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Mechanisms of Inhibition and Potentiation of α4β2 Nicotinic Acetylcholine Receptors by Members of the Ly6 Protein Family*

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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 α4β2 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 α4β2 function by distinct mechanisms.
Significance: Ly6 proteins greatly expand the range of properties of α4β2 nAChRs.

α4β2 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 α4β2 nAChRs. Using a FRET-based Ca^{2+} flux assay, we found that the maximum response of α4β2 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 α4β2 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 α4β2 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 α4β2 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 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 α7 homomers and α4β2 heteromers (1).

Several lines of evidence suggest that α4β2 combinations are the main nAChRs that contribute to nicotine addiction. α4β2 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, α4β2 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 α4β2 nAChRs appear to be 2-fold. First, after diffusing across cell membranes, nicotine binds to and stabilizes β2 subunits in the endoplasmic reticulum (ER), thus indirectly enhancing α4β2 co-assembly (23, 24). Second, nicotine is thought to convert α4β2 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

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[1] 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.

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

has been proposed that by promoting assembly of $\alpha4\beta2$ 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 $\alpha4_3\beta2_2$ toward high affinity $\alpha4_2\beta2_3$ receptors (29, 30). Finally, it has also been proposed that nicotine exposure imposes on $\alpha4\beta2$ 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 $\alpha4\beta2$ 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 $\alpha4\beta2$ 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 $\alpha4\beta2$ nAChRs in transfected HEKtsa cells. We show that several Ly6 proteins are able to form complexes with $\alpha4\beta2$ 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 $\alpha4\beta2$ nAChRs at the cell surface. The trafficking effect of Lynx2 is particularly notable in that it suppresses up-regulation of $\alpha4\beta2$ nAChRs following chronic exposure to nicotine. In addition to this novel antagonistic property, we also demonstrate that a previously uncharacterized Ly6 protein, Ly6g6e, potentiates $\alpha4\beta2$ signaling by slowing receptor desensitization. Our data suggest that the Ly6 proteins and the mechanisms they employ to modulate $\alpha4\beta2$ 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). α4-HA was generated by PCR amplifying α4 from GFP-α4-pciNeo (gift from Dr. H. Lester) with the following primers (5′-3′): F: AAAAGCTTACCATGGAGATCGGGGGCTCCG; R: TCTAGAGAGCTAGGTGCCGCTATTCC and subcloned into pcDNA3 in-frame with a C-terminal hemagglutinin (HA) tag (36) between the HindIII and Xba1 sites.

FRET-based Measurements of nAChR Activity—Measurements of $\alpha4\beta2$ nAChR activity were performed as previously described (37) with the following modifications: HEKtsa cells were transiently transfected with untagged α4-pciNeo and β2-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 μM 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 $NaHCO_3$, 1.2 mM $NaH_2PO_4H_2O$, 10 mM glucose, 5 mM HEPES, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 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 $\alpha4\beta2$ 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—HEKtsa cells were maintained at 37 °C and 5% CO_2 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 $CaCl_2$). 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: CGGCATCGCAGCAACTTTTTG; Lynx2-R: TTGCAAAGAGGGGTGTTGCAG; Ly6g6e-F: CTGTTACACCT-

GCAGCTTTGCC; Ly6g6e-R: GGTAGCAGAGTCATGAGGGGA 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× LDS sample buffer (Life Technologies). For α4-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× 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 $CaCl_2$, 1 $MgCl_2$, 20 glucose, pH 7.3. Patch pipettes (resistance 5–10 MΩ) 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 40x 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 Multiclamp 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 α4β2 nAChR Activity—The best characterized members of the mammalian Ly6 family, Lynx1 and Lynx2, have both been shown to form complexes with α4β2 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 α4β2 nAChR activity, we individually co-expressed them with mouse α4β2 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 α4β2 nAChRs are significantly less permeable to calcium than α7 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 α4β2 nAChRs at the cell surface, as previously described (24, 28, 42, 43). In addition, we used a mutant β2 subunit (β2-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. 1*C*). Thus, all subsequent α4β2 FRET assays were performed using the β2-dm ER-export mutant following 20 h of pretreatment with 1 μM nicotine.

Despite these enhancements, the FRET signals achieved with epibatidine stimulation of α4β2 nAChRs were still too low to plot reliable slopes of concentration-response curves, thus preventing quantification of EC_{50} 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 α4β2 activity at saturating concentrations of agonist. Using this assay we showed that the maximum response of α4β2 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 α4β2 nAChRs with Ly6g6e caused a 2-fold increase in the maximum FRET response to epibatidine (Fig. 1*A*, B). Thus, depending on the identity of the Ly6 proteins that were present, α4β2 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 α4β2 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 α4β2 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

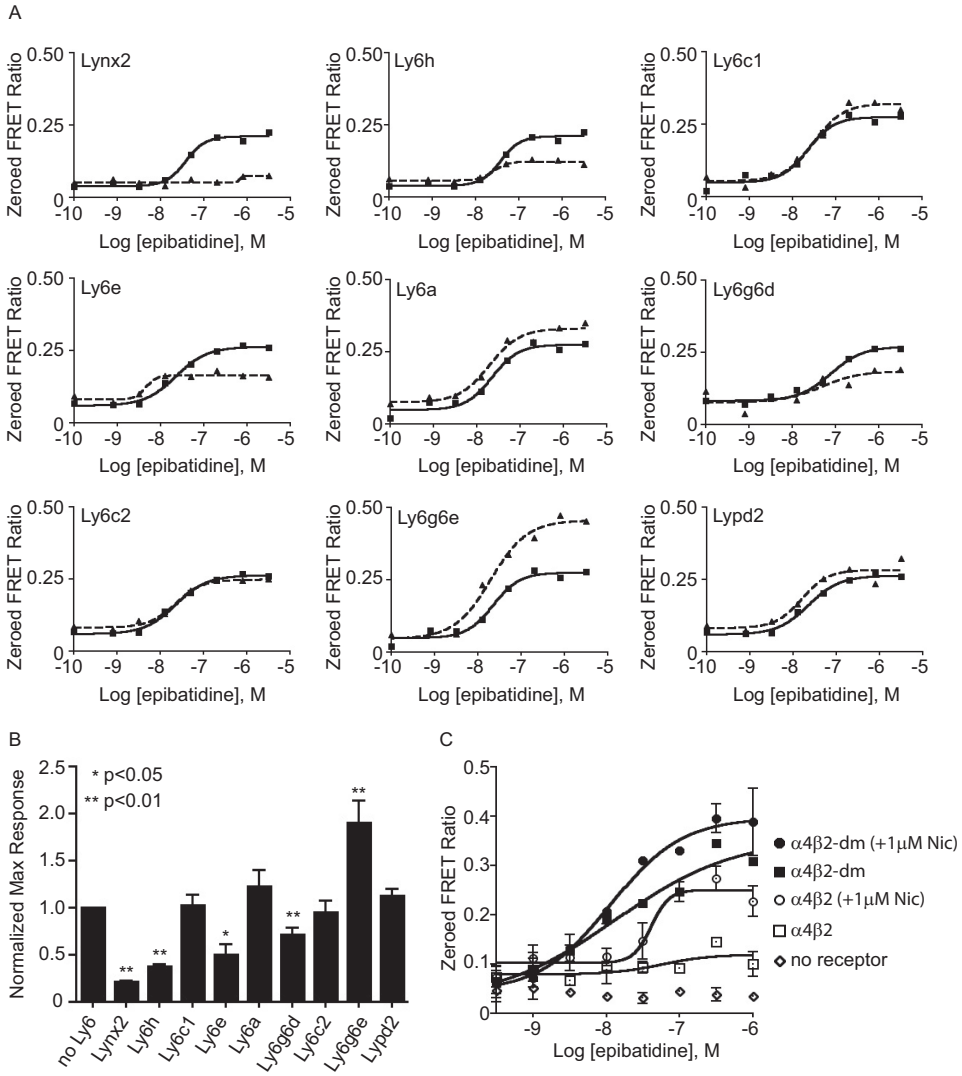

FIGURE 1. **Ly6 proteins modulate α4β2 nAChR activity.** *A*, representative concentration-response curves for activation of α4β2 nAChRs by epibatidine in the absence (*solid lines*) or presence (*dashed lines*) of various Ly6 proteins in transfected HEKtsa cells following 20 h pre-treatment with 1 μM nicotine. *B*, average maximum responses of α4β2 nAChRs to epibatidine. All measurements were normalized to control values for receptor alone. $n = 7$ for each condition. *, $p < 0.5$; **, $p < 0.01$ by one-way ANOVA with Dunnett's multiple comparison test. Error bars indicate S.E. *C*, nicotine pre-treatment (*Nic*) and β2 ER-export mutation (*dm*) both enhance α4β2 functional expression. Representative concentration-response curves of α4β2 nAChRs to epibatidine. *Open diamonds* (no receptor control) show that no signal is produced in the absence of transfected α4β2 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-α4 from these lysates followed by Western blotting for Myc revealed that all Ly6 proteins that suppressed α4β2 activity also co-immunoprecipitated with α4β2 nAChRs. Notably Ly6a, which had no functional effect on α4β2 nAChR activity in our flux assay, also did not co-immunoprecipitate with α4β2 subunits (Fig. 2, *A* and *B*). In the case of Ly6g6e, which potentiated rather than inhibited α4β2 activity, we did not detect an interaction following immunoprecipitation with total cellular α4β2. However, Ly6g6e (but not Ly6a) was selectively detected in complex with α4β2 from a membrane fraction enriched for cell surface expression using a C-terminal HA-tagged α4 (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-α4 or GFP-β2 alone, or with GFP-α4 plus untagged β2. Immunoprecipitation of GFP-tagged nAChR subunits followed by Western blotting for Myc revealed that Lynx2 selectively forms complexes with α4 compared with β2 subunits (Fig. 2*C*). We were unable to perform analogous experiments with Ly6g6e due to an inability to express α4 and β2 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 α4β2 nAChRs are believed to participate (6–8).

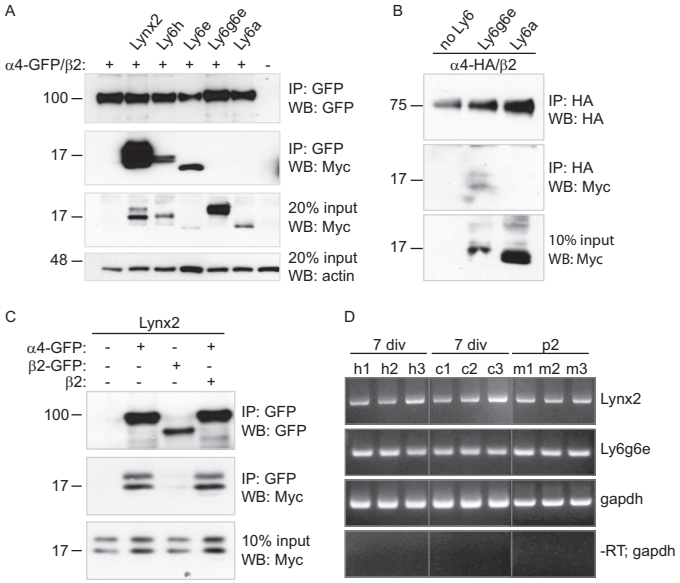

FIGURE 2. **α4β2 nAChRs form stable complexes with the Ly6 proteins that modulate their activities.** *A*, representative immunoblots of immunoprecipitated α4 subunits in HEKtsa cells transfected with β2, GFP-tagged α4, and variojus Myc-tagged Ly6 cDNAs. Ly6a (*A*, *lane 6*) was included as a negative control and does not co-immunoprecipitate with α4 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 α4 subunits in HEKtsa cells transfected with β2, HA-tagged α4, and Myc-tagged Ly6g6e or Ly6a cDNAs. Ly6a was included as a negative control and does not co-immunoprecipitate with HA-α4 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 HEKtsa cells transfected with Myc-tagged Lynx2 alone (*lane 1*) or in combination with GFP-tagged α4 (*lane 2*), GFP-tagged β2 (*lane 3*), or GFP-tagged α4 and untagged β2 (*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 α4β2 Expression at the Cell Surface—The striking changes in the maximal FRET responses of α4β2 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 α4β2 cell surface levels, we expressed GFP-α4β2 alone or with Ly6 proteins in HEKtsa cells and labeled the extracellular surfaces of all plasma membrane proteins by biotin conjugation. 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-α4 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 α4β2 at the cell surface. In contrast, Ly6g6e co-expression had no effect on α4 levels at the plasma membrane or throughout cells (Fig. 3*A*, B). Thus, the potentiation we observed in α4β2 signaling in the

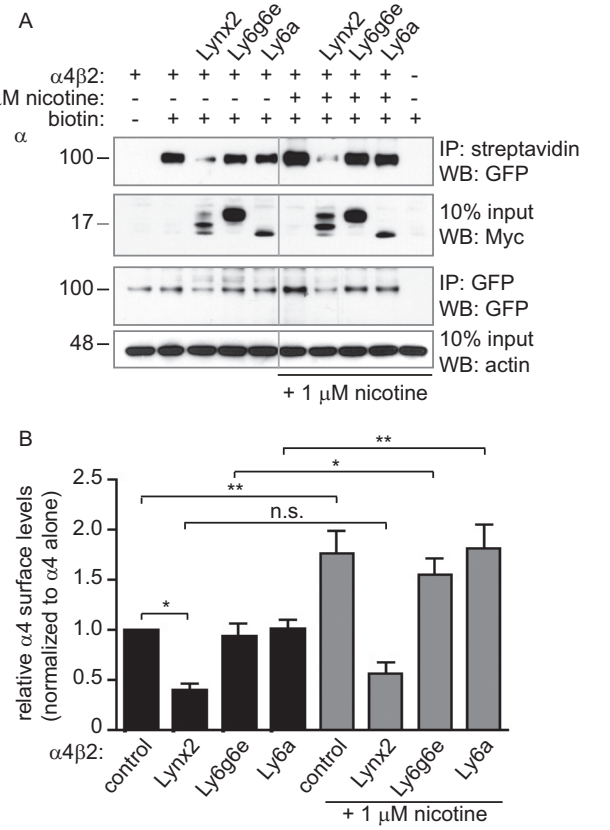

FIGURE 3. **Ly6 proteins regulate α4β2 nAChR localization at the plasma membrane.** *A*, representative immunoblots of streptavidin immunoprecipitations of surface biotinylated α4β2 nAChRs in transiently transfected HEKtsa cells. *Top panels*: streptavidin immunoprecipitation samples immunoblotted with anti-GFP antibody to assess surface α4-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 α4-GFP levels. *Bottom panels*: 10% input total cell lysates blotted with anti-actin antibody as a loading control. *B*, average α4β2 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 α4β2 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 α4β2 surface expression ($n = 8$). *, $p < 0.5$; **, $p < 0.01$ 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 α4β2 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 α4β2 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 α4 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 α4β2 export to the cell surface. In contrast, Ly6g6e and Ly6a had no effect on nicotine-mediated up-regulation of cell surface levels of α4 subunits (Fig. 3*A*, *right panel*, and 3*B*, *gray bars*).

Ly6g6e Functions at the Cell Surface to Potentiate α4β2 Activity—Since Ly6g6e does not alter receptor trafficking and seems to form a stable complex with α4β2 most efficiently at the cell surface, we reasoned that this Ly6 protein might potentiate the maximum response of activated α4β2 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 α4β2 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 α4β2 maximal response to agonist (Fig. 4*A*, *gray bars*). These results strongly support a role for Ly6g6e in modulating α4β2 activity. In contrast, the Lynx2-mediated suppression of α4β2 maximal response was maintained following PLC treatment, supporting our hypothesis that Lynx2 mediates much of its effects intracellularly to suppress surface expression of α4β2 nAChRs, as demonstrated by our biotin labeling experiments.

Ly6g6e Enhances Whole-cell α4β2 nAChR Currents—To further investigate the modulatory role of Ly6g6e on α4β2 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 α4β2 nAChR current amplitude and kinetics in individual cells. Based on our previous data, we hypothesized that Ly6g6e enhances α4β2 nAChRs through direct modulatory effects at the cell surface. Indeed, co-expression of Ly6g6e increased α4β2 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. 4*D*). Thus, the increased calcium currents identified by flux assay in Fig. 1 cannot be attributed to a change in ion selectivity of α4β2 nAChRs.

Ly6g6e Slows Desensitization of α4β2 nAChRs—Having ruled out changes in surface expression and changes in ion selectivity as probable mechanisms by which cell surface Ly6g6e potentiates α4β2-mediated currents, we examined changes in channel gating. Specifically, we measured the decay kinetics of whole-cell α4β2 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 α4β2 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. 5*D*).

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

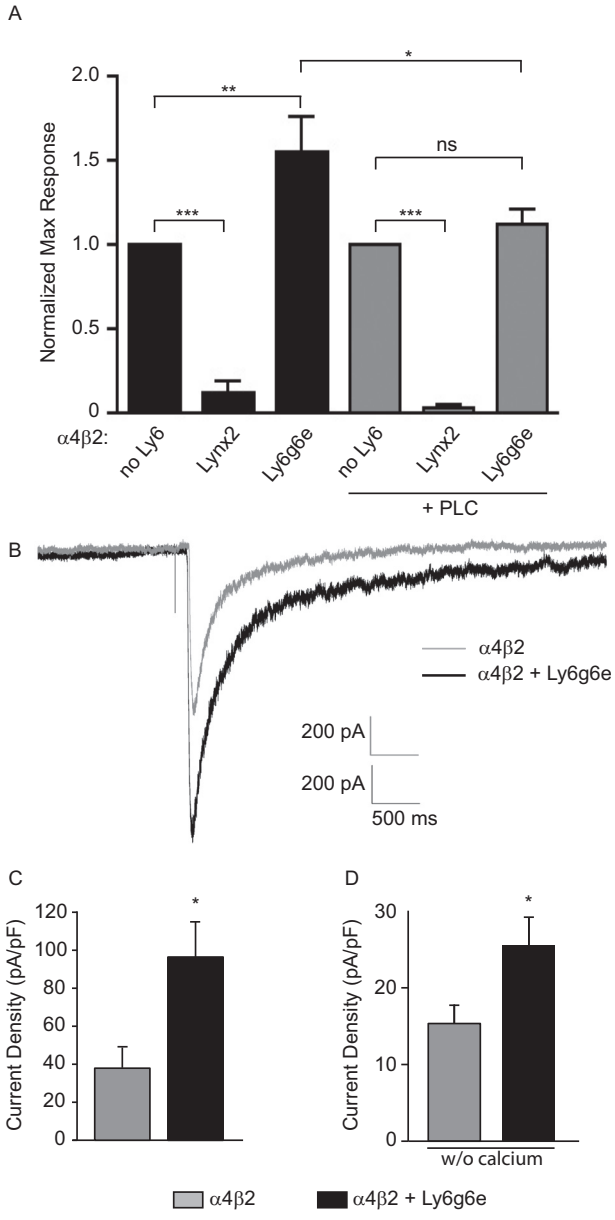

FIGURE 4. **Ly6g6e at the plasma membrane potentiates α4β2 activity.** *A*, average normalized maximum FRET responses to epibatidine in HEKtsa cells transiently transfected with α4 and β2 cDNAs in the absence *versus* the presence of Lynx2 or Ly6g6e. Lynx2 suppresses and Ly6g6e potentiates α4β2 activity 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 α4β2 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 α4 and β2 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 ≥ 5 for all conditions). *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test.

examined α4β2 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

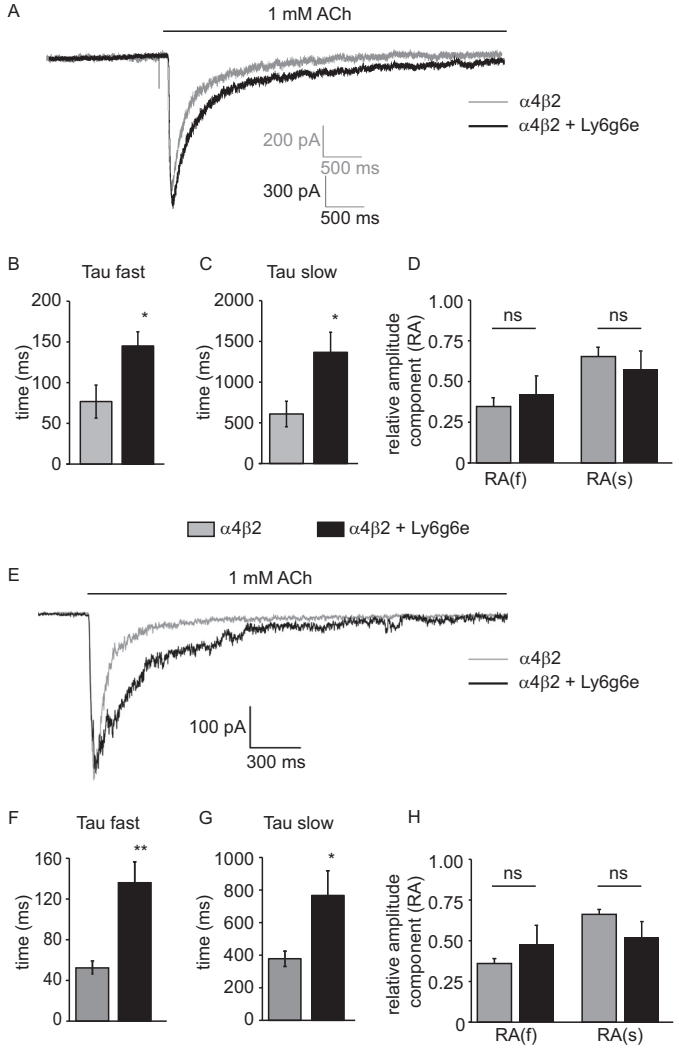

FIGURE 5. **Ly6g6e slows desensitization of α4β2 currents.** *A* and *D*, representative currents from HEKtsa cells transiently transfected with α4 and β2 cDNAs plus either empty vector (control; *gray trace*) or Ly6g6e cDNA (*black trace*). Cells were recorded either with (*A–D*; N ≥ 5) or without (*E–H*; N ≥ 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 α4β2 nAChR currents. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test.

associate with α4β2 nAChRs and to regulate current magnitude and kinetics.

Thus, Ly6g6e potentiates α4β2 signaling in two ways: by increasing peak current magnitude and by slowing desensitization. These results demonstrate a novel mechanism by which α4β2 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 function. 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 α4β2 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 α4β2 desensitization and an increase in the EC_{50} for receptor activation by agonist (33, 34, 39, 47). Evidence also exists for Lynx1 facilitating α4β2 receptor assembly (30).

Here we extend such studies by demonstrating that several additional Ly6 family members are capable of regulating α4β2 nAChR function. Using a high-throughput calcium flux assay as an initial screen for functional modulators of α4β2 nAChRs, we show that multiple members of the Ly6 family can suppress α4β2 activity. Using this screen, we also identify Ly6g6e as a previously unrecognized potentiator of α4β2 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 α4β2 receptors, and modulation of gating by local interactions with α4β2 at the cell surface.

In support of the first mechanism, we have shown that Lynx2 reduces the number of α4β2 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 α4 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 α4β2 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 α4β2 (Fig. 3, *A* and *B*) suggests that Lynx2 and nicotine might act at the same step in α4β2 maturation but in opposing ways.

In addition to the previously unrecognized influence of Lynx2 on trafficking of α4β2 nAChRs, we also demonstrated that Ly6g6e is a novel potentiator of α4β2 signaling. This effect is likely to be direct, since Ly6g6e and α4β2 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

of Lynx2, since only Lynx2's effects were resistant to PLC. Consistent with all these results, we found that Ly6g6e potentiates α4β2 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, α4β2 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 α4β2 nAChRs. Although the binding of Lynx2 to α4β2 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, α4β2 nAChRs would be up-regulated, but when Lynx2 completes with nicotine, α4β2 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 α4β2 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 α4β2 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 α4β2 nAChRs in one brain region will affect structurally related receptors as well as α4β2 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 α4β2 subunits, our work suggests that drugs that mimic Lynx2 or that antagonize interactions between Ly6g6e and α4β2 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 α4β2 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 α4β2 nAChRs. Ly6h in particular is nearly as effective as Lynx2 at suppressing α4β2 currents as measured in our flux assay. These results are somewhat surprising since a previous report suggested that Ly6h does not form complexes with α4β2. 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 α4β2 nAChRs function. Considering the overall limited primary sequence identity between these proteins, it will be interesting in future work to determine how they suppress α4β2 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 α7 nAChRs by some, though not all, of the Ly6 proteins shown to exert effects on α4β2 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.

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