

UC San Diego

UC San Diego Previously Published Works

Title

Mechanisms of Inhibition and Potentiation of $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors by Members of the Ly6 Protein Family*

Permalink

<https://escholarship.org/uc/item/5q64p271>

Journal

Journal of Biological Chemistry, 290(40)

ISSN

0021-9258

Authors

Wu, Meilin

Puddifoot, Clare A

Taylor, Palmer

et al.

Publication Date

2015-10-01

DOI

10.1074/jbc.m115.647248

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

Peer reviewed

Mechanisms of Inhibition and Potentiation of $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors by Members of the Ly6 Protein Family*

Received for publication, February 24, 2015, and in revised form, July 29, 2015. Published, JBC Papers in Press, August 14, 2015, DOI 10.1074/jbc.M115.647248

Meilin Wu^{†1}, Clare A. Puddifoot^{†1}, Palmer Taylor^{†#§¶||}, and William J. Joiner^{†#§¶||**2}

From the [†]Department of Pharmacology, [§]Biomedical Sciences Graduate Program, [¶]Neuroscience Graduate Program, ^{**}Center for Circadian Biology, and ^{||}Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, California 92093-0636

Background: Several Ly6 proteins inhibit $\alpha 4\beta 2$ nAChRs, but the underlying mechanisms and the properties of homologous modulatory proteins are not well understood.

Results: Lynx2 reduces cell-surface levels of receptors, whereas Ly6g6e slows receptor desensitization.

Conclusion: Ly6 proteins inhibit or potentiate $\alpha 4\beta 2$ function by distinct mechanisms.

Significance: Ly6 proteins greatly expand the range of properties of $\alpha 4\beta 2$ nAChRs.

$\alpha 4\beta 2$ nicotinic acetylcholine receptors (nAChRs) are abundantly expressed throughout the central nervous system and are thought to be the primary target of nicotine, the main addictive substance in cigarette smoking. Understanding the mechanisms by which these receptors are regulated may assist in developing compounds to selectively interfere with nicotine addiction. Here we report previously unrecognized modulatory properties of members of the Ly6 protein family on $\alpha 4\beta 2$ nAChRs. Using a FRET-based Ca^{2+} flux assay, we found that the maximum response of $\alpha 4\beta 2$ receptors to agonist was strongly inhibited by Ly6h and Lynx2 but potentiated by Ly6g6e. The mechanisms underlying these opposing effects appear to be fundamentally distinct. Receptor inhibition by Lynx2 was accompanied by suppression of $\alpha 4\beta 2$ expression at the cell surface, even when assays were preceded by chronic exposure of cells to an established chaperone, nicotine. Receptor inhibition by Lynx2 also was resistant to pretreatment with extracellular phospholipase C, which cleaves lipid moieties like those that attach Ly6 proteins to the plasma membrane. In contrast, potentiation of $\alpha 4\beta 2$ activity by Ly6g6e was readily reversible by pretreatment with phospholipase C. Potentiation was also accompanied by slowing of receptor desensitization and an increase in peak currents. Collectively our data support roles for Lynx2 and Ly6g6e in intracellular trafficking and allosteric potentiation of $\alpha 4\beta 2$ nAChRs, respectively.

Efforts to break the cycle of nicotine addiction have been facilitated by many years of research on the properties of the receptors on which nicotine acts. These receptors are aptly

* This work was supported by National Institutes of Health Grant R01 NS072431, Whitehall Foundation Grant WF20110560, and a PhRMA Foundation Research Starter Grant (to W. J. J.), and by National Institutes of Health Grant R01 GM18360 (to P. T.). The authors declare that they have no conflict of interest with the contents of this article.

[†] These authors contributed equally to this work.

² To whom correspondence should be addressed: Dept. of Pharmacology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA. Tel.: 858-534-3375; E-mail: wjoiner@ucsd.edu.

named nicotinic acetylcholine receptors (nAChRs)³ and mediate fast synaptic transmission in response to the endogenous neurotransmitter acetylcholine or to the exogenous agonist nicotine, which is the principle addictive substance in cigarette smoke. nAChRs are intrinsic membrane proteins that conduct Na^+ , K^+ , and Ca^{2+} ions through a central lumen that is opened upon extracellular binding of agonist to the interface between subunits. In mammalian neurons these subunits express as nine α and three β isoforms, which combine differentially to produce functional homopentamers or heteropentamers. The most abundantly expressed combinations in the mammalian CNS are $\alpha 7$ homomers and $\alpha 4\beta 2$ heteromers (1).

Several lines of evidence suggest that $\alpha 4\beta 2$ combinations are the main nAChRs that contribute to nicotine addiction. $\alpha 4\beta 2$ nAChRs have particularly high affinity for nicotine (2–5). They are expressed in regions of the brain implicated in addiction, including the ventral tegmental area (6–8). Knock-out of their individual subunits also drastically reduces high affinity nicotine binding in the brain as well as nicotine self-administration in mice (9, 10). Lastly, $\alpha 4\beta 2$ nAChRs are particularly up-regulated during chronic exposure to nicotine, as measured by changes in binding sites for radiolabeled agonist at the cell surface (11–15). This effect is thought to contribute to nicotine's addictive properties and possibly to its neuroprotective effects in Parkinson Disease (16–22).

The mechanisms underlying the up-regulation of $\alpha 4\beta 2$ nAChRs appear to be 2-fold. First, after diffusing across cell membranes, nicotine binds to and stabilizes $\beta 2$ subunits in the endoplasmic reticulum (ER), thus indirectly enhancing $\alpha 4\beta 2$ co-assembly (23, 24). Second, nicotine is thought to convert $\alpha 4\beta 2$ nAChRs from a low to high affinity conformation. Interestingly, this conversion process occurs even among receptors at the cell surface that have been isolated from pools of intracellular subunits (24, 25). Various hypotheses have been put forward to account for these two mechanisms. For example, it

³ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; PLC, phospholipase C; ACh, acetylcholine.

Novel Regulation of $\alpha 4\beta 2$ nAChRs by Ly6 Proteins

has been proposed that by promoting assembly of $\alpha 4\beta 2$ heteromers, nicotine acts like a chaperone for nAChRs (24, 26–28). Recent evidence also suggests that nicotine can alter the stoichiometry of nAChRs by shifting assembly from low affinity $\alpha 4_3\beta 2_2$ toward high affinity $\alpha 4_2\beta 2_3$ receptors (29, 30). Finally, it has also been proposed that nicotine exposure imposes on $\alpha 4\beta 2$ receptors a form of hysteresis in which receptors undergo conformational changes that leave them in a more activatable state (25).

Complicating these interpretations is the association of nAChRs with auxiliary subunits called Ly6 proteins, which have been identified in various cell types. These modulatory proteins are generally small and composed of single domains that are tethered to the outer leaflet of the plasma membrane by a glycosylphosphatidyl inositol (GPI) moiety, though in a few cases Ly6 proteins lack the GPI anchor and appear to be secreted (31, 32). Some Ly6 proteins, such as Lynx1 and Lynx2, have been shown to accelerate desensitization and reduce the sensitivity of nAChRs to agonist (33, 34). Recently evidence has emerged that the latter effect may in part be caused by Ly6-mediated alterations in subunit oligomerization and thus $\alpha 4\beta 2$ stoichiometry (30). Such changes could affect both receptor sensitivity to acute agonist as well as the percentage of receptors that are susceptible to up-regulation by chronic nicotine.

Because the Ly6 family is encoded by dozens of genes, most of which have not been studied in any detail, we hypothesized that some of these molecules might regulate $\alpha 4\beta 2$ receptors in ways that are relevant to nicotine addiction that have not been previously recognized. In this study, we tested this hypothesis by examining the acute functional and chronic cell biological effects of 9 different mammalian Ly6 proteins on $\alpha 4\beta 2$ nAChRs in transfected HEK293 cells. We show that several Ly6 proteins are able to form complexes with $\alpha 4\beta 2$ nAChRs and to suppress the maximal response of these receptors to agonist. We also show that Lynx2 has a previously unrecognized ability to reduce levels of $\alpha 4\beta 2$ nAChRs at the cell surface. The trafficking effect of Lynx2 is particularly notable in that it suppresses up-regulation of $\alpha 4\beta 2$ nAChRs following chronic exposure to nicotine. In addition to this novel antagonistic property, we also demonstrate that a previously uncharacterized Ly6 protein, Ly6g6e, potentiates $\alpha 4\beta 2$ signaling by slowing receptor desensitization. Our data suggest that the Ly6 proteins and the mechanisms they employ to modulate $\alpha 4\beta 2$ nAChRs are far more varied than previously recognized, with important implications for nicotine-mediated changes in nervous system function.

Experimental Procedures

Molecular Biology—Mouse Ly6 genes were cloned from expressed sequence tags (ESTs) (Open Biosystems) or from adult CD1 mouse brain cDNA as previously described (35). $\alpha 4$ -HA was generated by PCR amplifying $\alpha 4$ from GFP- $\alpha 4$ -pciNeo (gift from Dr. H. Lester) with the following primers (5'-3'): F: AAAAGCTTACCATTGGAGATCGGGGGCTCCG; R: TCTAGAGAGCTAGGTGCCGCTATTCC and subcloned into pcDNA3 in-frame with a C-terminal hemagglutinin (HA) tag (36) between the HindIII and XbaI sites.

FRET-based Measurements of nAChR Activity—Measurements of $\alpha 4\beta 2$ nAChR activity were performed as previously

described (37) with the following modifications: HEK293 cells were transiently transfected with untagged $\alpha 4$ -pciNeo and $\beta 2$ -dm-pciNeo (gifts from Dr. Henry Lester) plus the calcium FRET reporter TN-XXL supplemented with either Ly6-pcDNA3 or pcDNA3 alone at a ratio of 1:1:2:5, respectively. One day after transfection, cells were replated into clear-bottom 96-well plates coated with poly-L-lysine (Sigma) (one column of 8 wells per transfection reaction) allowing for treatment of identically transfected cells with 8 concentrations of agonist. Cells were then exposed to 1 μ M nicotine (Tocris, R&D) overnight. 20 h post nicotine addition, the media was removed and replaced with artificial cerebral spinal fluid (ACSF: 121 mM NaCl, 5 mM KCl, 26 mM NaHCO₃, 1.2 mM NaH₂PO₄·H₂O, 10 mM glucose, 5 mM HEPES, 2.4 mM CaCl₂, 1.3 mM MgCl₂, pH 7.4) and assayed for response to increasing concentrations of epibatidine. Peak response for each well was recorded and used to generate concentration-response curves. For phospholipase C (PLC) experiments, cells were pre-incubated in Optimem (Life Technologies) with or without 0.35 units/ml PLC (Life Technologies) for 30 min at 37 °C prior to assay. Following PLC treatment, cells were placed in ACSF, and agonist responses were measured as described above. Maximum responses were calculated from concentration-response curves averaged over at least four experiments, each performed in triplicate, with results normalized to the maximum response of $\alpha 4\beta 2$ nAChRs alone. One-way ANOVA repeated measures analysis with Dunnett's multiple comparison post-test was used for statistical analysis.

Cell Culture and Cell Surface Biotinylation—HEK293 cells were maintained at 37 °C and 5% CO₂ in culture medium consisting of 10% fetal bovine serum (Omega), 1% penicillin/streptomycin (Mediatech) and 1% L-glutamine (Sigma) in low glucose DMEM supplemented with 2 mM L-Glutamine (Mediatech). Cells were grown to 50–80% confluence for transfection with X-tremegene HP reagent (Roche) at a 2:1 ratio of transfection reagent to DNA in Optimem (Life Technologies). Transfection mixture was removed 24 h after transfection and replaced with normal growth medium. Cells were lysed 48 h after transfection in SDS lysis/IP buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.05% SDS) with Complete Protease Inhibitors (Roche). For surface biotinylation assays, 48 h after transfection cells were rinsed twice in PBS (pH 8.0) and incubated in PBS (pH 8.0) with 0.3 mg/ml NHS-biotin (Pierce) for 45 min on ice. The biotinylation reaction was quenched by two five-minute incubations in quenching buffer (50 mM Tris base, pH 7.4, 275 mM NaCl, 6 mM KCl, 2 mM CaCl₂). Labeled cells were rinsed once in PBS prior to lysis with 250 mM NaCl, 50 mM Tris HCl, pH 7.4, 5 mM EDTA, 0.5% Triton X-100, and Complete Protease Inhibitors (Roche).

Primary Rat Neuron Cultures and RT-PCR—Primary hippocampal and cortical neurons were prepared from P2 rat tissue obtained by tissue transfer protocol T14121 from Joan Heller Brown as previously described (35). mRNA was extracted from these cells, and RT-PCR was performed as previously described (35) with the following primers (5'-3'): Lynx2-F: CGGCATCGCAGCAACTTTTGTG; Lynx2-R: TTGCAAAGAGGGGTGTTGCAG; Ly6g6e-F: CTGTTACACCT-

GCAGCTTTGCC; Ly6g6e-R: GGTAGCAGAGTCATGAG-GGGA AG.

Immunoprecipitation and Immunoblotting—For streptavidin immunoprecipitations and co-immunoprecipitation assays, 500–700 μg of total cell lysate was incubated in a total volume of 1 ml RIPA buffer (150 mM NaCl, 25 mM Hepes, pH 7.5, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate) with Complete Protease Inhibitors (Roche) and rabbit anti-GFP (Life Technologies) antibody or streptavidin-conjugated agarose beads (Pierce) overnight at 4 °C on a rotating platform. The following day, protein-G conjugated magnetic beads (NEB) were added to anti-GFP immunoprecipitation samples and incubated at 4 °C for 2–5 h. Beads were washed three times in 1 ml of RIPA buffer and resuspended in 2 \times LDS sample buffer (Life Technologies). For $\alpha 4$ -HA surface-labeled co-immunoprecipitations, transfected cells were washed once with ice-cold PBS and then incubated with rabbit anti-HA antibodies (Rockland) diluted in Optimem for 1 h on ice. Unbound antibody was removed by washing with ice-cold PBS. Cells were lysed as described above. 10% of the lysate was reserved for input sample and the remainder was diluted to 1 ml total volume in IP buffer (10 mM Tris pH. 7.5, 100 mM NaCl, 0.5% Triton X-100, 0.05% SDS). Lysate was incubated with protein G magnetic beads (NEB) for 2 h at 4 °C on a rotating platform, washed twice in 1 ml of IP buffer, and then resuspended in 2 \times LDS sample buffer (Life Technologies). Western blot analyses of cell lysates and co-immunoprecipitation complexes were performed as previously described (38). For input conditions, 10–20% of the amount of protein used for IP was loaded per lane as indicated in figure legends. Proteins were detected using the following antibodies: rabbit anti-GFP (Life Technologies), mouse anti-Myc (Santa Cruz Biotechnologies), mouse anti-HA (Covance), and mouse anti-actin (Millipore EMD).

Electrophysiology—Whole-cell patch clamp electrophysiology was used to measure agonist-evoked currents from HEKtsa cells ~ 48 h after transfection as previously described (35). pBOB GFP was included with wild-type nAChR and Ly6 cDNAs to visualize transfected cells for recordings. Coverslips containing transiently transfected HEKtsa cells were transferred into a recording chamber containing (in mM): 140 NaCl, 3 KCl, 25 HEPES, 1 CaCl₂, 1 MgCl₂, 20 glucose, pH 7.3. Patch pipettes (resistance 5–10 M Ω) were filled with internal recording solution containing (in mM): 100 K-gluconate, 30 KCl, 5 EGTA, and 10 HEPES, pH 7.3 (300 mOsm). Cells were visualized using a 40 \times water immersion lens (Olympus). Cells were voltage clamped at -60 mV, and whole-cell currents were evoked by application of 1 mM acetylcholine adjacent to the patched cell at a constant flow rate of 4 ml/min at room temperature (22–25 °C) using a ValveLink8.2 Pinch Valve Perfusion System (Automate). Data were acquired using a Multi-clamp 700B amplifier (Molecular Devices, Union City, CA) at a sampling rate of 10 kHz and filtered at 2 kHz. Currents were subsequently analyzed using P-Clamp 10.4 (Molecular Devices, Union City, CA).

Results

Several Ly6 Proteins Modulate $\alpha 4\beta 2$ nAChR Activity—The best characterized members of the mammalian Ly6 family,

Lynx1 and Lynx2, have both been shown to form complexes with $\alpha 4\beta 2$ nAChRs in transfected HEK-293 cells and to modulate receptor activity when co-expressed in oocytes (33, 34), as well as in knock-out mice (34, 39). Since these are only two examples of a large protein family with over 40 related members (40), we decided to test additional members for functional modulation of nAChR activity.

We previously cloned 9 Ly6 genes from mouse brain ESTs or total mouse brain cDNA (35). To test if the encoded Ly6 proteins could modulate $\alpha 4\beta 2$ nAChR activity, we individually co-expressed them with mouse $\alpha 4\beta 2$ nAChRs in transiently transfected HEKtsa cells. Using the calcium-sensitive ratiometric FRET reporter, TN-XXL, we measured calcium influx into transfected cells in response to increasing concentrations of the agonist epibatidine as previously described (37). Since $\alpha 4\beta 2$ nAChRs are significantly less permeable to calcium than $\alpha 7$ nAChRs (reviewed in Ref. 41), we pre-treated transfected cells with 1 μM nicotine for 20 h prior to assaying for receptor activity to increase the levels of functional $\alpha 4\beta 2$ nAChRs at the cell surface, as previously described (24, 28, 42, 43). In addition, we used a mutant $\beta 2$ subunit ($\beta 2$ -dm) which has been reported to enhance export from the endoplasmic reticulum (ER) (44). The combination of these two factors, *i.e.* nicotine pre-treatment and enhanced ER export, resulted in a nearly 4-fold increase in agonist-specific FRET signal (Fig. 1C). Thus, all subsequent $\alpha 4\beta 2$ FRET assays were performed using the $\beta 2$ -dm ER-export mutant following 20 h of pretreatment with 1 μM nicotine.

Despite these enhancements, the FRET signals achieved with epibatidine stimulation of $\alpha 4\beta 2$ nAChRs were still too low to plot reliable slopes of concentration-response curves, thus preventing quantification of EC₅₀ values. However, maximum FRET responses were highly reproducible, allowing us to utilize this assay as a high-throughput method of screening many Ly6 proteins for up- or down-regulation of $\alpha 4\beta 2$ activity at saturating concentrations of agonist. Using this assay we showed that the maximum response of $\alpha 4\beta 2$ to epibatidine decreased by over 50% in the presence of Lynx2 or Ly6h, and to a lesser but still significant extent in the presence of Ly6e and Ly6g6d, compared with controls measured in the absence of Ly6 proteins. In contrast, co-expression of $\alpha 4\beta 2$ nAChRs with Ly6g6e caused a 2-fold increase in the maximum FRET response to epibatidine (Fig. 1A, B). Thus, depending on the identity of the Ly6 proteins that were present, $\alpha 4\beta 2$ nAChR signaling could be suppressed or potentiated.

Modulatory Ly6 Proteins Form Stable Complexes with nAChRs and Express in Important Brain Regions—Because we found that several Ly6 proteins can functionally modulate the activity of $\alpha 4\beta 2$ nAChRs, we hypothesized that receptors and modulators should be able to form stable complexes. To test for such putative interactions, we first tagged each Ly6 construct with a Myc epitope just before the encoded C-terminal attachment site to GPI. We then co-expressed Myc-tagged Ly6 constructs with functional GFP-tagged $\alpha 4\beta 2$ nAChRs (43) in transiently transfected HEKtsa cells. We note that in Western blots of lysates from these cells some Ly6 proteins appear to have multiple bands. These are consistent with variable states of maturation, including removal of the N-terminal signal peptide prior to exit from the ER and cleavage of the C-terminal peptide

Novel Regulation of $\alpha 4\beta 2$ nAChRs by Ly6 Proteins

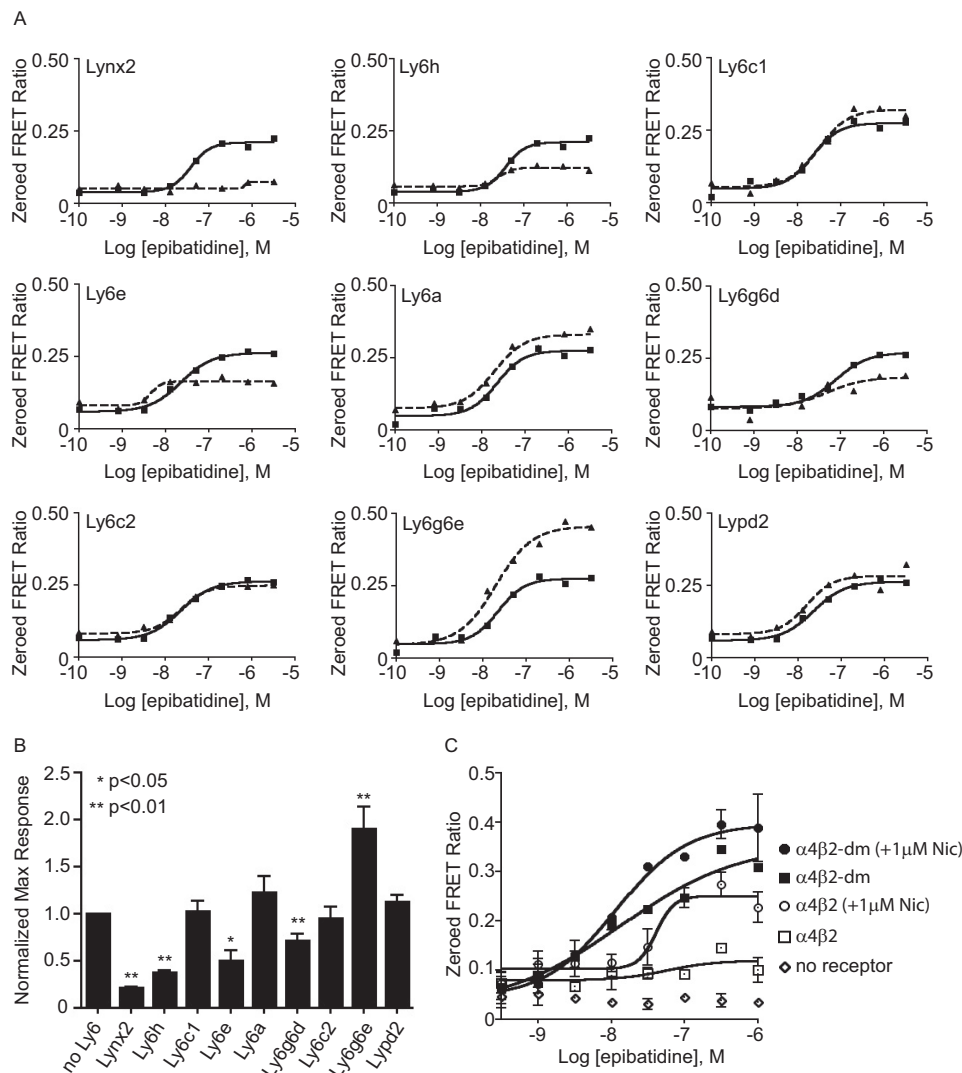


FIGURE 1. Ly6 proteins modulate $\alpha 4\beta 2$ nAChR activity. *A*, representative concentration-response curves for activation of $\alpha 4\beta 2$ nAChRs by epibatidine in the absence (solid lines) or presence (dashed lines) of various Ly6 proteins in transfected HEK293 cells following 20 h pre-treatment with 1 μ M nicotine. *B*, average maximum responses of $\alpha 4\beta 2$ nAChRs to epibatidine. All measurements were normalized to control values for receptor alone. $n = 7$ for each condition. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with Dunnett's multiple comparison test. Error bars indicate S.E. *C*, nicotine pre-treatment (Nic) and $\beta 2$ ER-export mutation (dm) both enhance $\alpha 4\beta 2$ functional expression. Representative concentration-response curves of $\alpha 4\beta 2$ nAChRs to epibatidine. Open diamonds (no receptor control) show that no signal is produced in the absence of transfected $\alpha 4\beta 2$ subunits.

prior to attachment of the GPI moiety. In addition, several Ly6 proteins are predicted to be further modified by glycosylation, resulting in additional differences in migration.

Immunoprecipitation of GFP- $\alpha 4$ from these lysates followed by Western blotting for Myc revealed that all Ly6 proteins that suppressed $\alpha 4\beta 2$ activity also co-immunoprecipitated with $\alpha 4\beta 2$ nAChRs. Notably Ly6a, which had no functional effect on $\alpha 4\beta 2$ nAChR activity in our flux assay, also did not co-immunoprecipitate with $\alpha 4\beta 2$ subunits (Fig. 2, *A* and *B*). In the case of Ly6g6e, which potentiated rather than inhibited $\alpha 4\beta 2$ activity, we did not detect an interaction following immunoprecipitation with total cellular $\alpha 4\beta 2$. However, Ly6g6e (but not Ly6a) was selectively detected in complex with $\alpha 4\beta 2$ from a membrane fraction enriched for cell surface expression using a C-terminal HA-tagged $\alpha 4$ (Fig. 2*B*). These results support a direct association between Ly6 proteins and the nAChRs that they regulate.

We also tested the selectivity of Ly6 proteins for nAChR subunits by co-expressing Myc-tagged Lynx2 with GFP- $\alpha 4$ or GFP- $\beta 2$ alone, or with GFP- $\alpha 4$ plus untagged $\beta 2$. Immunoprecipitation of GFP-tagged nAChR subunits followed by Western blotting for Myc revealed that Lynx2 selectively forms complexes with $\alpha 4$ compared with $\beta 2$ subunits (Fig. 2*C*). We were unable to perform analogous experiments with Ly6g6e due to an inability to express $\alpha 4$ and $\beta 2$ independently at the cell surface, where Ly6g6e interacts with nAChRs.

Since Lynx2 and Ly6g6e had the most extreme influence on receptor function, we examined the expression profiles of their transcripts in select brain regions using RT-PCR. We found that both Lynx2 and Ly6g6e are expressed in cultured primary rat hippocampal and cortical neurons as well as the midbrain (Fig. 2*D*). Notably, these brain regions are thought to be important for cognition and nicotine addiction, which are processes in which $\alpha 4\beta 2$ nAChRs are believed to participate (6–8).

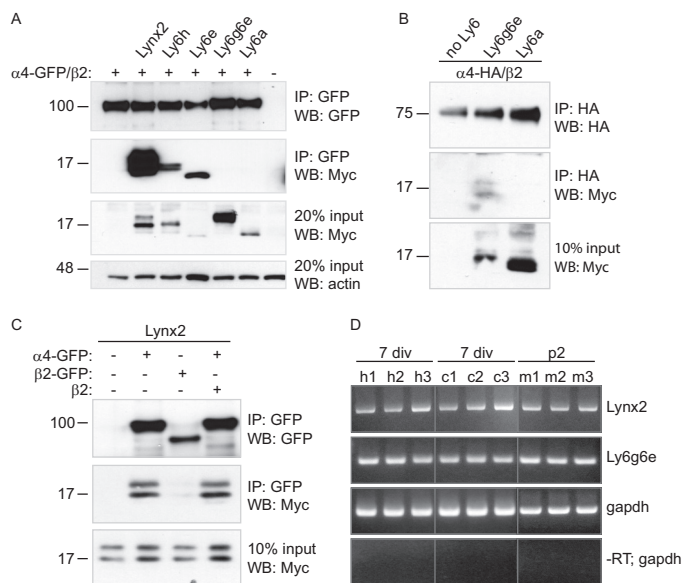


FIGURE 2. $\alpha 4\beta 2$ nAChRs form stable complexes with the Ly6 proteins that modulate their activities. *A*, representative immunoblots of immunoprecipitated $\alpha 4$ subunits in HEK293 cells transfected with $\beta 2$, GFP-tagged $\alpha 4$, and various Myc-tagged Ly6 cDNAs. Ly6a (*A*, lane 6) was included as a negative control and does not co-immunoprecipitate with $\alpha 4$ subunits. *Top panel*: anti-GFP immunoprecipitation samples blotted with anti-GFP. *Upper-middle panel*: anti-GFP immunoprecipitation samples blotted with anti-Myc. *Lower-middle panel*: 20% input total cell lysates blotted with anti-Myc. *Bottom panel*: input samples blotted with anti-actin as a loading control. *B*, representative immunoblots of surface labeled $\alpha 4$ subunits in HEK293 cells transfected with $\beta 2$, HA-tagged $\alpha 4$, and Myc-tagged Ly6g6e or Ly6a cDNAs. Ly6a was included as a negative control and does not co-immunoprecipitate with HA- $\alpha 4$ at the cell surface. *Top panel*: anti-HA surface immunoprecipitation samples blotted with anti-HA. *Middle panel*: anti-HA surface immunoprecipitation samples blotted with anti-Myc. *Bottom panel*: 10% input total cell lysates blotted with anti-Myc. *C*, representative immunoblots of immunoprecipitated complexes from HEK293 cells transfected with Myc-tagged Lynx2 alone (*lane 1*) or in combination with GFP-tagged $\alpha 4$ (*lane 2*), GFP-tagged $\beta 2$ (*lane 3*), or GFP-tagged $\alpha 4$ and untagged $\beta 2$ (*lane 4*). *Top panel*: anti-GFP immunoprecipitation samples blotted with anti-GFP. *Middle panel*: anti-GFP immunoprecipitation samples blotted with anti-Myc. *Bottom panel*: 10% input total cell lysates blotted with anti-Myc. *D*, RT-PCR analysis of Lynx2 and Ly6g6e expression in cultured primary rat hippocampal (h1, 2, 3) and cortical (c1, 2, 3) neurons and freshly dissected P2 rat midbrain (m1, 2, 3). Gapdh is included as a loading control and gapdh (–RT) as a negative control.

Lynx2 Suppresses $\alpha 4\beta 2$ Expression at the Cell Surface—The striking changes in the maximal FRET responses of $\alpha 4\beta 2$ nAChRs in the presence of certain Ly6 proteins could result from regulation of receptor levels at the cell surface, receptor gating, or receptor ion selectivity (since our assay is based on intracellular Ca^{2+}). To determine the effects of Ly6 proteins on $\alpha 4\beta 2$ cell surface levels, we expressed GFP- $\alpha 4\beta 2$ alone or with Ly6 proteins in HEK293 cells and labeled the extracellular surfaces of all plasma membrane proteins by biotinylation. We then immunoprecipitated either plasma membrane localized receptors with streptavidin or total cellular receptors with anti-GFP antibody. Western blot analysis of biotinylated surface-expressed GFP- $\alpha 4$ revealed a significant decrease in receptor levels without major effects on total cellular receptor expression when Lynx2 was present (Fig. 3, *A* and *B*). This result suggests that the decreased response to agonist stimulation is largely attributable to reduced abundance of $\alpha 4\beta 2$ at the cell surface. In contrast, Ly6g6e co-expression had no effect on $\alpha 4$ levels at the plasma membrane or throughout cells (Fig. 3*A*, *B*). Thus, the potentiation we observed in $\alpha 4\beta 2$ signaling in the

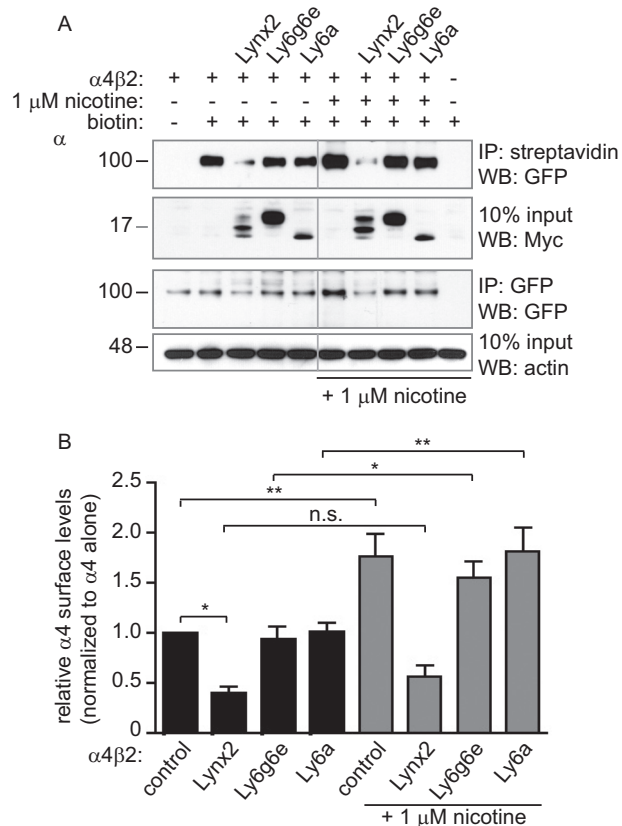


FIGURE 3. Ly6 proteins regulate $\alpha 4\beta 2$ nAChR localization at the plasma membrane. *A*, representative immunoblots of streptavidin immunoprecipitations of surface biotinylated $\alpha 4\beta 2$ nAChRs in transiently transfected HEK293 cells. *Top panels*: streptavidin immunoprecipitation samples immunoblotted with anti-GFP antibody to assess surface $\alpha 4$ -GFP levels. *Upper middle panels*: 10% input total cell lysates blotted with anti-Myc antibody for Ly6 protein expression. *Lower middle panels*: anti-GFP immunoprecipitation samples blotted with anti-GFP antibody for total $\alpha 4$ -GFP levels. *Bottom panels*: 10% input total cell lysates blotted with anti-actin antibody as a loading control. *B*, average $\alpha 4\beta 2$ surface protein levels quantified by measured pixel density of streptavidin immunoprecipitated receptors normalized to immunoprecipitation from total cellular lysates ($n = 8$). Control condition was from cells transfected with empty vector. Co-expression of Lynx2 reduces $\alpha 4\beta 2$ surface expression in the absence of nicotine (*left panels*) and blocks receptor up-regulation following 20 h pretreatment with 1 μM nicotine (*right panels*). In contrast, Ly6g6e and Ly6a have no effect on $\alpha 4\beta 2$ surface expression ($n = 8$). *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with Bonferroni's multiple comparison test. Error bars indicate S.E.

presence of Ly6g6e cannot be caused by increased receptor density at the cell surface. Similarly, Ly6a, which had no effect on $\alpha 4\beta 2$ activity in our FRET-based flux assay, also does not change receptor levels at the cell surface (Fig. 3, *A* and *B*).

Since chronic nicotine exposure has been shown to increase export of $\alpha 4\beta 2$ nAChRs to the cell surface (24, 28, 42, 43), we examined the impact of modulatory Ly6 proteins on receptor chaperoning by nicotine. As expected, pre-incubation with 1 μM nicotine for 20 h prior to biotin labeling and cell lysis resulted in an increase in $\alpha 4$ levels at the cell surface (Fig. 3*A*, *right panel*, and 3*B*, *gray bars*). However, co-expression of Lynx2 severely blunted this nicotine-induced up-regulation, suggesting that Lynx2 interferes with the pathway by which nicotine chaperones $\alpha 4\beta 2$ export to the cell surface. In contrast, Ly6g6e and Ly6a had no effect on nicotine-mediated up-regulation of cell surface levels of $\alpha 4$ subunits (Fig. 3*A*, *right panel*, and 3*B*, *gray bars*).

Novel Regulation of $\alpha 4\beta 2$ nAChRs by Ly6 Proteins

Ly6g6e Functions at the Cell Surface to Potentiate $\alpha 4\beta 2$ Activity—Since Ly6g6e does not alter receptor trafficking and seems to form a stable complex with $\alpha 4\beta 2$ most efficiently at the cell surface, we reasoned that this Ly6 protein might potentiate the maximum response of activated $\alpha 4\beta 2$ nAChRs instead by altering the biophysical properties of receptors at the plasma membrane. We thus hypothesized that removal of Ly6g6e selectively from the cell surface would restore normal activity to $\alpha 4\beta 2$ nAChRs. To test this hypothesis, we applied soluble phospholipase C (PLC) to the extracellular solution surrounding transiently transfected HEKtsa cells 30 min prior to agonist application. PLC cleaves GPI-linked molecules, including Ly6 proteins, from the outer leaflet of the plasma membrane (45). Consistent with our hypothesis, PLC pretreatment nearly abolished the Ly6g6e-mediated potentiation of $\alpha 4\beta 2$ maximal response to agonist (Fig. 4A, gray bars). These results strongly support a role for Ly6g6e in modulating $\alpha 4\beta 2$ activity. In contrast, the Lynx2-mediated suppression of $\alpha 4\beta 2$ maximal response was maintained following PLC treatment, supporting our hypothesis that Lynx2 mediates much of its effects intracellularly to suppress surface expression of $\alpha 4\beta 2$ nAChRs, as demonstrated by our biotin labeling experiments.

Ly6g6e Enhances Whole-cell $\alpha 4\beta 2$ nAChR Currents—To further investigate the modulatory role of Ly6g6e on $\alpha 4\beta 2$ function, we used whole-cell voltage clamp to record acetylcholine (ACh)-evoked currents in transiently transfected HEKtsa cells in the absence or presence of Ly6g6e. In contrast to our flux assays in Fig. 1, which enabled us to screen for changes in the total agonist-evoked calcium influx in a population of cells, electrophysiology allowed us to analyze the effect of Ly6g6e on $\alpha 4\beta 2$ nAChR current amplitude and kinetics in individual cells. Based on our previous data, we hypothesized that Ly6g6e enhances $\alpha 4\beta 2$ nAChRs through direct modulatory effects at the cell surface. Indeed, co-expression of Ly6g6e increased $\alpha 4\beta 2$ nAChR current amplitude in response to a saturating concentration of acetylcholine (1 mM; Fig. 4, B and C). Thus, the potentiation cannot be explained by a shift in the agonist EC_{50} . The potentiation also persisted in the absence of extracellular calcium (Fig. 4D). Thus, the increased calcium currents identified by flux assay in Fig. 1 cannot be attributed to a change in ion selectivity of $\alpha 4\beta 2$ nAChRs.

Ly6g6e Slows Desensitization of $\alpha 4\beta 2$ nAChRs—Having ruled out changes in surface expression and changes in ion selectivity as probable mechanisms by which cell surface Ly6g6e potentiates $\alpha 4\beta 2$ -mediated currents, we examined changes in channel gating. Specifically, we measured the decay kinetics of whole-cell $\alpha 4\beta 2$ nAChR currents in the presence and absence of Ly6g6e. Following application of 1 mM acetylcholine, transfected cells displayed fast activating (<15 ms time to peak) inward currents followed by a biphasic decay to baseline that could be approximated by the sum of two exponentials. We found that co-expression of Ly6g6e significantly slowed both kinetic components of $\alpha 4\beta 2$ nAChR desensitization (Fig. 5, A–C), tau fast (τ_f) and tau slow (τ_s), without affecting the relative contribution of each component to the total decay (Fig. 5D).

To determine whether chronic exposure to nicotine might influence the gating effects of Ly6g6e that we observed, we next

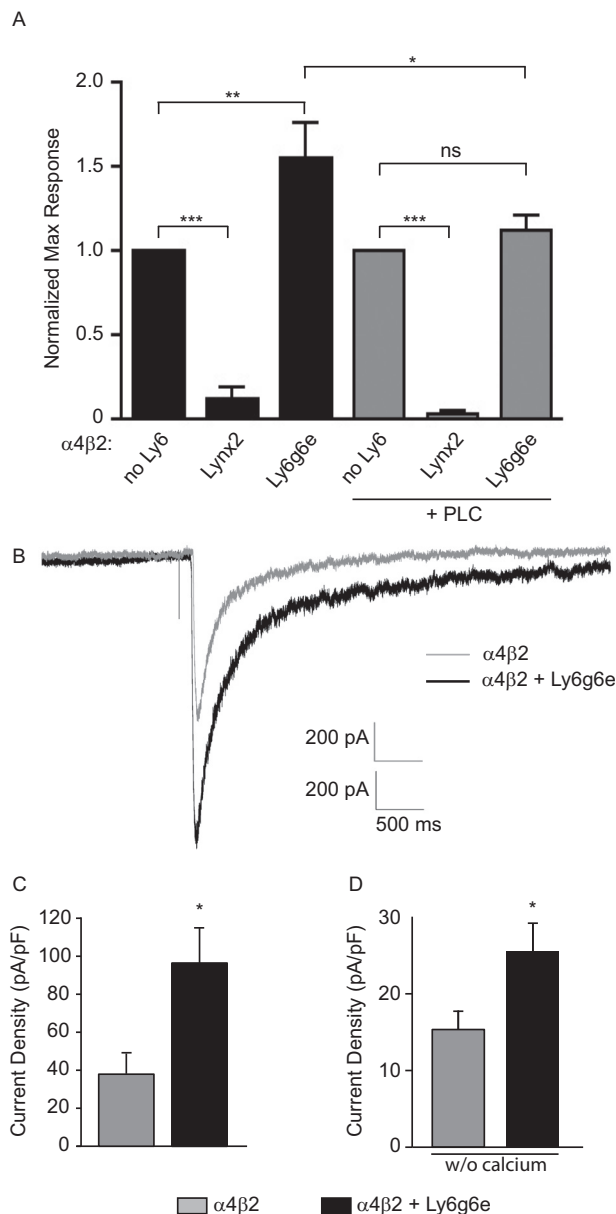


FIGURE 4. Ly6g6e at the plasma membrane potentiates $\alpha 4\beta 2$ activity. A, average normalized maximum FRET responses to epibatidine in HEKtsa cells transiently transfected with $\alpha 4$ and $\beta 2$ cDNAs in the absence versus the presence of Lynx2 or Ly6g6e. Lynx2 suppresses and Ly6g6e potentiates $\alpha 4\beta 2$ activity in response to epibatidine in the absence of exogenously applied PLC (black bars). Pretreatment of cells with PLC abolishes Ly6g6e-dependent potentiation but has no effect on Lynx2-dependent suppression of $\alpha 4\beta 2$ activity (gray bars). $n = 4$ for all conditions. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one way ANOVA with Bonferroni's multiple comparison test. B, representative ACh-evoked whole-cell currents from HEKtsa cells transiently co-transfected with $\alpha 4$ and $\beta 2$ cDNAs plus either empty vector (control; gray trace) or Ly6g6e cDNA (black trace). C and D, ACh-evoked whole-cell currents were increased in the presence of Ly6g6e both with (C) and without (D) external calcium ($N \geq 5$ for all conditions). *, $p < 0.05$; **, $p < 0.01$ by Student's t test.

examined $\alpha 4\beta 2$ nAChR currents in the absence of nicotine pretreatment. In this situation, the current amplitude was reduced, probably due to a decrease in the surface level of receptor. Nonetheless, we still observed an increase in both the fast and slow decay components in the presence of Ly6g6e (Fig. 5, E–H), consistent with our previous results. Hence, Ly6g6e and chronic nicotine do not interfere with each other's capacity to

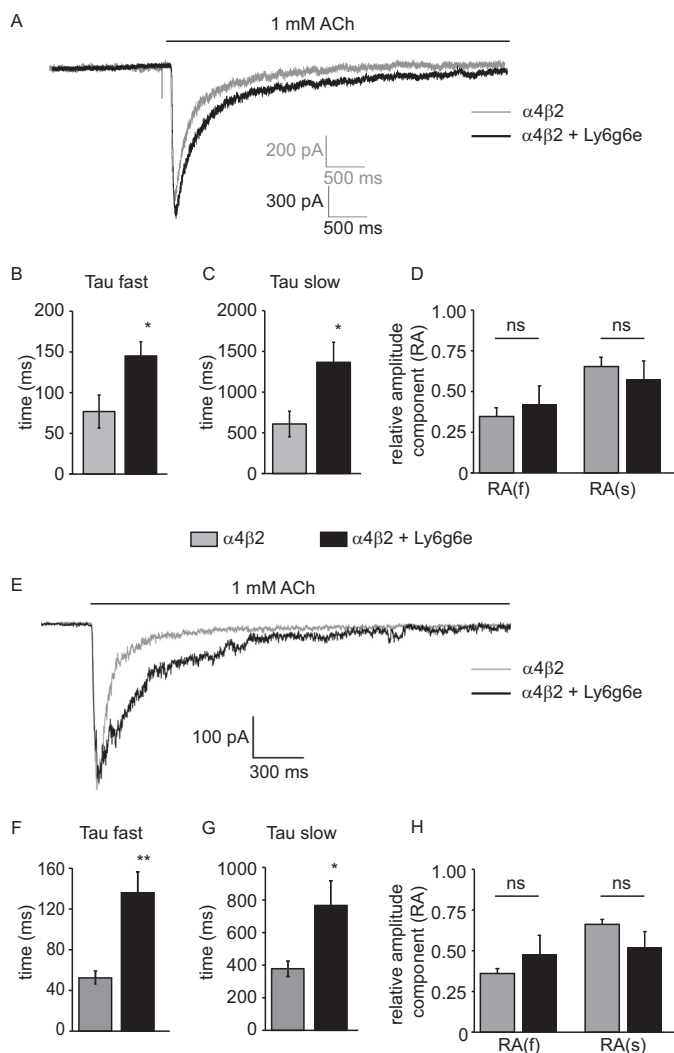


FIGURE 5. Ly6g6e slows desensitization of $\alpha 4\beta 2$ currents. *A* and *D*, representative currents from HEK293 cells transiently transfected with $\alpha 4$ and $\beta 2$ cDNAs plus either empty vector (control; gray trace) or Ly6g6e cDNA (black trace). Cells were recorded either with (*A–D*; $N \geq 5$) or without (*E–H*; $N \geq 8$) pretreatment with 1 μM nicotine for 20 h prior to recording. Traces were normalized to peak amplitudes to illustrate changes in desensitization kinetics. The total decaying current in this situation was approximated by the sum of two exponentials using the equation $I = [A_f \times \exp(-t/\tau_f)] + [A_s \times \exp(-t/\tau_s)]$, where τ_f (tau fast) and τ_s (tau slow) are the fast and slow time constants, and A_f and A_s are the corresponding amplitudes of the fast and slow components, respectively. Fits of receptor desensitization revealed increases in both tau fast (*B*, *F*) and tau slow components (*C*, *G*) by Ly6g6e. *D* and *H*, relative amplitude components of the fast and slow decay were unchanged in the presence of Ly6g6e. Pretreatment with nicotine did not interfere with the ability of Ly6g6e to alter kinetics of $\alpha 4\beta 2$ nAChR currents. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test.

associate with $\alpha 4\beta 2$ nAChRs and to regulate current magnitude and kinetics.

Thus, Ly6g6e potentiates $\alpha 4\beta 2$ signaling in two ways: by increasing peak current magnitude and by slowing desensitization. These results demonstrate a novel mechanism by which $\alpha 4\beta 2$ nAChRs can be modulated involving protein-protein interactions with the endogenous prototoxin, Ly6g6e, at the cell surface.

Discussion

As a global modulator of neuronal signaling, nAChR activity must be exquisitely regulated to maintain normal brain func-

tion. Increasing evidence suggests that at least some of this regulation is mediated by auxiliary proteins in the Ly6 family. For example, previous work has identified two Ly6 family members, Lynx1 and Lynx2, as important endogenous antagonists of $\alpha 4\beta 2$ signaling that serve to promote cell survival and behavioral plasticity (34, 39, 46). These functions have been attributed to the biophysical properties of Lynx1 and Lynx2, which include acceleration of $\alpha 4\beta 2$ desensitization and an increase in the EC_{50} for receptor activation by agonist (33, 34, 39, 47). Evidence also exists for Lynx1 facilitating $\alpha 4\beta 2$ receptor assembly (30).

Here we extend such studies by demonstrating that several additional Ly6 family members are capable of regulating $\alpha 4\beta 2$ nAChR function. Using a high-throughput calcium flux assay as an initial screen for functional modulators of $\alpha 4\beta 2$ nAChRs, we show that multiple members of the Ly6 family can suppress $\alpha 4\beta 2$ activity. Using this screen, we also identify Ly6g6e as a previously unrecognized potentiator of $\alpha 4\beta 2$ currents. With subsequent detailed analysis, we also demonstrate that the different effects we observed can occur by two distinct mechanisms: regulation of subcellular trafficking of $\alpha 4\beta 2$ receptors, and modulation of gating by local interactions with $\alpha 4\beta 2$ at the cell surface.

In support of the first mechanism, we have shown that Lynx2 reduces the number of $\alpha 4\beta 2$ nAChRs at the cell surface. Several reasons suggest that the inhibition of nAChR currents that we measured by flux assay is largely due to this effect, as opposed to previously described actions of Lynx2, such as acceleration of desensitization and decreased receptor affinity for agonist (34). For example, Lynx2 reduces $\alpha 4$ expression at the plasma membrane by over 80% without affecting total cellular expression of nAChRs. Because the magnitude of this effect is so extreme, very few receptors must reside at the cell surface to be antagonized. The majority of the inhibition of $\alpha 4\beta 2$ activity by Lynx2 that we measured also seems unlikely to be caused by changes in receptor stoichiometry, like those attributed to Lynx1, which are thought to reduce receptor affinity for agonist (30, 34). While we cannot rule out such a role for Lynx2, inhibition by this protein should be surmountable by increasing the concentration of agonist, whereas the inhibition we measured is not. This inhibition by Lynx2 is also notably resistant to PLC treatment, suggesting that it results not from direct antagonism or enhanced endocytosis of receptors at the plasma membrane but instead from reduced trafficking of receptors to the cell surface. Indeed, our finding that Lynx2 interferes with nicotine's ability to enhance cell surface levels of $\alpha 4\beta 2$ (Fig. 3, *A* and *B*) suggests that Lynx2 and nicotine might act at the same step in $\alpha 4\beta 2$ maturation but in opposing ways.

In addition to the previously unrecognized influence of Lynx2 on trafficking of $\alpha 4\beta 2$ nAChRs, we also demonstrated that Ly6g6e is a novel potentiator of $\alpha 4\beta 2$ signaling. This effect is likely to be direct, since Ly6g6e and $\alpha 4\beta 2$ nAChRs can form a stable complex at the cell surface; Ly6g6e modulates receptor kinetics; and potentiation of receptor activity can be abolished by acute exogenous treatment of cells with soluble PLC, which dissociates GPI-linked proteins from the outer leaflet of the plasma membrane. This last result also rules out a trafficking role for Ly6g6e that might compete with and oppose the effects

Novel Regulation of $\alpha 4\beta 2$ nAChRs by Ly6 Proteins

of Lynx2, since only Lynx2's effects were resistant to PLC. Consistent with all these results, we found that Ly6g6e potentiates $\alpha 4\beta 2$ activity in two ways: enhancement of peak current amplitude and slowing of receptor desensitization. Although we hypothesize that Ly6g6e shifts equilibria between previously characterized receptor conformations (48, 49), we cannot rule out the possibility that Ly6g6e might have alternative or additional actions, such as stabilizing otherwise short-lived poorly characterized open states with higher single channel conductance or increased calcium permeability. Indeed, both alternatives have been proposed to support potentiating effects of other Ly6 proteins on nAChRs (33, 50). In our case, however, any potential changes in calcium permeability cannot explain the potentiating effects of Ly6g6e since we find that these effects persist in the absence of extracellular calcium.

Our work raises several important questions. For example, what is the structural basis of Ly6-nAChR interactions? If there is a single major binding site for Ly6 proteins on nAChRs then it may overlap with the binding site for nicotine for several reasons. First, $\alpha 4\beta 2$ receptors that escape the trafficking limitations of Lynx2 to reside at the cell surface still exhibit competitive antagonism between Lynx2 and agonist (34). Second, Lynx2 blocks the ability of nicotine to potentiate cell surface expression of $\alpha 4\beta 2$ nAChRs. Although the binding of Lynx2 to $\alpha 4\beta 2$ could occur downstream or in parallel to the binding event for nicotine and thus diminish nicotine's activity, an alternative explanation is that Lynx2 competes with nicotine for a binding site on receptors. That is, when only nicotine is bound, $\alpha 4\beta 2$ nAChRs would be up-regulated, but when Lynx2 competes with nicotine, $\alpha 4\beta 2$ potentiation by drug would not occur. Lastly, Ly6 proteins are structurally homologous to three-fingered snake toxins such as α -cobratoxin and α -bungarotoxin, which are thought to interact with nAChRs at subunit-subunit interfaces where agonists and competitive antagonists bind (51–54). Thus, Ly6 proteins are likely to bind to heteromeric nAChRs at similar sites. Modeling based on the crystal and NMR structures of ACh binding protein and Lynx1, respectively, also supports such a binding site for Ly6 proteins on nAChRs (47).

Another important question is whether our understanding of the actions of Ly6 proteins on nAChRs can be utilized to interfere with chronic nicotine's potentiation of $\alpha 4\beta 2$ signaling in ways that could be therapeutically beneficial for ameliorating either the pleasurable effects of nicotine or the cravings associated with nicotine withdrawal. Although we have no data to directly support such a hypothesis, it is worth noting that efforts to break the cycle of nicotine addiction include treatment of smokers with $\alpha 4\beta 2$ receptor agonists, antagonists and partial agonists (55–58). One drawback to most of these molecules is lack of structural and regional selectivity: *i.e.* drugs that act directly on $\alpha 4\beta 2$ nAChRs in one brain region will affect structurally related receptors as well as $\alpha 4\beta 2$ nAChRs in many other brain regions, thus potentially leading to undesirable side effects. One solution to this problem might be to develop drugs that mimic or interfere with the effects of Ly6 proteins that exist in complexes with nAChRs in selected brain regions. For example, we have detected Lynx2 and Ly6g6e transcript in the midbrain, which is believed to be involved in nicotine reward and

withdrawal. Although it is not yet known how broadly the encoded proteins co-localize with $\alpha 4\beta 2$ subunits, our work suggests that drugs that mimic Lynx2 or that antagonize interactions between Ly6g6e and $\alpha 4\beta 2$ could potentially nullify potentiated nicotinic signaling in the midbrain caused by chronic nicotine exposure.

Our findings also expand the number of Ly6 proteins known to regulate $\alpha 4\beta 2$ nAChRs by diverse mechanisms. Besides our discovery that Ly6g6e is a novel potentiator, we also identified four previously undescribed inhibitors, all of which form stable complexes with $\alpha 4\beta 2$ nAChRs. Ly6h in particular is nearly as effective as Lynx2 at suppressing $\alpha 4\beta 2$ currents as measured in our flux assay. These results are somewhat surprising since a previous report suggested that Ly6h does not form complexes with $\alpha 4\beta 2$. It is likely that our expression system, co-immunoprecipitation conditions, or position of our epitope tag on Ly6h was more permissive for detecting protein-protein interactions than the analogous conditions in the previous report (34). Other than Lynx2, we have not studied the mechanisms by which our identified inhibitors of $\alpha 4\beta 2$ nAChRs function. Considering the overall limited primary sequence identity between these proteins, it will be interesting in future work to determine how they suppress $\alpha 4\beta 2$ activity and which structural determinants are required for these effects. Based on the crystal structure of acetylcholine binding protein and the NMR structure of soluble Lynx1, modeling suggests that one of the loops in the Ly6 domain of Lynx1 might be responsible for specific interactions with nAChRs (47). Examination of alignments of Ly6 sequences (40) reveals a high degree of variability in these loops, which might predict a large array of possible interacting surfaces with varying affinities for different nAChRs. On a related note, we have also detected regulation of $\alpha 7$ nAChRs by some, though not all, of the Ly6 proteins shown to exert effects on $\alpha 4\beta 2$ receptors (35). We suggest that the combinatorial possibilities of different nAChRs interacting with various Ly6 proteins with different biophysical properties and receptor affinities greatly expands the dynamic range over which cholinergic signaling can be regulated.

Author Contributions—M. W., C. A. P., and W. J. J. designed experiments and wrote the manuscript. P. T. provided advice on experimental design, interpretation of results and preparation of the manuscript. M. W. performed molecular biology, biochemistry, and flux assays. C. A. P. performed electrophysiological recordings.

Acknowledgments—We thank Henry Lester for generously providing $\alpha 4$, GFP- $\alpha 4$, and $\beta 2$ -dm expression plasmids. We also thank John Yamauchi and Kimberly Gomez for assistance with the FRET-based calcium flux assay and Joan Heller Brown for providing rat tissue for neuronal culture.

References

1. Gotti, C., Clementi, F., Fornari, A., Gaimarri, A., Guiducci, S., Manfredi, I., Moretti, M., Pedrazzi, P., Pucci, L., and Zoli, M. (2009) Structural and functional diversity of native brain neuronal nicotinic receptors. *Biochem. Pharmacol.* **78**, 703–711
2. Conroy, W. G., and Berg, D. K. (1998) Nicotinic receptor subtypes in the developing chick brain: appearance of a species containing the $\alpha 4$, $\beta 2$, and $\alpha 5$ gene products. *Mol. Pharmacol.* **53**, 392–401

3. Flores, C. M., Rogers, S. W., Pabreza, L. A., Wolfe, B. B., and Kellar, K. J. (1992) A subtype of nicotinic cholinergic receptor in rat brain is composed of $\alpha 4$ and $\beta 2$ subunits and is up-regulated by chronic nicotine treatment. *Mol. Pharmacol.* **41**, 31–37
4. Whiting, P. J., and Lindstrom, J. M. (1988) Characterization of bovine and human neuronal nicotinic acetylcholine receptors using monoclonal antibodies. *J. Neurosci.* **8**, 3395–3404
5. Zoli, M., Léna, C., Picciotto, M. R., and Changeux, J. P. (1998) Identification of four classes of brain nicotinic receptors using β mutant mice. *J. Neurosci.* **18**, 4461–4472
6. Azam, L., Chen, Y., and Leslie, F. M. (2007) Developmental regulation of nicotinic acetylcholine receptors within midbrain dopamine neurons. *Neuroscience* **144**, 1347–1360
7. Whiteaker, P., Cooper, J. F., Salminen, O., Marks, M. J., McClure-Begley, T. D., Brown, R. W. B., Collins, A. C., and Lindstrom, J. M. (2006) Immunolabeling demonstrates the interdependence of mouse brain $\alpha 4$ and $\beta 2$ nicotinic acetylcholine receptor subunit expression. *J. Comp. Neurol.* **499**, 1016–1038
8. Yang, K., Hu, J., Lucero, L., Liu, Q., Zheng, C., Zhen, X., Jin, G., Lukas, R. J., and Wu, J. (2009) Distinctive nicotinic acetylcholine receptor functional phenotypes of rat ventral tegmental area dopaminergic neurons. *J. Physiol.* **587**, 345–361
9. Picciotto, M. R., Addy, N. A., Mineur, Y. S., and Brunzell, D. H. (2008) It is not “either/or”: activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. *Prog. Neurobiol.* **84**, 329–342
10. Pons, S., Fattore, L., Cossu, G., Tolu, S., Porcu, E., McIntosh, J. M., Changeux, J. P., Maskos, U., and Fratta, W. (2008) Crucial role of $\alpha 4$ and $\alpha 6$ nicotinic acetylcholine receptor subunits from ventral tegmental area in systemic nicotine self-administration. *J. Neurosci.* **28**, 12318–12327
11. Benwell, M. E., Balfour, D. J., and Anderson, J. M. (1988) Evidence that tobacco smoking increases the density of (-)-[3H]nicotine binding sites in human brain. *J. Neurochem.* **50**, 1243–1247
12. Breese, C. R., Marks, M. J., Logel, J., Adams, C. E., Sullivan, B., Collins, A. C., and Leonard, S. (1997) Effect of smoking history on [3H]nicotine binding in human postmortem brain. *J. Pharmacol. Exp. Ther.* **282**, 7–13
13. Marks, M. J., Burch, J. B., and Collins, A. C. (1983) Effects of chronic nicotine infusion on tolerance development and nicotinic receptors. *J. Pharmacol. Exp. Ther.* **226**, 817–825
14. Perry, D. C., Dávila-García, M. I., Stockmeier, C. A., and Kellar, K. J. (1999) Increased nicotinic receptors in brains from smokers: membrane binding and autoradiography studies. *J. Pharmacol. Exp. Ther.* **289**, 1545–1552
15. Schwartz, R. D., and Kellar, K. J. (1983) Nicotinic cholinergic receptor binding sites in the brain: regulation *in vivo*. *Science* **220**, 214–216
16. Lester, H. A., Xiao, C., Srinivasan, R., Son, C. D., Miwa, J., Pantoja, R., Banghart, M. R., Dougherty, D. A., Goate, A. M., and Wang, J. C. (2009) Nicotine is a selective pharmacological chaperone of acetylcholine receptor number and stoichiometry. Implications for drug discovery. *AAPS J.* **11**, 167–177
17. Liu, Q., Emadi, S., Shen, J.-X., Sierks, M. R., and Wu, J. (2013) Human $\alpha 4\beta 2$ nicotinic acetylcholine receptor as a novel target of oligomeric α -synuclein. *PLoS ONE* **8**, e55886
18. Lorenz, R., Samnick, S., Dillmann, U., Schiller, M., Ong, M. F., Faßbender, K., Buck, A., and Spiegel, J. (2014) Nicotinic $\alpha 4\beta 2$ acetylcholine receptors and cognitive function in Parkinson’s disease. *Acta Neurol. Scand.* **130**, 164–171
19. Perez, X. A., Bordia, T., McIntosh, J. M., and Quik, M. $\alpha 6\text{ss}2^*$ and $\alpha 4\text{ss}2^*$ nicotinic receptors both regulate dopamine signaling with increased nigrostriatal damage: relevance to Parkinson’s disease. *Mol. Pharmacol.* **78**, 971–980
20. Picciotto, M. R., and Zoli, M. (2008) Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease. *Front. Biosci.* **13**, 492–504
21. Quik, M., Campos, C., Bordia, T., Strachan, J.-P., Zhang, J., McIntosh, J. M., Letchworth, S., and Jordan, K. (2013) $\alpha 4\beta 2$ Nicotinic receptors play a role in the nAChR-mediated decline in L-dopa-induced dyskinesias in parkinsonian rats. *Neuropharmacology* **71**, 191–203
22. Quik, M., O’Neill, M., and Perez, X. A. (2007) Nicotine neuroprotection against nigrostriatal damage: importance of the animal model. *Trends Pharmacol. Sci.* **28**, 229–235
23. Darsow, T., Booker, T. K., Piña-Crespo, J. C., and Heinemann, S. F. (2005) Exocytic trafficking is required for nicotine-induced up-regulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J. Biol. Chem.* **280**, 18311–18320
24. Govind, A. P., Walsh, H., and Green, W. N. (2012) Nicotine-induced up-regulation of native neuronal nicotinic receptors is caused by multiple mechanisms. *J. Neurosci.* **32**, 2227–2238
25. Vallejo, Y. F., Buisson, B., Bertrand, D., and Green, W. N. (2005) Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. *J. Neurosci.* **25**, 5563–5572
26. Kuryatov, A., Luo, J., Cooper, J., and Lindstrom, J. (2005) Nicotine acts as a pharmacological chaperone to up-regulate human $\alpha 4\beta 2$ acetylcholine receptors. *Mol. Pharmacol.* **68**, 1839–1851
27. Sallette, J., Pons, S., Devillers-Thiery, A., Soudant, M., Prado de Carvalho, L., Changeux, J.-P., and Corringer, P. J. (2005) Nicotine upregulates its own receptors through enhanced intracellular maturation. *Neuron* **46**, 595–607
28. Xiao, C., Nashmi, R., McKinney, S., Cai, H., McIntosh, J. M., and Lester, H. A. (2009) Chronic nicotine selectively enhances $\alpha 4\beta 2^*$ nicotinic acetylcholine receptors in the nigrostriatal dopamine pathway. *J. Neurosci.* **29**, 12428–12439
29. Nelson, M. E., Kuryatov, A., Choi, C. H., Zhou, Y., and Lindstrom, J. (2003) Alternate stoichiometries of $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *Mol. Pharmacol.* **63**, 332–341
30. Nichols, W. A., Henderson, B. J., Yu, C., Parker, R. L., Richards, C. I., Lester, H. A., and Miwa, J. M. (2014) Lynx1 Shifts $\alpha 4\beta 2$ Nicotinic Receptor Subunit Stoichiometry by Affecting Assembly in the Endoplasmic Reticulum. *J. Biol. Chem.* **289**, 31423–31432
31. Adermann, K., Wattler, F., Wattler, S., Heine, G., Meyer, M., Forssmann, W. G., and Nehls, M. (1999) Structural and phylogenetic characterization of human SLURP-1, the first secreted mammalian member of the Ly-6/uPAR protein superfamily. *Protein Sci.* **8**, 810–819
32. Arredondo, J., Chernyavsky, A. I., Jolkovsky, D. L., Webber, R. J., and Grando, S. A. (2006) SLURP-2: A novel cholinergic signaling peptide in human mucocutaneous epithelium. *J. Cell. Physiol.* **208**, 238–245
33. Ibañez-Tallon, I., Miwa, J. M., Wang, H. L., Adams, N. C., Crabtree, G. W., Sine, S. M., and Heintz, N. (2002) Novel modulation of neuronal nicotinic acetylcholine receptors by association with the endogenous prototoxin lynx1. *Neuron* **33**, 893–903
34. Tekinay, A. B., Nong, Y., Miwa, J. M., Lieberam, I., Ibanez-Tallon, I., Greengard, P., and Heintz, N. (2009) A role for LYNX2 in anxiety-related behavior. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4477–4482
35. Puddifoot, C. A., Wu, M., Sung, R. J., and Joiner, W. J. (2015) Ly6h regulates trafficking of $\alpha 7$ nicotinic acetylcholine receptors and nicotine-induced potentiation of glutamatergic signaling. *J. Neurosci.* **35**, 3420–3430
36. Wu, M., Robinson, J. E., and Joiner, W. J. (2014) SLEEPLESS Is a Bifunctional Regulator of Excitability and Cholinergic Synaptic Transmission. *Curr. Biol.* **24**, 621–629
37. Yamauchi, J. G., Nemezc, Á., Nguyen, Q. T., Muller, A., Schroeder, L. F., Talley, T. T., Lindstrom, J., Kleinfeld, D., and Taylor, P. (2011) Characterizing ligand-gated ion channel receptors with genetically encoded Ca²⁺ sensors. *PLoS ONE* **6**, e16519
38. Wu, M., Li, J., Engleka, K. A., Zhou, B., Lu, M. M., Plotkin, J. B., and Epstein, J. A. (2008) Persistent expression of Pax3 in the neural crest causes cleft palate and defective osteogenesis in mice. *J. Clin. Invest.* **118**, 2076–2087
39. Miwa, J. M., Stevens, T. R., King, S. L., Caldarone, B. J., Ibanez-Tallon, I., Xiao, C., Fitzsimonds, R. M., Pavlides, C., Lester, H. A., Picciotto, M. R., and Heintz, N. (2006) The prototoxin lynx1 acts on nicotinic acetylcholine receptors to balance neuronal activity and survival *in vivo*. *Neuron* **51**, 587–600
40. Galat, A., Gross, G., Drevet, P., Sato, A., and Ménez, A. (2008) Conserved structural determinants in three-fingered protein domains. *FEBS J* **275**, 3207–3225
41. Dani, J. A., and Bertrand, D. (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu. Rev. Pharmacol. Toxicol.* **47**, 699–729
42. Nashmi, R., Dickinson, M. E., McKinney, S., Jareb, M., Labarca, C., Fraser,

Novel Regulation of $\alpha 4\beta 2$ nAChRs by Ly6 Proteins

- S. E., and Lester, H. A. (2003) Assembly of $\alpha 4\beta 2$ nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. *J. Neurosci.* **23**, 11554–11567
43. Srinivasan, R., Pantoja, R., Moss, F. J., Mackey, E. D. W., Son, C. D., Miwa, J., and Lester, H. A. (2011) Nicotine up-regulates $\alpha 4\beta 2$ nicotinic receptors and ER exit sites via stoichiometry-dependent chaperoning. *J. Gen. Physiol.* **137**, 59–79
44. Srinivasan, R., Richards, C. L., Xiao, C., Rhee, D., Pantoja, R., Dougherty, D. A., Miwa, J. M., and Lester, H. A. (2012) Pharmacological chaperoning of nicotinic acetylcholine receptors reduces the endoplasmic reticulum stress response. *Mol. Pharmacol.* **81**, 759–769
45. Koh, K., Joiner, W. J., Wu, M. N., Yue, Z., Smith, C. J., and Sehgal, A. (2008) Identification of SLEEPLESS, a sleep-promoting factor. *Science* **321**, 372–376
46. Morishita, H., Miwa, J. M., Heintz, N., and Hensch, T. K. (2010) Lynx1, a cholinergic brake, limits plasticity in adult visual cortex. *Science* **330**, 1238–1240
47. Lyukmanova, E. N., Shenkarev, Z. O., Shulepko, M. A., Mineev, K. S., D'Hoedt, D., Kasheverov, I. E., Filkin, S. Y., Krivolapova, A. P., Janickova, H., Dolezal, V., Dolgikh, D. A., Arseniev, A. S., Bertrand, D., Tsetlin, V. I., and Kirpichnikov, M. P. (2011) NMR structure and action on nicotinic acetylcholine receptors of water-soluble domain of human LYNX1. *J. Biol. Chem.* **286**, 10618–10627
48. Auerbach, A., and Akk, G. (1998) Desensitization of mouse nicotinic acetylcholine receptor channels. A two-gate mechanism. *J. Gen. Physiol.* **112**, 181–197
49. Paradiso, K. G., and Steinbach, J. H. (2003) Nicotine is highly effective at producing desensitization of rat $\alpha 4\beta 2$ neuronal nicotinic receptors. *J. Physiol.* **553**, 857–871
50. Darvas, M., Morsch, M., Racz, I., Ahmadi, S., Swandulla, D., and Zimmer, A. (2009) Modulation of the Ca²⁺ conductance of nicotinic acetylcholine receptors by Lypd6. *Eur. Neuropsychopharmacol.* **19**, 670–681
51. Bourne, Y., Talley, T. T., Hansen, S. B., Taylor, P., and Marchot, P. (2005) Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake α -neurotoxins and nicotinic receptors. *EMBO J.* **24**, 1512–1522
52. Dellisanti, C. D., Yao, Y., Stroud, J. C., Wang, Z.-Z., and Chen, L. (2007) Crystal structure of the extracellular domain of nAChR $\alpha 1$ bound to α -bungarotoxin at 1.94 Å resolution. *Nat. Neurosci.* **10**, 953–962
53. Huang, S., Li, S.-X., Bren, N., Cheng, K., Gomoto, R., Chen, L., and Sine, S. M. (2013) Complex between α -bungarotoxin and an $\alpha 7$ nicotinic receptor ligand-binding domain chimaera. *Biochem. J.* **454**, 303–310
54. Tsetlin, V. I. (2015) Three-finger snake neurotoxins and Ly6 proteins targeting nicotinic acetylcholine receptors: pharmacological tools and endogenous modulators. *Trends Pharmacol. Sci.* **36**, 109–123
55. Biala, G., Staniak, N., and Budzynska, B. (2010) Effects of varenicline and mecamylamine on the acquisition, expression, and reinstatement of nicotine-conditioned place preference by drug priming in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **381**, 361–370
56. Coe, J. W., Brooks, P. R., Vetelino, M. G., Wirtz, M. C., Arnold, E. P., Huang, J., Sands, S. B., Davis, T. I., Lebel, L. A., Fox, C. B., Shrikhande, A., Heym, J. H., Schaeffer, E., Rollema, H., Lu, Y., Mansbach, R. S., Chambers, L. K., Rovetti, C. C., Schulz, D. W., Tingley, F. D., 3rd, and O'Neill, B. T. (2005) Varenicline: an $\alpha 4\beta 2$ nicotinic receptor partial agonist for smoking cessation. *J. Med. Chem.* **48**, 3474–3477
57. Cohen, C., Bergis, O. E., Galli, F., Lochead, A. W., Jegham, S., Biton, B., Leonardon, J., Avenet, P., Sgard, F., Besnard, F., Graham, D., Coste, A., Oblin, A., Curet, O., Voltz, C., Gardes, A., Caille, D., Perrault, G., George, P., Soubrie, P., and Scatton, B. (2003) SSR591813, a novel selective and partial $\alpha 4\beta 2$ nicotinic receptor agonist with potential as an aid to smoking cessation. *J. Pharmacol. Exp. Ther.* **306**, 407–420
58. Rollema, H., Chambers, L. K., Coe, J. W., Glowa, J., Hurst, R. S., Lebel, L. A., Lu, Y., Mansbach, R. S., Mather, R. J., Rovetti, C. C., Sands, S. B., Schaeffer, E., Schulz, D. W., Tingley, F. D., 3rd, and Williams, K. E. (2007) Pharmacological profile of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonist varenicline, an effective smoking cessation aid. *Neuropharmacology* **52**, 985–994