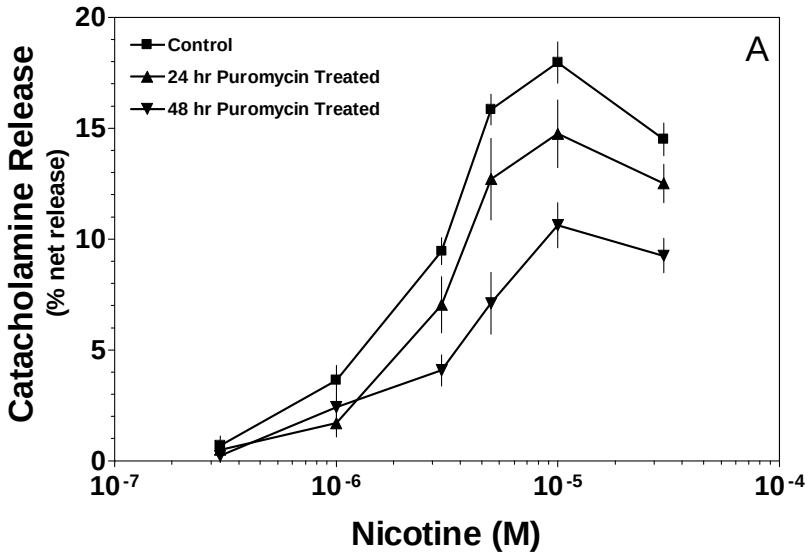

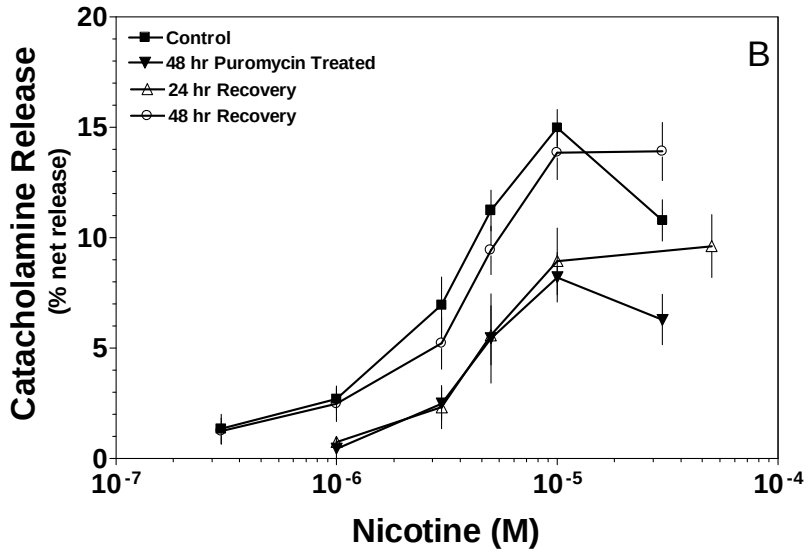

Figure 4

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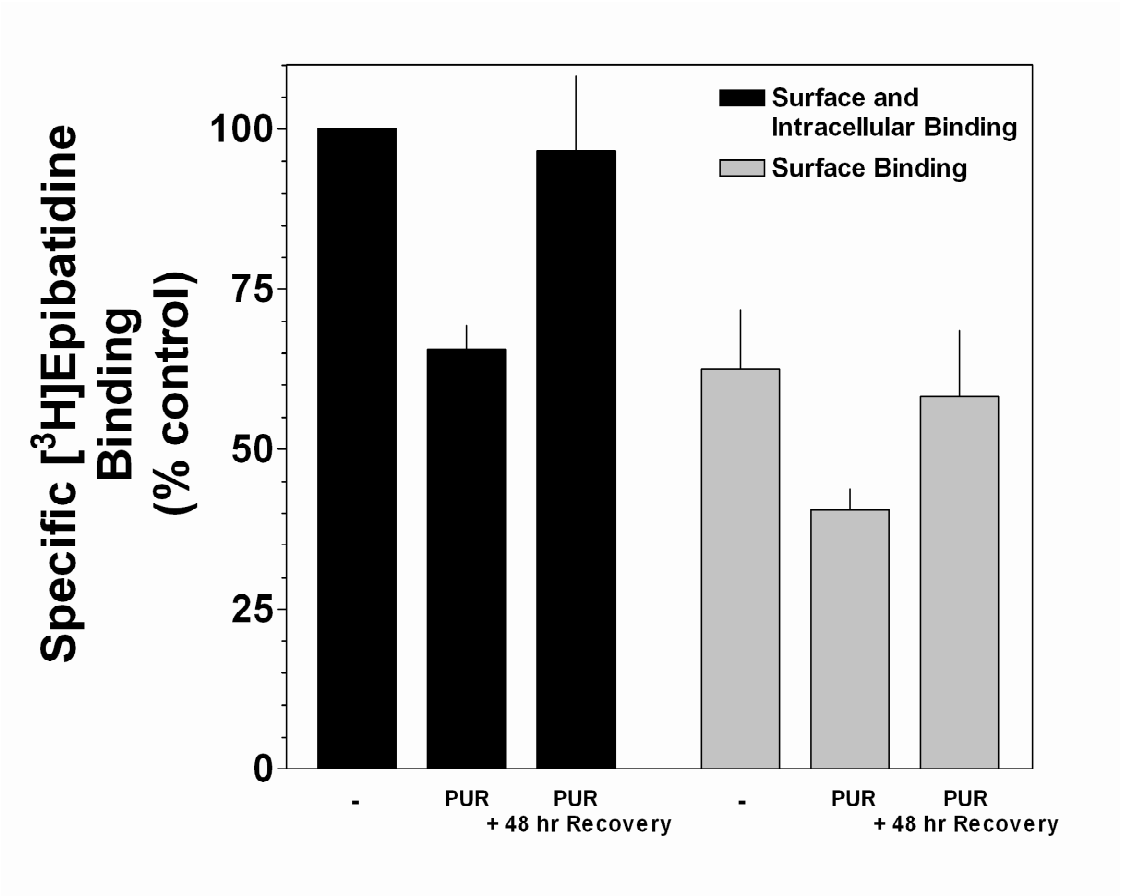

Figure 5A

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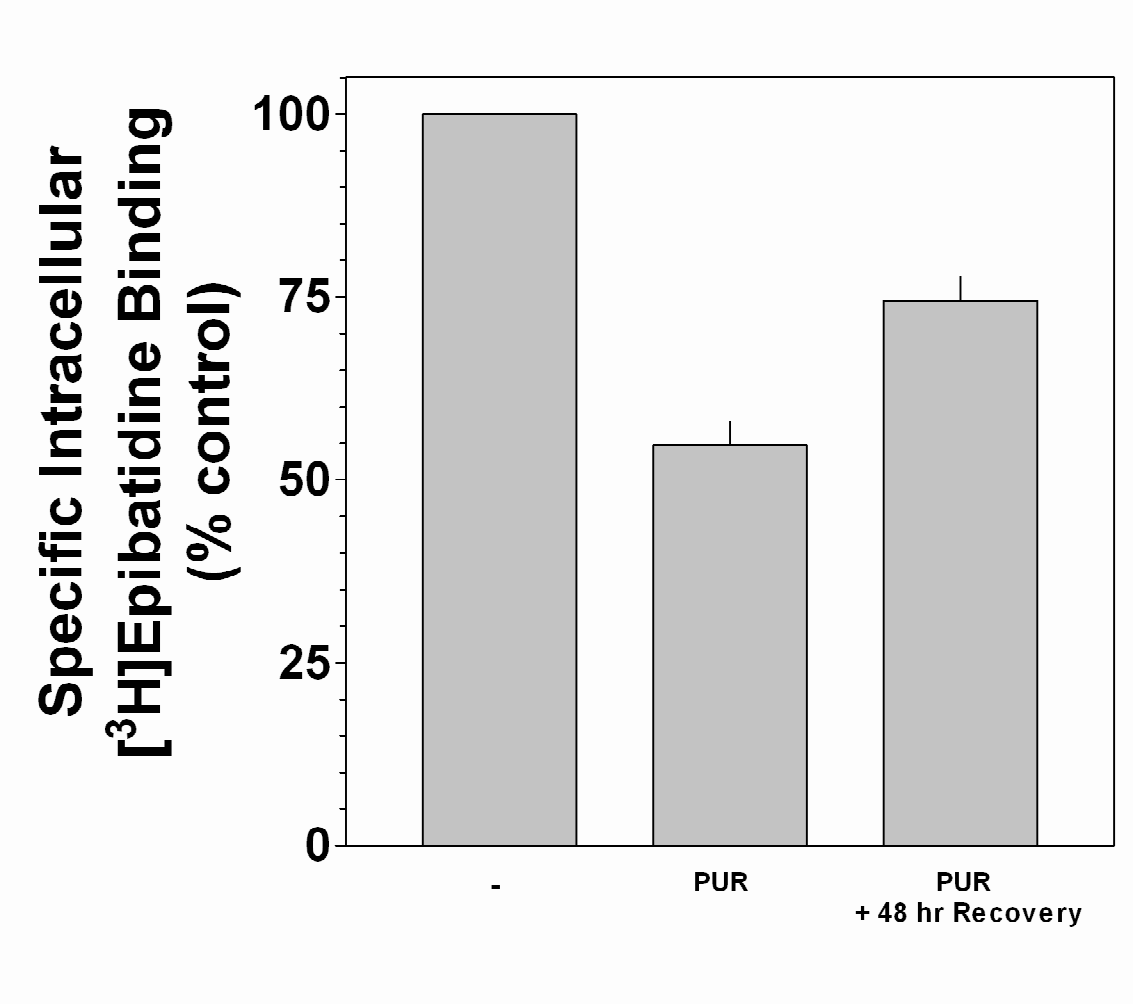

Figure 5B

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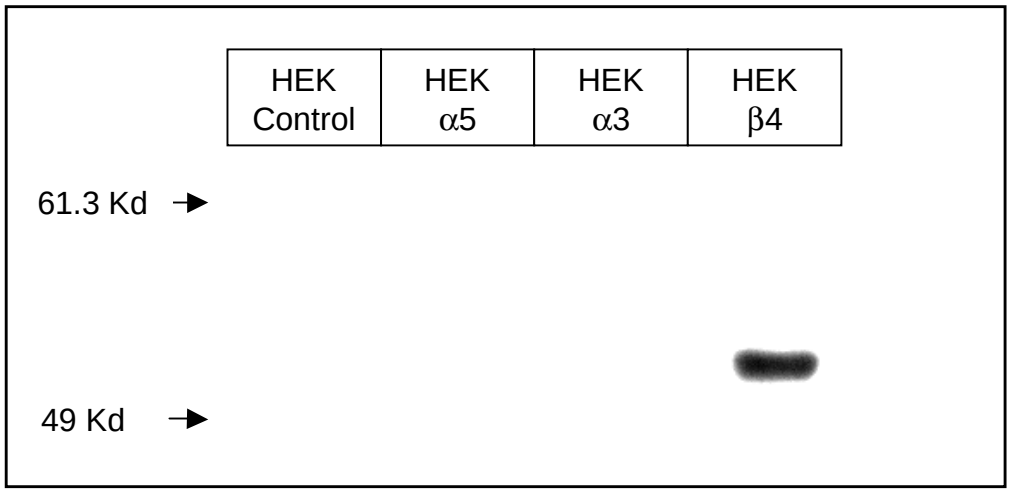

Figure 6

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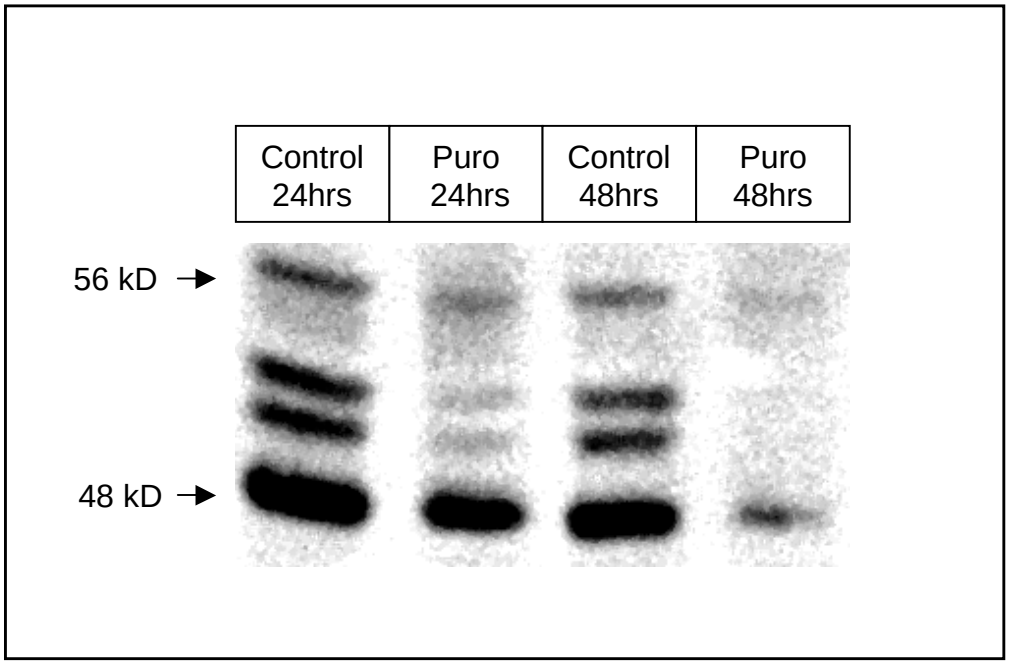

Figure 7

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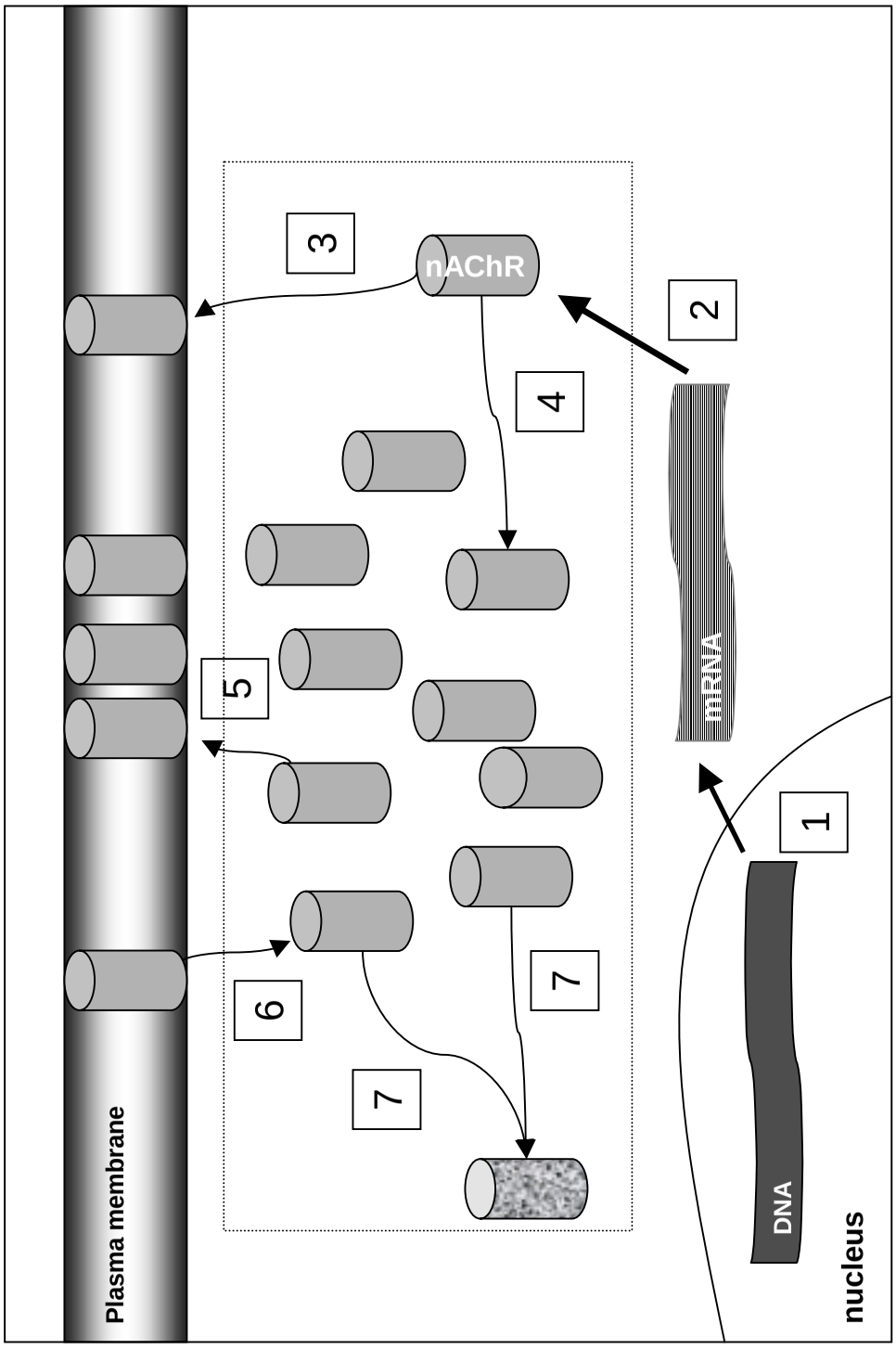

Figure 8