Organelle-Specific Single-Molecule Imaging of $\alpha_4\beta_2$ Nicotinic Receptors Reveals the Effect of Nicotine on Receptor Assembly and Cell-Surface Trafficking

Ashley M. Fox-Lee  
*University of Kentucky*

Faruk H. Moonschi  
*University of Kentucky*, faruk.moonschi@uky.edu

Christopher I. Richards  
*University of Kentucky*, chris.richards@uky.edu

*Right click to open a feedback form in a new tab to let us know how this document benefits you.*

Follow this and additional works at: https://uknowledge.uky.edu/chemistry_facpub

Part of the [Biochemistry, Biophysics, and Structural Biology Commons](https://uknowledge.uky.edu/biochemistry), and the [Chemistry Commons](https://uknowledge.uky.edu/chemistry)

**Repository Citation**

Fox-Lee, Ashley M.; Moonschi, Faruk H.; and Richards, Christopher I., "Organelle-Specific Single-Molecule Imaging of $\alpha_4\beta_2$ Nicotinic Receptors Reveals the Effect of Nicotine on Receptor Assembly and Cell-Surface Trafficking" (2017). *Chemistry Faculty Publications*. 113.  
https://uknowledge.uky.edu/chemistry_facpub/113

This Article is brought to you for free and open access by the Chemistry at UKnowledge. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
Organelle-Specific Single-Molecule Imaging of α4β2 Nicotinic Receptors Reveals the Effect of Nicotine on Receptor Assembly and Cell-Surface Trafficking

Notes/Citation Information
Published in The Journal of Biological Chemistry, v. 292, no. 51, p. 21159-21169.

This research was originally published in The Journal of Biological Chemistry. Ashley M. Fox-Loe, Faruk H. Moonschi, and Christopher I. Richards. Organelle-Specific Single-Molecule Imaging of α4β2 Nicotinic Receptors Reveals the Effect of Nicotine on Receptor Assembly and Cell-Surface Trafficking. J. Biol. Chem. 2017; 292:21159-21169. © 2017 by The American Society for Biochemistry and Molecular Biology, Inc.

The copyright holder has granted the permission for posting the article here.

Digital Object Identifier (DOI)
https://doi.org/10.1074/jbc.M117.801431
Organelle-specific single-molecule imaging of \( \alpha 4\beta 2 \) nicotinic receptors reveals the effect of nicotine on receptor assembly and cell-surface trafficking

Ashley M. Fox-Loe¹, Faruk H. Moonschi¹, and Christopher I. Richards²

From the Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506

Edited by F. Anne Stephenson

Nicotinic acetylcholine receptors (nAChRs) assemble in the endoplasmic reticulum (ER) and traffic to the cell surface as pentamers composed of \( \alpha \) and \( \beta \) subunits. Many nAChR subtypes can assemble with varying subunit ratios, giving rise to multiple stoichiometries exhibiting different subcellular localization and functional properties. In addition to the endogenous neurotransmitter acetylcholine, nicotine also binds to and activates nAChRs and influences their trafficking and expression on the cell surface. Currently, no available technique can specifically elucidate the stoichiometry of nAChRs in the ER versus those in the plasma membrane. Here, we report a method involving single-molecule fluorescence measurements to determine the structural properties of these membrane proteins after isolation in nanoscale vesicles derived from specific organelles. These cell-derived nanovesicles allowed us to separate single membrane receptors while maintaining them in their physiological environment. Sorting the vesicles according to the organelle of origin enabled us to determine localized differences in receptor structural properties, structural influence on transport between organelles, and changes in receptor assembly within intracellular organelles. These organelle-specific nanovesicles revealed that one structural isoform of the \( \alpha 4\beta 2 \) nAChR was preferentially trafficked to the cell surface. Moreover, nicotine altered nAChR assembly in the ER, resulting in increased production of the receptor isoform that traffics more efficiently to the cell surface. We conclude that the combined effects of the increased assembly of one nAChR stoichiometry and its preferential trafficking likely drive the up-regulation of nAChRs on the cell surface upon nicotine exposure.

Received for publication, June 9, 2017, and in revised form, October 20, 2017. Published, Papers in Press, October 26, 2017, DOI 10.1074/jbc.M117.801431

This work was supported in part by the National Institute on Drug Abuse, National Institutes of Health Training Grants DA016176 (A. M. F.-L.) and DA038817. The authors declare that they have no conflicts of interest with respect to their authorship and does not necessarily represent the official views of the National Institutes of Health.

¹ Both authors contributed equally to this work.
² To whom correspondence should be addressed: Dept. of Chemistry, University of Kentucky, 505 Rose St., Lexington, KY 40506. Tel.: 859-218-0971; E-mail: chris.richards@uky.edu.
³ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ER, endoplasmic reticulum; ID, integrated density; N2a, neuroblastoma 2a; PM, plasma membrane; PMID, plasma membrane integrated density; %PM, percentage on plasma membrane; SEP, superflippic pHfluorin; TIRF, total internal reflection fluorescence; PMCA, plasma membrane calcium ATPase; ROI, region of interest.
Altered stoichiometry of nAChRs

Figure 1. Schematic showing the generation of organelle-specific nanovesicles containing a single nAChR. Cells expressing fluorescently labeled membrane receptors are expressed throughout the ER and on the plasma membrane. Nitrogen cavitation is used to fragment the cells forming small membrane domains from cellular organelles. These membrane domains spontaneously form nanoscale vesicles. The domains and subsequent vesicles are small enough that there is a low probability of more than one receptor being encapsulated. The resulting vesicles have the same membrane properties as the organelle of origin, thus maintaining a physiological environment. Differences in the densities between the organelle membranes are used to separate them via gradient centrifugation. Vesicles are isolated on glass substrates for TIRF imaging.

membrane receptors in cell-derived nanoscale vesicles composed of original membranes enables the separation of receptors based on organelle (25, 26). Investigation of oligomeric proteins from specific organelles at a single-molecule level provides a way to distinguish between different structural and functional populations of these proteins, allowing the effect of changes in assembly on protein trafficking to be directly studied.

Here we report a novel approach that enables us to perform organelle-specific single-molecule studies of membrane proteins. We can effectively select populations from the ER and the plasma membrane to quantify properties such as the distribution of stoichiometric assemblies of oligomeric proteins. We applied this technique to study nicotine-induced changes in the assembly of α4β2 nAChRs in the ER and changes in trafficking to the cell surface.

Results

Ligand-induced up-regulation of α4β2 receptors

Nicotine and several other nicotinic receptor ligands have been shown to up-regulate the number of receptors on the cell surface. It has been hypothesized that this up-regulation is connected to changes in receptor stoichiometry (18, 27). We first evaluated a series of ligands to determine whether they altered the expression and trafficking of α4β2 by using a pH-sensitive fluorophore, super ecliptic pHluorin (SEP) (11, 28, 29). The SEP label was genetically incorporated into the protein sequence of the receptor so that it was on the luminal side of the ER and the extracellular side of the plasma membrane (Fig. 2, A and B). SEP is fluorescent at neutral pH and quenched at acidic pH. Thus, receptors in the ER and plasma membrane will exhibit fluorescence, whereas receptors in the Golgi and trafficking vesicles are not fluorescent. Using total internal reflection fluorescence microscopy, we measured ligand-induced up-regulation of α4β2 expression on the plasma membrane as an increase in plasma membrane integrated density (PMID) and a change in the distribution between the ER and plasma membrane (%PM) as compared with control cells (26, 30). Exposure to nicotine or cytisine resulted in a 2.5-fold increase, varenicline yielded a 2-fold increase, and bupropion resulted in a 1.5-fold increase in α4β2 expression on the cell surface (Fig. 2C). Additionally, the intracellular distribution of α4β2 between the plasma membrane and peripheral ER (%PM) shifted toward the plasma membrane upon exposure to each of these ligands (Fig. 2D). Comparisons between the ligands showed that nicotine provides the highest level of up-regulation in terms of expression and distribution toward the plasma membrane.

Ligand-induced changes in α4β2 assembly

To determine whether ligands that up-regulated nicotinic receptors also changed their assembly, we then generated whole-cell nanovesicles from cells expressing α4GFP β2wt nAChRs (Fig. 3A) and examined the distribution of the two possible α4β2 isoforms, (α4GFP)4(β2)4 and (α4GFP)3(β2)2. Nanovesicles were derived from cells both in the presence and absence of the nicotinic ligands. Single receptors were then isolated into membrane-derived vesicles via nitrogen cavitation, immobilized on a glass surface, and imaged using total internal reflection fluorescence (TIRF) microscopy. A representative image of isolated vesicles is shown in Fig. 3B. We then determined the number of photobleaching events (31–33) from the intensity time traces recorded for the fluorescence of each nanovesicle (Fig. 3, C and D). The assignment of the stoichiometric distribution is complicated by the fact that a small fraction of GFP exists in a non-fluorescent state (25, 34). Additionally, the observed distribution of photobleaching events arises from a combination of (α4GFP)4(β2)4 and (α4GFP)3(β2)2 stoichiometries. To account for these factors, the observed distribution was fit to two binomial distributions, corresponding to the distributions of the photobleaching events of (α4GFP)4(β2)4 and (α4GFP)3(β2)2. They were weighted iteratively to determine the contribution of each stoichiometry. Results from these unsorted vesicles provided a measure of the whole-cell distribution of α4β2 stoichiometries. In the absence of any ligand, we observed a distribution of 41% (α4)4(β2)4 and 59% (α4)3(β2)2 (Fig. 4A). The presence of nicotine shifted the distribution of stoichiometry to 59% (α4)2(β2)3 and 41% (α4)3(β2)2 (Fig. 4B). A comparison of all ligands showed that each altered the assembly toward (α4)3(β2)3 with cytisine increasing to 50%, varenicline increasing to 54%, and bupropion increasing to 55%
of the high-sensitivity stoichiometry (Fig. 4, C–E). The observed distribution of bleaching steps is shown in Table 1.

**Organelle-specific stoichiometry of α4β2**

We isolated nanovesicles and then sorted them via gradient centrifugation into those derived from the ER and plasma membrane (Fig. 1). Differences in the density of endogenous ER and plasma membrane receptor populations. B, when the extracellular solution was replaced with a pH 5.4 solution, all SEP on the plasma membrane transition to a non-fluorescent state, and only the receptors within the ER are visible. C, the integrated density of α4β2 on the plasma membrane increased from $-2.5 \times 10^6$ in the absence of any compound to $7 \times 10^6$ in the presence of nicotine (Nic) or cytisine (Cyt). Varenicline (Var) and bupropion (Bup) both resulted in a 2-fold increase in the integrated density on the plasma membrane demonstrating ligand-induced up-regulation. D, the percentage of the receptors present on the plasma membrane increased from 21.5% for control cells to 30–40% for all nicotinic receptor ligands, showing a shift in distribution of receptors toward the plasma membrane ($n = 61, 47, 42, 38, 51$). The data are mean values ± S.D.). **, $p < 0.001$. Integrated density (average fluorescence intensity × area) is the total gray values background within a region of interest that encompasses a cell. PMID is obtained by subtracting the integrated density of pH 5 image of a cell from pH 7 image of the same cell.

Because nicotinic receptors are synthesized in the ER, differences in assembly are reflected in the stoichiometry of this population. Receptors are trafficked to the cell surface after assembly; thus, a change in the stoichiometry on the plasma membrane reflects preferential trafficking or increased stability on the cell surface. We performed separate single-molecule studies on both the ER and plasma membrane-specific nanovesicles. Single-molecule photobleaching analysis relies on the observation of single step bleaching events of GFP where each bleaching event corresponds to a single subunit. Our results using GFP-labeled α subunits showed that the predominately expressed stoichiometry of α4β2 nAChRs depends on the subcellular region. Receptors encapsulated in nanovesicles derived from the ER show that in the absence of nicotine, α4β2 nAChRs predominately assemble with the low-sensitivity stoichiometry of $(α4)_2(β2)_2$. The distribution of photobleaching events from the ER resident α4β2 nAChRs fit to a theoretical distribution
Altered stoichiometry of nAChRs

Figure 3. Single-molecule photobleaching to determine nAChR stoichiometry. A, representative mouse N2a cell expressing GFP-labeled nicotinic receptors in TIRF. B, representative TIRF image of isolated nanovesicles containing individual GFP-labeled receptors on a glass substrate. C and D, representative time traces of two (C) and three (D) photobleaching steps for GFP-labeled nicotinic receptors, corresponding to two or three GFP-labeled α4 subunits, respectively.

Figure 4. Whole-cell evaluation of (α4)2(β2)1 versus (α4)3(β2)2 assembly upon exposure smoking cessation agents. Expected distributions of one, two, and three photobleaching steps were obtained by weighting two binomial distributions. A χ² goodness-of-fit test was used to verify expected and observed distributions of two and three GFP-labeled α4 subunits. A, in the absence of a pharmacological agent, the α4β2 population exists as 41% (α4)2(β2)1 and 59% (α4)3(β2)2. B, 500 nM nicotine alters the ratio of isoforms to 59% (α4)2(β2)1 and 41% (α4)3(β2)2. C, 500 nM cytisine shifts the stoichiometry to 50% high-sensitivity receptors. D, 500 nM varenicline shifts the distribution to 54% high-sensitivity receptors. E, 500 nM bupropion shifts the stoichiometry to 55% high-sensitivity receptors, (α4)3(β2)2. The error bars for the subunit distribution are based on counting events and are calculated as the square root of the counts.
Altered stoichiometry of nAChRs

Table 1

The observed distribution of bleaching steps for whole cell nanovesicles expressing α4β2

| Vesicles counted | One step | Two steps | Three steps | Four steps |
|------------------|----------|-----------|-------------|------------|
| Control          | 767      | 53        | 386         | 328        | 54         |
| Nicotine         | 192      | 22        | 112         | 58         | 9          |
| Cytisine         | 357      | 41        | 187         | 130        | 17         |
| Varenicline      | 833      | 94        | 459         | 280        | 30         |
| Bupropion        | 1089     | 102       | 598         | 389        | 43         |

Figure 5. Western blots verifying separation of ER and plasma membrane–derived nanovesicles. Anti-calnexin was used as an ER marker to identify nanovesicles originating from the ER. Anti-PMCA was used to detect nanovesicles derived from the plasma membrane. Calnexin is detected in higher density fractions when vesicles are formed at 250 p.s.i. (A) and at 600 p.s.i. (B). Minimal PMCA is detected at fragmentation of 250 p.s.i. (C) but are localized to lower density fractions upon swelling with a hypotonic solution and higher cavitational pressure of 600 p.s.i. (D). ER-specific nanovesicles are collected from fraction 2 after 250 p.s.i. Plasma membrane–specific nanovesicles are collected from fraction 7 after formation at 600 p.s.i.

Nicotine changes the stoichiometry of α4β2 in the ER

We next prepared ER and plasma membrane–specific vesicles from cells expressing α4β2 in the presence of 500 nM nicotine. We observed a clear nicotine-induced shift in the stoichiometry of ER resident α4β2 receptors (Fig. 6C). When nicotine was present, single-molecule bleaching step analysis showed the majority of endoplasmic α4β2 assembled as the high-sensitivity isoform, fitting a 55% (α4)2(β2)3 and 45% (α4)3(β2)2 distribution (Fig. 6C). This shift from the stoichiometry seen in the absence of nicotine indicates that nicotine drives the assembly of the high-sensitivity isoform, (α4)2(β2)3. Although some groups have previously hypothesized that nicotine alters the assembly of α4β2 receptors (8, 9, 36, 37), these organelle-specific single-molecule studies allowed us to directly observe the process of nicotine altering the assembly of receptors in the ER for the first time. In addition to nicotine altering the assembly of α4β2 within the ER, the percentage of the high-sensitivity (α4)2(β2)3 stoichiometry on the plasma membrane was also increased (Fig. 6D). After exposure to nicotine, the distribution of photobleaching events observed from plasma membrane–resident α4β2 nicotinic receptors was fit to a distribution of 70% (α4)2(β2)3 and 30% (α4)3(β2)2 (Fig. 6D).

Biased transfection to validate nicotine-induced shifts in receptor stoichiometry

Our studies indicate that nicotine induces a shift toward the assembly of the high-sensitivity stoichiometry in both the ER and the plasma membrane. Biased transfection has previously been used to induce a shift stoichiometry in that mimics that seen with nicotine (38, 39). We performed a set of control studies to verify that in our experiments we could observe a shift in stoichiometry in the ER and the plasma membrane. We transfected HEK-293T cells with a 1:10 ratio of α4-GFP:β2-wt plasmids and generated vesicles from whole cells, the ER, and plasma membrane. Single-molecule photobleaching analysis studies of the unsorted receptors showed 73% (α4)2(β2)3 and 27% (α4)3(β2)2 (Fig. 7A). The ER–originated receptors exhibited 67% (α4)2(β2)3 and 33% (α4)3(β2)2 (Fig. 7B). The plasma membrane population exhibited 82% (α4)2(β2)3 and 18% (α4)3(β2)2 (Fig. 7C). These control studies verify that our technique is capable of independently measuring organelle-specific shifts in stoichiometry.

Discussion

We have developed a new technique that allows us to perform organelle-specific single-molecule studies on membrane proteins. We utilized this novel method to determine that changes in nicotinic receptor stoichiometry related to nicotine-induced up-regulation (40) are likely driven by both changes in the assembly in the ER and the preferential trafficking of the high-sensitivity stoichiometry. The consequences of these changes are an increase in the number of receptors both in the ER and on the plasma membrane, as well as a shift in stoichiometric distribution toward the high-sensitivity assembly. Previous studies to measure ER specific changes in stoichiometry have primarily been limited by a lack of existing techniques that are capable of directly quantifying subcellular specific structural assemblies of complex proteins in a physiological cellular environment. The isolation of membrane proteins in organelle-specific nanovesicles provides a snapshot of membrane protein assembly in each subcellular location at the time the vesicles are generated. We observed that nicotine, cytisine, varenicline, and bupropion all up-regulated the number of receptors on the cell surface. We also observed that these same compounds all altered the stoichiometry of α4β2 nAChRs toward the high-sensitivity stoichiometry. Nicotine induced the largest increase in membrane expression and the largest shift toward the high-sensitivity stoichiometry, (α4)2(β2)3. The only previous single-molecule study of α4β2 stoichiometry on the plasma membrane showed that cytisine elicited a shift toward the low-sensitivity stoichiometry in contrast to our findings (17). This previous study only examined the stoichiometry in the very tip of filopodia projected into 150–200-nm apertures. This restricted studies to a specialized surface domain likely accounting for the differences seen here. Our studies sample
Altered stoichiometry of nAChRs

Figure 6. Single-molecule bleaching step analysis shows organelle-specific differences in α4β2 nAChR isoforms. A, the observed ratio of vesicles showing one, two, or three steps was 0.057, 0.43, and 0.51, respectively (blue columns). These observed values were then fit to a 30:70 (high-sensitivity:low-sensitivity) stoichiometry. The fit was verified using a χ² goodness-of-fit analysis. B, the expression of α4β2 nAChRs on the plasma membrane fit binomial distributions weighted for 56% (α4)(β2)3 and 44% (α4)(β2)2. The observed fraction of vesicles showing one, two, or three bleaching steps was 0.12, 0.56, and 0.32, respectively. C, for ER resident receptors in the presence of nicotine, the observed fraction of one, two, and three bleaching steps were 0.086, 0.59, and 0.33, respectively. These observed values were then fit to a 55:45 distribution. D, the observed fraction of vesicles with one, two, or three bleaching steps were 0.11, 0.67, and 0.22, respectively. This was fit to a 70:30 distribution. The error bars for the subunit distribution are based on counting events and are calculated as the square root of the counts.

| Vesicles | One step | Two steps | Three steps | Four steps |
|----------|----------|-----------|-------------|------------|
| ER no drug | 458 | 26 | 199 | 233 | 14 |
| PM no drug | 545 | 26 | 199 | 233 | 1 |
| ER + 500 nM nicotine | 465 | 40 | 273 | 152 | 27 |
| PM + 500 nM nicotine | 883 | 100 | 592 | 191 | 18 |

Table 2

The observed distribution of bleaching steps for organelle-specific nanovesicles expressing α4β2

We then performed organelle-specific single-molecule studies of α4β2 nAChRs in the presence and absence of nicotine to resolve the connection between increased expression and changes in stoichiometry. Comparing the distribution between the low-sensitivity stoichiometry and high-sensitivity stoichiometry in the ER and plasma membrane showed a much larger fraction of receptors exhibiting the high-sensitivity stoichiometry on the plasma membrane. This strongly suggests that the high-sensitivity stoichiometry traffics to the cell surface more efficiently than the low-sensitivity stoichiometry. We also observed a shift in the ER stoichiometry toward the high-sensitivity isoform in the presence of nicotine. This suggests that nicotine induces an intracellular change in the assembly of the α4β2 nAChR. Biased transfection can shift the production of the α4β2 toward the high-sensitivity isoform. Single-molecule photobleaching event analysis of vesicles from biased transfections experiments confirmed a shift toward the high-sensitivity stoichiometry in vesicles originating from the ER. We observed an even larger shift toward the high-sensitivity stoichiometry on the plasma membrane. The presence of a higher proportion of the high-sensitivity subtype in the plasma membrane compared with the ER verifies the preferential trafficking of the (α4)2(β2)3 stoichiometry from the ER to plasma membrane.

Fig. 8 summarizes the fitted values from Fig. 6 to illustrate the shift in stoichiometry. The observed differences in organelle stoichiometry show that endogenous assembly in the ER favors the low-sensitivity stoichiometry but that the high-sensitivity isoform is preferentially trafficked from the ER to the plasma membrane. Despite having a lower fraction in the ER, this preferential trafficking results in a larger fraction of high-sensitivity receptors on the plasma membrane. Recent work by several groups has proposed that nicotine acts as a pharmacological chaperone either altering the assembly of nAChRs or influencing the trafficking. Previous studies have also shown that this increases the numbers of α4β2 nAChRs in the ER and the plasma membrane. In these studies, we confirm that increased numbers of receptors in the ER and altered assembly likely play
Observed distribution was validated using a expected distribution of one, two, and three photobleaching steps was determined by weighting two binomial distributions, and the fit of expected and observed distribution was validated using a χ² goodness-of-fit test. The assigned weights represent the proportion of the high- and low-sensitivity stoichiometries. A, the observed photobleaching distribution of the receptors obtained from the whole-cell homogenate fit with the expected distribution obtained with 73% (α4β2), and 27% (α4β2). B, the ER originated receptors exhibited a photobleaching step distribution which agreed with 67% (α4β2), and 33% (α4β2) stoichiometries. C, the stoichiometry for receptors from the plasma membrane was 82% (α4β2), and 18% (α4β2). The error bars for the subunit distribution are based on counting events and are calculated as the square root of the counts.

Figure 7. Biased transfection of α4β2 to shift assembly toward the high-sensitivity ((α4)2(β2)4) subtype in both the ER and the plasma membrane. The expected distribution of one, two, and three photobleaching steps was determined by weighting two binomial distributions, and the fit of expected and observed distribution was validated using a χ² goodness-of-fit test. The assigned weights represent the proportion of the high- and low-sensitivity stoichiometries. A, the observed photobleaching distribution of the receptors obtained from the whole-cell homogenate fit with the expected distribution obtained with 73% (α4β2), and 27% (α4β2). B, the ER originated receptors exhibited a photobleaching step distribution which agreed with 67% (α4β2), and 33% (α4β2) stoichiometries. C, the stoichiometry for receptors from the plasma membrane was 82% (α4β2), and 18% (α4β2). The error bars for the subunit distribution are based on counting events and are calculated as the square root of the counts.

Figure 8. Organelle-specific single-molecule studies reveal a combination of endogenous preferential trafficking, and an intracellular increase in assembly may be responsible for nicotine-induced up-regulation. A, organelle-specific single-molecule photobleaching step studies of stoichiometry show that in the absence of nicotine, α4β2 predominantly assembles into the 3α stoichiometry (blue) (70%). B, in the absence of nicotine, the 2α stoichiometry (green) is preferentially trafficked to the cell surface, resulting in a higher proportion of receptors on the cell surface having the 2α stoichiometry. C, in the presence of nicotine, the intracellular assembly of α4β2 is altered to favor the high-sensitivity, 2α isof orm (green). D, the increase in availability of the preferentially trafficked stoichiometry, (α4)2(β2)4, leads to an even higher proportion of the 2α stoichiometry (green) on the plasma membrane (70%).

Altered stoichiometry of nAChRs

Roles in plasma membrane up-regulation but are only part of the mechanism. Upon addition of nicotine, the assembly of subunits into a pentamer within the ER is altered to a higher ratio of high-sensitivity receptors that can then be efficiently trafficked to the cell surface. This suggests a mechanism of nicotine-induced plasma membrane up-regulation that is tied to increased numbers of receptors in the ER, preferential trafficking, and a change in assembly. The shift in assembly of α4β2 nAChRs within the ER upon exposure to nicotine toward the preferentially trafficked high-sensitivity isoform is likely responsible in part for the nicotine-induced up-regulation that has been previously observed. It is possible that residues in the M1–M2 and M3–M4 loops on the intracellular side of each of the subunits of α4β2 regulate preferential trafficking to the cell surface. These intracellular loops contain a number of ER retention and ER exit motifs, as well as sites that undergo post-translational modification in the secretory pathway (9, 41). These same processes are also responsible for targeted trafficking to neuronal subcellular regions. Additionally, recycling from the Golgi back to the ER has been shown to be necessary for nicotine-induced up-regulation of some nicotinic receptor subtypes (16). It is likely that differences in post-translational modification sites of the intracellular regions of α4 and β2 lead to the observed differences in trafficking between the two stoichiometries. Employment of our organelle-specific single-molecule method enabled the distinction between changes in trafficking compared with changes in assembly of α4β2 nicotinic receptors to partially delineate the underlying mechanism of nicotine-induced up-regulation.

Experimental procedures

Plasmid constructs for fluorescently labeled nicotinic receptors (SEP and GFP) were generated as previously reported (26). The α4–SEP construct was made by fusing the DNA sequence of SEP to the 3' end of the DNA sequence of the α4 subunit. GFP constructs were made by inserting the label between the M3 and M4 transmembrane segments of the α4 subunit. Both constructs have been shown to produce functional receptors in previous studies (25, 42, 43).

Cell culture

Undifferentiated mouse neuroblastoma 2a (N2a) cells were employed to study the trafficking of α4β2 nAChRs. N2a cells were cultured and maintained with an N2a growth medium (equal volume mixture of DMEM and OptiMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin) at 37 °C with 5% CO₂ in a humidified incubator. Approximately 90,000 N2a cells were plated on a poly-d-lysine–coated glass-bottomed dish (35 mm in diameter; Cell E&G, San Diego, CA). The coated dish was prepared by incubating it with 0.1% poly-d-lysine in sterile deionized water at 37 °C for 1 h. The unbound poly-d-lysine was removed by rinsing with sterile deionized water, and the dish was dried for 2 h in a biosafety hood. After 16–24 h, the N2a cells were transfected with 500 ng
of each α4-SEP and β2-wt plasmids with 2 μl of Lipofectamine 2000 as described previously (26). Briefly, cells were transfected for 24 h, followed by a 24-h incubation in growth media prior to imaging. Transfection mix was prepared by incubating a mixture of 250 μl of OptiMEM and 2 μl of Lipofectamine 2000 transfection agent for 5 min at room temperature, followed by a 25-min room temperature incubation upon combination with a mixture of 250 μl of OptiMEM and 500 ng of each plasmid DNA. The 500 μl of transfection mix was added to preplated cells in 1.5 ml OptiMEM. After 24 h, transfection medium was replaced with N2a growth medium for an additional 24-h incubation. Transfected cells were imaged 48 h after initial transfection. When applicable, 500 nm of each nictinic ligand, (−)-nicotine hydrogen tartrate salt (−98%), bupropion hydrochloride (>98%), varenicline tartrate, or (−)-cytisine (>99%), was added to the transfection medium and replenished later in the growth medium. Transfection efficiency was generally 80% and was not significantly altered by the presence of any of the ligands.

**Total internal reflection fluorescence**

The TIRF microscope system employed to visualize SEP or GFP molecules was previously described (26, 44). Briefly, a 488-nm DPSS laser excitation source was directed toward the back aperture of an objective (60×, 1.49 NA) mounted on an inverted microscope (Olympus IX81). The angle of excitation light was adjusted to obtain total internal reflection through the objective using a stepper motor that translated the beam across the back aperture of the objective. The emission was collected though the objective, and a dichroic mirror was used to direct the light to an EMCCD camera (Andor).

**Receptor expression and distribution**

For *in vivo* fluorescence imaging studies, the growth medium of the transfected N2a cells was replaced with an extracellular solution (10 mM HEPES, 10 mM D-glucose, 150 mM NaCl, 4 mM KCl, 2 mM MgCl₂, and 2 mM CaCl₂) of pH 7.4. The dish was then mounted on a translational stage, and the cells were located by exciting the SEP molecules with a 488-nm laser (~1 milliwatt) source. Images of cells were captured using an EMCCD camera with a 200-ms exposure time. The extracellular solution was then replaced with an identical solution of pH 5.4, followed by a 10-min stage-top incubation before capturing images of the same cells. An open source software, ImageJ (National Institutes of Health) was employed to analyze the images. Background was subtracted using the rolling ball background subtraction with a diameter of 25 pixels. A freehand region of interest (ROI) was drawn around a cell, and an intensity based threshold was used to obtain an integrated density for each cell. The integrated density of the cell at pH 5.4 (ER ID) is subtracted from the integrated density of the same cell at pH 7.4 (total ID) to calculate the relative number of receptors on the plasma membrane or PMID. The percentage of receptors located on the plasma membrane within the TIRF region of excitation (%PM) is calculated by dividing the PMID by the total ID at pH 7.4, multiplied by 100. The data are reported as the means ± S.D.

**Nanovesicle preparation**

HEK-293T cells were cultured and maintained with a growth medium (DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin) at 37 °C temperature with 5% CO₂ in a humidified incubator. Three million HEK-293T cells were plated in a Matrigel-coated T75 flask 16–24 h prior to transfection. The cells were transfected with 14 μl of Lipofectamine 2000 and 3.5 μg of each plasmid as previously described (25, 26). For biased expression experiments, a 1:10 transfection ratio of α4-GFP:β2-wt using 1 μg of α4-GFP and 10 μg of β2-wt was employed. Briefly, a mixture of 250 μl of OptiMEM and the above mentioned amount of α4-GFP and β2-wt plasmids was prepared. Separately, 14 μl of Lipofectamine 2000 was added to 250 μl of OptiMEM and incubated for 5 min at room temperature before being added to the DNA mixture. This new mixture was incubated at room temperature for 25 min. Afterward, the transfection mixture was added to the flask of HEK-293T cells. The following day, vesicles were prepared from transfected cells as previously described (25). Briefly, the cells underwent nitrogen cavitation at 250 p.s.i. for 5 min while suspended in 5 ml of sucrose-HEPES buffer supplemented with a protease inhibitor (250 mM sucrose, 10 mM HEPES, 1 Pierce protease inhibitor mini tablet per 10 ml of buffer (ThermoScientific), pH 7.5). Cell lysate was then centrifuged at 4000 × g for 10 min. Supernatant was collected and centrifuged at 10,000 × g for 20 min. Supernatant was again collected and centrifuged at 100,000 × g for 1 h. The pellet was resuspended in 800 μl of sucrose-HEPES buffer (250 mM sucrose, 10 mM HEPES, pH 7.5). Nanovesicles were stored at −80 °C until use.

**Generation of ER and plasma membrane vesicles**

HEK-293T cells were transfected as described above. After transfection for 24 h, transfection mix was removed, and the cells were rinsed once with PBS. To generate nanoscale plasma membrane vesicles containing a single nACHR, transfected cells were first swollen for 20 min in a hypotonic solution (10 mM NaCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 0.2 mM CaCl₂, pH 7.4) at 0 °C. To prepare both plasma membrane and ER-derived vesicles, the cells were treated with 5 ml of 1× Versene (Invitrogen), incubated at 37 °C for 5 min, and pelleted by centrifugation at 200 × g for 5 min, as previously described. The cell pellet was resuspended in 3 ml of sucrose buffer plus protease inhibitors (250 mM sucrose, 10 mM HEPES, 1 Pierce protease inhibitor mini tablet per 10 ml of buffer (ThermoScientific), pH 7.5) before undergoing nitrogen cavitation in a nitrogen decompressor (Parr Instrument Company, Moline, IL). To generate ER nanovesicles, the cells were pressurized to ~250 p.s.i. for 20 min. At this pressure, plasma membrane rupturing is minimal, and therefore nanoscale vesicle formation from this organelle is negligible. To generate plasma membrane nanovesicles, the cells were pressurized to ~600 p.s.i. for 20 min. The cell lysate was collected and dispensed onto an OptiPrep gradient.

**Separation of organelle-specific vesicles**

A 9-fraction OptiPrep (60% (w/v) iodoxanol in H₂O; Accurate Chemical & Scientific Corp., Westbury, NY) gradient was used.
to purify organelle-specific nanovesicles. Gradient solutions of OptiPrep were prepared by diluting the 60% stock solution to 30, 20, and 10% in sucrose-HEPES buffer (250 mM sucrose, 10 mM HEPES, pH 7.5) and stored at 4 °C. The gradient was prepared in an Ultra-Clear centrifuge tube (Beckman Coulter), with 3 ml of the densest fraction added first. ER or plasma membrane nanovesicles containing cell lysate, based on nitrogen pressure during cavitation, was dispensed on top of the 10% fraction, before centrifugation at 112,000 × g for 1.5 h. After centrifugation, nine 1–1.5-ml fractions, with density interfaces in the same fraction, were collected using a peristaltic pump. Tubing connected to the pump was vertically inserted into the centrifuge tube so that the highest density fraction is collected first. After fractionation, OptiPrep was removed from nanovesicles by centrifugation at 10,000 × g for 1 h.

**Western blot analysis**

Resuspended OptiPrep fractions containing membrane proteins were ran on a prepackaged NuPAGE 4–12% Bis-Tris gel (Life Technologies), followed by transfer to a nitrocellulose membrane. The membrane was first blocked for 1 h with a PBST solution (5% nonfat milk, 0.1% Tween in PBS). Primary antibodies specific for calnexin (Santa Cruz, calnexin antibody (H-70), catalog no. sc-11397) or PMCA (Santa Cruz, PMCA antibody (D-1), catalog no. sc-271193) were added to the membrane in a 1:1000 dilution and incubated overnight at 4 °C. Endogenous calnexin is solely found in the membrane of the ER, whereas PMCA is expressed on the plasma membrane, thus providing a means to identify fractions that consist of exclusively ER or plasma membranes. After overnight incubation, primary antibodies were removed by four repeated 5-min washes with PBST. Secondary rabbit antibody (calnexin) or mouse antibody (PMCA) (Jackson ImmunoResearch) was added in a 1:5000 dilution and incubated for 1 h at room temperature, followed by another series of four repeated 5-min washes with PBST. Bands were visualized by addition of Western blotting substrate for chemiluminescence (Clarity; Bio-Rad) on a Chemi-Doc system (Bio-Rad). To validate these results, blots were repeated with a completely different set of antibodies. For primary antibodies, we used 1:2000 diluted rabbit monoclonal anti-calnexin (catalog no. ab92573, Abcam) for ER identification and 1:2000 diluted rabbit monoclonal anti-Na,K-ATPase (catalog no. ab76020, Abcam) for plasma membrane identification. In both sets of orthogonal studies, the ER and plasma membrane bands matched the expected molecular weights.

**Imaging nanovesicles**

A 35-mm glass-bottomed dish was cleaned by sonicating the dish in 5 M NaOH solution for 30 min at 45 °C and then in 0.1 M HCl solution for 30 min at 45 °C. The dish was rinsed with water, sprayed with 100% ethanol three times after each step, and then dried using compressed air. Finally, the dishes were treated in an oxygen plasma (21% oxygen for ~5 min). A biotinylated anti-GFP antibody functionalized glass-bottomed dish was prepared by incubating a cleaned dish at room temperature with 1 mg/ml Silane-PEG-Biotin in 95% ethanol for 30 min, 0.1 mg/ml NeutrAvidin in PBS (1× PBS, pH 7.4) solution for 5 min, and finally 1 μg/ml biotinylated anti-GFP antibody in PBS for 15 min. Between each of the steps, the dish was rinsed three times with 1× PBS solution. Vesicles were immobilized on the biotinylated anti-GFP antibody functionalized dish by adding 50–200-fold diluted vesicles in PBS for 30 min at room temperature. The unbound vesicles were removed by rinsing with PBS, and ~1 ml of PBS solution was added to the dish. The microscope setup employed to capture images for SEP based studies was also utilized to obtain movies of about 1000 frames (100 ms of exposure time) during 488-nm laser excitation (~3 milliwatts).

**Data analysis**

A customized software package was written in Matlab to populate time traces from the movies collected with immobilized vesicles. Briefly, the first 10 frames of a movie were combined together to make a composite frame, which was utilized to find peaks with a user defined threshold level. A 3-pixel by 3-pixel ROI was selected for each peak position to obtain the mean intensity of the ROI. A 5-pixel by 5-pixel ring around the peak was selected, and the mean value of the pixels located on the ring was considered as background that was subtracted from the mean value of the ROI of the corresponding frame to obtain a background subtracted mean intensity of the ROI. Time traces for all peaks were stored in a temporary file. During the initial evaluation, a time trace of the temporary file was accepted if the difference of the mean of the intensities of first 20 frames and last 20 frames was more than twice the standard deviation of last 20 frames. All time traces for the qualified molecules were collected and stored for further analysis.

A photobleaching step was counted only if it lasted at least 1 s and the intensity levels of a step and the next lower level had a difference of at least twice the standard deviation of the lower level. A time trace was considered to arise from a single-molecule if it showed at least one clear bleaching step. Each set of data was independently analyzed at least twice, and the results were compared.

**Data fitting**

The probability of observing a photobleaching event from a GFP molecule is less than 1. Therefore, a binomial distribution was employed to determine the distribution of the number of photobleaching events observed from a population of GFP-labeled receptors. A general equation for observing k number of photobleaching events from n number of GFP-labeled receptors can be written as,

\[
F(k; n, p) = \frac{n!}{k!(n-k)!} p^k (1-p)^{n-k} \quad \text{(Eq. 1)}
\]

where \( p \) is the probability of observing a photobleaching event from a GFP-labeled subunit, which has been previously determined as 0.90 (25).

A matrix \( M_2 \) with the probabilities of obtaining one, two, and three photobleaching events from two GFP containing α4β2 nAChRs (i.e. (α4-GFP)2(β2-wt)3) can be written as follows.

\[
M_2 = [F(1; 2, 0.9), F(2; 2, 0.9), 0] \quad \text{(Eq. 2)}
\]

**Altered stoichiometry of nAChRs**

\[
\begin{align*}
\alpha_1 & = \frac{9}{2}, \\
\alpha_2 & = \frac{9}{1}, \\
\alpha_3 & = \frac{9}{1}, \\
\alpha_4 & = \frac{9}{1}, \\
\beta & = \frac{9}{1}, \\
\gamma & = \frac{9}{1},
\end{align*}
\]
Similarly, matrix $M_{3y}$, containing the probabilities of observing one, two, and three photobleaching events from three GFP containing $\alpha_4\beta_2$ nAChRs (i.e. $(\alpha_4\text{-GFP})_3(\beta_2\text{-wt})_2$), can be expressed as shown in Equation 3.

$$M_3 = \begin{bmatrix} F(1;3,0.9), F(2;3,0.9), F(3;3,0.9) \end{bmatrix} \quad (Eq. 3)$$

Because the probability of obtaining zero photobleaching events from two or three GFP-labeled $\alpha_4\beta_2$ nAChRs can be greater than zero, the probability distributions $M_2$ and $M_3$ were normalized as follows,

$$M'_2 = \begin{bmatrix} F(1;2,0.9), F(2;2,0.9), 0 \end{bmatrix} \quad (Eq. 4)$$

$$M'_3 = \begin{bmatrix} F(1;3,0.9), F(2;3,0.9), F(3;3,0.9) \end{bmatrix} \quad (Eq. 5)$$

where $M'_2$ and $M'_3$ are normalized probability distribution matrices corresponding to $M_2$ and $M_3$, respectively; $S_2 = F(1;2,0.9) + F(2;2,0.9) + 0$, and $S_3 = F(1;3,0.9) + F(2;3,0.9) + F(3;3,0.9)$. Because the experimentally observed distribution emerged from a mixture of $(\alpha_4\text{-GFP})_2(\beta_2\text{-wt})_2$ and $(\alpha_4\text{-GFP})_4(\beta_2\text{-wt})_2$ stoichiometries, a theoretical probability distribution ($T_{pd}$) was computed by providing a weight to each normalized probability matrix as follows,

$$T_{pd} = a_2 \times M'_2 + a_3 \times M'_3 \quad (Eq. 6)$$

where $a_2$ and $a_3$ are the weights assigned to $M'$ and $M'$ distributions, respectively, and $a_2 + a_3 = 1$. Therefore, $a_2$ and $a_3$ are the proportions of $(\alpha_4\text{-GFP})_2(\beta_2\text{-wt})_2$ and $(\alpha_4\text{-GFP})_4(\beta_2\text{-wt})_2$ stoichiometries, respectively. This probability distribution ($T_{pd}$) was multiplied by total number of observed one, two, and three photobleaching events to generate a theoretical distribution. A $\chi^2$ goodness-of-fit test was employed to compare theoretical and observed distributions. The error bars for subunit distribution are based on counting events and are calculated as the square root of the counts (31). The values of $a_2$ and $a_3$ were iteratively assigned and a $\chi^2$ goodness-of-fit test statistics was calculated for each set of $a_2$ and $a_3$. A customized Matlab script was written to calculate $a_2$ and $a_3$ from the best $\chi^2$ goodness-of-fit test statistics.

Author contributions—A. M. F.-L., F. H. M., and C. I. R. designed the studies. A. M. F.-L. and F. H. M. performed the experiments. F. H. M. wrote the analysis software for the single-molecule studies. All authors reviewed the results and approved the final version of the manuscript.

References
1. Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., and Rogers, S. W. (2009) Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 89, 73–120
2. Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P., and Lindstrom, J. (1991) Neuronal nicotinic acetylcholine-receptors expressed in Xenopus oocytes have a pentameric quaternary structure. J. Biol. Chem. 266, 11192–11198
3. Lukas, R. J., Changeux, J. P., Le Novère, N., Albuquerque, E. X., Balfour, D. J., Berg, D. K., Bertrand, D., Chiappinelli, V. A., Clarke, P. B., Collins, A. C., Dani, J. A., Grady, S. R., Kellar, K. J., Lindstrom, J. M., Marks, M. J., et al. (1999) International Union of Pharmacology: XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol. Rev. 51, 397–401
4. Eaton, J. B., Lucero, L. M., Stratton, H., Chang, Y., Cooper, J. F., Lindstrom, J. M., Lukas, R. J., and Whiteaker, P. (2014) The unique $\alpha_2\beta_1$–$\alpha_2$ agonist binding site in $(\alpha_4\beta_3)(\beta_2\text{-wt})_2$ subtype nicotinic acetylcholine receptors permits differential agonist desensitization pharmacology versus the $(\alpha_2)(\beta_2)$, subtype. J. Pharmacol. Exp. Ther. 348, 46–58
5. Chatterjee, S., Santos, N., Holgate, J., Haass-Koffler, C. L., Hopf, F. W., Kharazia, V., Lester, H., Bonci, A., and Bartlett, S. E. (2013) The $\alpha_5$ subunit regulates the expression and function of $\alpha_4$-containing neuronal nicotinic acetylcholine receptors in the ventral-tegmental area. PLoS One 8, e68300
6. Zwart, R., and Vrijenberg, H. P. (1998) Four pharmacologically distinct subtypes of $\alpha_4\beta_2$ nicotinic acetylcholine receptor expressed in Xenopus laevis oocytes. Mol. Pharmacol 54, 1124–1131
7. Moroni, M., Zwart, R., Sher, E., Cassels, B. K., and Bermudez, I. (2006) $\alpha_4\beta_2$ nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. Mol. Pharmacol. 70, 755–768
8. 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
9. Srinivasan, R., Pantoja, R., Moss, F. J., Mackey, E. D., 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
10. Turner, J. R., Castellano, L. M., and Blendy, J. A. (2011) Parallel anxiolytic-like effects and upregulation of neuronal nicotinic acetylcholine receptors following chronic nicotine and varenicline. Nicotinic Tab. Res. 13, 41–46
11. Richards, C. I., Srinivasan, R., Xiao, C., Mackey, E. D., Miwa, J., and Lester, H. A. (2011) Trafficking of $\alpha_4^+$ nicotinic receptors revealed by superecliptic phluorin. J. Biol. Chem. 286, 31241–31249
12. Ngolob, J., Liu, L., Zhao-Shea, R., Gao, G., Gardner, P. D., and Tapper, A. R. (2015) Functional upregulation of $\alpha_4^+$ nicotinic acetylcholine receptors in VTA GABAergic neurons increases sensitivity to nicotine reward. J. Neurasci. 35, 8570–8578
13. Walsh, H., Govind, A. P., Mastro, R., Hoda, J. C., Bertrand, D., Vallejo, Y., and Green, W. N. (2008) Upregulation of nicotinic receptors by nicotine varies with receptor subtype. J. Biol. Chem. 283, 6022–6032
14. Mukhin, A. G., Kimes, A. S., Chefer, S. I., Matochik, J. A., Contoreggi, C. S., Horti, A. G., Vaupe1, D. B., Pavlova, O., and Stein, E. A. (2008) Greater nicotinic acetylcholine receptor density in smokers than in nonsmokers: a PET study with 2-18F-FA-85380. J. Nucl. Med. 49, 1628–1635
15. Srinivasan, R., Henderson, B. J., Lester, H. A., and Richards, C. I. (2014) Pharmacological chaperoning of nAChRs: a therapeutic target for Parkinson’s disease. Pharmacol. Res. 83, 20–29
16. Henderson, B. J., Srinivasan, R., Nichols, W. A., Dilworth, C. N., Gutierrez, D. F., Mackey, E. D., McKinney, S., Drenan, R. M., Richards, C. I., and Lester, H. A. (2014) Nicotine exploits a COP1-mediated process for chaperone-mediated up-regulation of its receptors. J. Gen. Physiol. 143, 51–66
17. Richards, C. I., Luong, K., Srinivasan, R., Turner, S. W., Dougherty, D. A., Kozlach, J., and Lester, H. A. (2012) Live-cell imaging of single receptor composition using zero-mode waveguide nanostructures. Nano Lett. 12, 3690–3694
18. Fasoli, F., Moretti, M., Zoli, M., Pistillo, F., Crespi, A., Clementi, F., Mc Clare-Begley, T., Marks, M. J., and Gotti, C. (2016) In vivo chronic nicotine exposure differentially and reversibly affects upregulation and stoichiometry of $\alpha_4\beta_2$ nicotinic receptors in cortex and thalamus. Neuropharmacology 108, 324–331
19. Jain, A., Liu, R., Ramani, B., Arauz, E., Ishitsuka, Y., Ragunathan, K., Park, J., Chen, J., Xiang, Y. K., and Ha, T. (2011) Probing cellular protein complexes using single-molecule pull-down. Nature 473, 484–488
20. Ulbrich, M. H., and Isaccof, E. Y. (2007) Subunit counting in membrane-bound proteins. Nat. Methods 4, 319–321
21. Lee, J., and Lee, T.-H. (2017) Single-molecule investigations on histone H2A-H2B dynamics in the nucleosome. Biochemistry 56, 977–985
Altered stoichiometry of nAChRs

22. Banerjee, P. R., Moosa, M. M., and Deniz, A. A. (2016) Two-dimensional crowding uncovers a hidden conformation of α-synuclein. *Angew. Chem. Int. Ed. Engl.* **55**, 12789–12792

23. Husbands, A. Y., Aggarwal, H. T., and Timmermans, M. C. (2016) In planta single-molecule pull-down reveals tetrameric stoichiometry of HD-ZIPIII:LITTLE ZIPPER complexes. *Plant Cell* **28**, 1783–1794

24. Rodgers, M. L., Paulson, J., and Hoskins, A. A. (2015) Rapid isolation and single-molecule analysis of ribonucleoproteins from cell lysate by SNAP-SiMPull. *RNA* **21**, 1031–1041

25. Moonschi, F. H., Effinger, A. K., Zhang, X., Martin, W. E., Fox, A. M., Heidary, D. K., DeRouchey, J. E., and Richards, C. I. (2015) Cell-derived vesicles for single-molecule imaging of membrane proteins. *Angew. Chem. Int. Ed. Engl.* **54**, 481–484

26. Fox, A. M., Moonschi, F. H., and Richards, C. I. (2015) The nicotine metabolite, cotinine, alters the assembly and trafficking of a subset of nicotinic acetylcholine receptors. *J. Biol. Chem.* **290**, 24403–24412

27. Marks, M. J., O’Neill, H. C., Wynalda-Camozzi, K. M., Ortiz, N. C., Simmons, E. E., Short, C. A., Butt, C. M., McIntosh, J. M., and Grady, S. R. (2015) Chronic treatment with varenicline changes expression of four nAChR binding sites in mice. *Neuropharmacology* **99**, 142–155

28. Khiroug, S. S., Pryazhnikov, E., Coleman, S. K., Jeromin, A., Keinänen, K., and Khiroug, L. (2009) Dynamic visualization of membrane-inserted fraction of pHfluorin-tagged channels using repetitive acidification technique. *BMC Neurosci.* **10**, 141

29. Lin, D. T., Makino, Y., Sharma, K., Hayashi, T., Neve, R., Takamiya, K., and Huganir, R. L. (2009) Regulation of AMPA receptor extrasynaptic insertion by 4.1N, phosphorylation and palmitoylation. *Nat. Neurosci.* **12**, 879–887

30. Zhang, Z., Baksh, M. M., Finn, M. G., Heidary, D. K., and Richards, C. I. (2017) Direct measurement of trafficking of the cystic fibrosis transmembrane conductance regulator to the cell surface and binding to a chemical membrane conductor. *Biochemistry* **56**, 240–249

31. Bharill, S., Fu, Z., Palty, R., and Isacoff, E. Y. (2014) Stoichiometry and specific assembly of best ion channels. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 6491–6496

32. Durisic, N., Godin, A. G., Wever, C. M., Heyes, C. D., Lakadamyali, M., and Dent, J. A. (2012) Stoichiometry of the human glycine receptor revealed by direct subunit counting. *J. Neurosci.* **32**, 12915–12920

33. Hastie, P., Ulbrich, M. H., Wang, H. L., Arant, R. J., Lau, A. G., Zhang, Z., Isacoff, E. Y., and Chen, L. (2013) AMPA receptor/TARP stoichiometry visualized by single-molecule subunit counting. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 5163–5168

34. Foo, Y. H., Naredi-Rainer, N., Lamb, D. C., Ahmed, S., and Wohland, T. (2012) Factors affecting the quantification of biomolecular interactions by fluorescence cross-correlation spectroscopy. *Biophys. J.* **102**, 1174–1183

35. Li, X., and Donowitz, M. (2014) Fractionation of subcellular membrane vesicles of epithelial and non-epithelial cells by OptiPrep density gradient ultracentrifugation. *Methods Mol. Biol.* **1174**, 85–99

36. Kuryatov, A., Luo, J., Cooper, J., and Lindstrom, J. (2009) Nicotine acts as a pharmacological chaperone to up-regulate human α4β2 acetylcholine receptors. *Mol. Pharmacol.* **68**, 1839–1851

37. Mazzo, F., Pistillo, F., Grazierio, G., Clementi, F., Borgese, N., Gotti, C., and Colombo, S. F. (2013) Nicotine-modulated subunit stoichiometry affects stability and trafficking of α3 β4 nicotinic receptor. *J. Neurosci.* **33**, 12316–12328

38. Morales-Perez, C. L., Noviello, C. M., and Hibbs, R. E. (2016) X-ray structure of the human α4β2 nicotinic receptor. *Nature* **538**, 411–415

39. Morales-Perez, C. L., Noviello, C. M., and Hibbs, R. E. (2016) Manipulation of subunit stoichiometry in heteromeric membrane proteins. *Structure* **24**, 797–805

40. Henderson, B. J., and Lester, H. A. (2015) Inside-out neuropharmacology of nicotinic drugs. *Neuropharmacology* **96**, 178–193

41. Amici, S. A., McKay, S. B., Wells, G. B., Robson, J. I., Nasir, M., Ponath, P., and Anand, R. (2012) A highly conserved cytoplasmic cysteine residue in the α4 nicotinic acetylcholine receptor is palmitoylated and regulates protein expression. *J. Biol. Chem.* **287**, 23119–23127

42. Fox-Loe, A. M., Dwoskin, L. P., and Richards, C. I. (2016) Nicotinic acetylcholine receptors as targets for tobacco cessation therapeutics: cutting-edge methodologies to understand receptor assembly and trafficking. *Neuromethods* **117**, 119–132

43. Srinivasan, R., Richards, C. I., 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

44. Araki, Y., Lin, D. T., and Huganir, R. L. (2010) Plasma membrane insertion of the AMPA receptor GluA2 subunit is regulated by NSF binding and Q/R editing of the ion pore. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 11080–11085
Organelle-specific single-molecule imaging of α4β2 nicotinic receptors reveals the effect of nicotine on receptor assembly and cell-surface trafficking
Ashley M. Fox-Loe, Faruk H. Moonschi and Christopher I. Richards

J. Biol. Chem. 2017, 292:21159-21169. doi: 10.1074/jbc.M117.801431 originally published online October 26, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.801431

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 44 references, 23 of which can be accessed free at http://www.jbc.org/content/292/51/21159.full.html#ref-list-1