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Nicotinic Acetylcholine Receptors as Targets for Tobacco Cessation Therapeutics: Cutting-Edge Methodologies to Understand Receptor Assembly and Trafficking

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Abstract

Tobacco dependence is a chronic relapsing disorder and nicotine, the primary alkaloid in tobacco, acts at nicotinic receptors to stimulate dopamine release in brain, which is responsible for the reinforcing properties of nicotine, leading to addiction. Although the majority of tobacco users express the desire to quit, only a small percentage of those attempting to quit are successful using the currently available pharmacotherapies. Nicotine upregulates the number of specific nicotinic receptors on the neuronal cell surface. An increase in receptor trafficking or preferential stoichiometric assembly of receptor subunits involves changes in assembly, endoplasmic reticulum export, vesicle transport, decreased degradation, desensitization, enhanced maturation of functional pentamers, and pharmacological chaperoning. Understanding these changes on a mechanistic level is important to the development of nicotinic receptors as drug targets. For this reason, cutting-edge methodologies are being developed and employed to pinpoint distinct changes in localization, assembly, export, vesicle trafficking, and stoichiometry in order to further understand the physiology of these receptors and to evaluate the action of novel therapeutics for smoking cessation.

Keywords

Nicotine addiction; Tobacco-use cessation; Alpha4beta2 nicotinic receptors; Trafficking; Receptor assembly; Superecliptic pHluorin; Total internal reflection fluorescence; Cytisine; Varenicline; Cotinine

1 Introduction

Tobacco addiction and dependence is a chronic relapsing disorder in which compulsive drug use persists despite significant negative consequences [1 – 3]. Nicotine, the primary alkaloid in tobacco, acts at nicotinic receptors (nAChRs) to initiate actions leading to addiction to tobacco products. Although the majority of tobacco users express the desire to quit, only a small percentage of those attempting to quit are successful with the currently available pharmacotherapies, including nicotine replacement, varenicline, bupropion, and cytosine [4 – 6]. Available tobacco cessation therapeutics have been targeted, thus far, at a limited number of nAChR subtypes. A greater understanding of nicotinic receptors as druggable targets is needed for the discovery and development of new efficacious cessation therapeutics to help those who desire to quit.

Nicotinic receptors are members of the cys-loop superfamily of ligand-gated ion channels, formed by the assembly of five individual subunits around a central hydrophilic pore [7, 8]. nAChRs primarily mediate fast synaptic transmission in the periphery and modulate neurotransmitter function in the central nervous system [9, 10]. Nicotinic receptors are pentameric, consisting of a combination of alpha (α 2– α 10) and beta (β 2– β 4) subunits [7, 8, 11, 12], with each combination forming a unique subtype that differs from other subtypes in pharmacological and kinetic properties, as well as localization to different areas of the body and brain.

Due to the pentameric structure and variety of subunits that can be assembled, nicotinic receptors can form homopentamers or heteropentamers. Homopentamers consist of five identical subunits of either α 7 or α 9 subunits [13 – 16]. Heteropentamers are a combination of alternating alpha and beta subunits. The fifth position in a heteropentamer is occupied by an additional alpha or beta subunit, giving rise to the potential for different stoichiometries for the same subtype of nAChR. For example, a heteropentamer consisting of two types of subunits can exist as either (α)₃(β)₂ or (α)₂(β)₃ depending on an alpha or beta being incorporated into the fifth position. These two stoichiometries, although consisting of the same subtype, often differ in terms of sensitivity to agonists, expression, and rates of desensitization. The fifth position in a heteropentamer can be occupied by an auxiliary subunit such as α 5 or β 3 [17], and incorporation of an auxiliary subunit can further alter receptor trafficking and function [18, 19].

Additionally, pharmacological agents can preferentially induce the expression of one stoichiometry over another, as well as alter trafficking and expression of these receptors [19 – 23]. Beyond the traditionally characterized functional response (cation-selective flux through the ion channel) induced by nAChR ligands, nicotine and other receptor agonists and antagonists upregulate the number of receptors on the neuronal cell surface [20, 24 – 27]. Upregulation is defined as an increase in number, trafficking, or preferential stoichiometric assembly of receptors, and involves changes in receptor assembly, endoplasmic reticulum export, and vesicle transport [18, 20, 27, 28]. Although the mechanism of nAChR upregulation is not completely understood, current theories of upregulation include decreased receptor degradation, desensitization of surface receptors, enhanced maturation of functional pentamers, and pharmacological chaperoning [7, 8, 11, 17, 21, 27, 29 – 31]. Studies have shown that upregulation is also a post-translational event, evident by a lack in increase of receptor subunit mRNA [30]. In addition to nicotine, drugs classified as nAChR agonists, partial agonists (e.g., cytisine and varenicline), and antagonists (e.g., mecamylamine) also upregulate some subtypes of nAChRs [21, 30, 32], including those composed of α 4 and β 2 subunits.

In this regard, α 4 β 2 is probably the best studied nAChR subtype, being the most predominantly expressed and upregulated [13, 33, 34]. Available tobacco-use cessation drugs have targeted the α 4 β 2 subtype. The α 4 β 2 partial agonist, cytisine, is a natural product from *Cytisus laborinum*, and is marketed in Europe as a smoking cessation therapeutic [21, 35 – 37]. Cytisine has similar efficacy as a cessation agent compared to other FDA-approved smoking cessation agents available in the USA [5, 6]. Varenicline, a synthetic analog of cytisine, is available as a smoking cessation agent in the USA [32].

Varenicline also targets $\alpha 4\beta 2$ nAChRs, acting as a partial agonist, but also acts as a full agonist at $\alpha 7$ nAChRs, and exhibits weak actions at $\alpha 3\beta 2$ and $\alpha 6\beta 2$ [38]. Dianicline, another compound acting as a partial agonists at $\alpha 4\beta 2$ receptors, has a similar pharmacological profile to varenicline [39, 40]. Interestingly, sazetidine A, an analog of nicotine, acts as a partial agonist at the $(\alpha 4)_2(\beta 2)_3$ subtype and as an antagonist at the $(\alpha 4)_3(\beta 2)_2$ subtype [41, 42]. Thus, different stoichiometries of nAChR can interact differently with pharmacological agents, which play an important role when targeting specific subtypes for drug development.

Cotinine, the primary metabolite of nicotine and an nAChR partial agonist, upregulates a subset of $\alpha 4\beta 2$ nAChRs [19]. Interestingly, cotinine does not alter the density or trafficking of $\alpha 6\beta 2$, $\alpha 4\beta 2\alpha 5$, or $\alpha 3\beta 4$ nAChRs [7]. This may be because these subtypes exhibit a higher basal plasma membrane density, such that trafficking is already efficient. Thus, cotinine appears to specifically upregulate $\alpha 4\beta 2$ nAChRs. Both cotinine and nicotine increase the expression of the high-sensitivity receptor, $(\alpha 4)_2(\beta 2)_3$ [19, 22, 43], while the partial agonist cytosine results in preferential assembly of the low-sensitivity receptor, $(\alpha 4)_3(\beta 2)_2$ [44, 45]. The increase in low-sensitivity receptors is possibly the result of an additional cytosine-binding site at the α - α interface of $(\alpha 4)_3(\beta 2)_2$. High- and low-sensitivity stoichiometries of $\alpha 4\beta 2$ exhibit different ligand affinities, cation flux, and desensitization rates [18, 46], likely contributing to nicotine addiction and conceivably the treatment of nicotine addiction. An important aspect of drug-induced changes is that surface activation does not appear to be required; drug concentration only needs to be high enough to interact with the specific subtype in the endoplasmic reticulum [17, 27].

Although $\alpha 4\beta 2$ is clearly upregulated when exposed to nicotine, the situation is not as clear for $\alpha 6\beta 2$; upregulation, downregulation, and no change in $\alpha 6\beta 2$ have been reported following exposure to nicotine [47 – 50]. Activation of both $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ - containing nAChR subtypes ($\alpha 4\beta 2$, $\alpha 4\alpha 5\beta 2$, $\alpha 6\beta 2$, $\alpha 4\alpha 6\beta 2$, $\alpha 6\beta 2\beta 3$, and $\alpha 4\alpha 6\beta 2\beta 3$) has been reported to mediate nicotine-induced dopamine release within the neuronal circuitry associated with the reinforcing properties of nicotine [51 – 54]. $\alpha 4\beta 2$ - and $\alpha 6\beta 2$ -containing nAChR subtypes are viable targets for the development of new tobacco cessation agents. Based on these findings, subtype selective $\alpha 6\beta 2$ nAChR antagonists (e.g., *N,N'*-dodecane-1,12-diyl-bis-3-picolinium bromide, bPiDDB, and r-bPiDDB, in which the two tetrahydro-3-picolino headgroups are chemically reduced to provide tetrahydro-3-ethylpyridino head groups) have been discovered that inhibit nicotine-induced dopamine release and intravenous nicotine self-administration in rats, with no effect on sucrose-maintained responding [55]. bPiDDB and r-bPiDDB may be efficacious new tobacco-use cessation agents that act by blocking the reinforcing properties of nicotine. However, potential effects of these novel antagonists on nAChR subtype expression, distribution, assembly, and trafficking have not been evaluated as yet.

Changes in expression, distribution, and assembly of nAChRs are a result of exposure to both agonists and antagonists. Understanding these changes on a mechanistic level is important to the development of nAChRs as drug targets. For this reason, cutting-edge methodologies are being developed and employed to pinpoint distinct changes in

localization, assembly, export, vesicle trafficking, and stoichiometry in order to further understand the physiology of these receptors.

2 Novel Methods for Investigating Ligand Effects on nAChR Subtypes

In the last two decades, the use of fluorescence microscopy to investigate cellular systems has expanded rapidly. Fluorescence techniques are used currently to study a wide range of biological events including receptor diffusion, protein-protein interactions, and protein expression. More recently, techniques have enabled the study of proteins as they are transported through the secretory pathway. Ligand-induced upregulation leads to an increased number of receptors on the cell surface likely either through higher rates of trafficking or longer residence time on the plasma membrane. Techniques that can directly measure changes in trafficking rates, subcellular distribution, and expression levels on the plasma membrane provide insight into the mechanism of upregulation.

A key milestone in fluorescence microscopy was the discovery of green fluorescent protein (GFP) [56]. Genetically encoded fluorescent proteins have made it possible to quantify expression of proteins and pinpoint localization within a live cell [19, 20, 23, 27]. The primary amino acid sequence of GFP can be directly incorporated into the sequence of a protein of interest, allowing GFP fluorescence to serve as a direct measurement of gene expression and protein dynamics. Collected fluorescence emission is proportional to the concentration of protein expressed. Mutagenesis of the primary GFP sequence led to a wide variety of fluorescent proteins ranging in excitation or emission wavelength, photostability, and pH sensitivity, allowing the choice of fluorophore to be tailored to a particular application [56]. Fluorescent proteins have been expressed successfully in live cultured cells, neurons, and even in mice [19, 20, 23, 57], which allows for the possible applications of fluorescence microscopy to study subcellular protein localization and trafficking, intermolecular interactions, and single-molecule experiments in a variety of in vivo systems.

Superecliptic pHluorin (SEP), a pH-sensitive variant of GFP, is a particularly useful fluorescent protein to study vesicle dynamics and receptor localization [19, 23, 58]. This fluorophore has been incorporated into numerous neurotransmitter receptor subunits, including AMPA, GABA, and nicotinic receptors [23, 58, 59]. The pH dependence corresponds to a change in fluorescence emission based on the local environment of the fluorophore (Fig. 1). At neutral pH, SEP fluoresces when excited with 488 nm excitation, but fails to fluoresce under acidic condition of $\text{pH} < 6$ [58]. Therefore, manipulation of extracellular pH or existing pH gradients within a cell modifies the fluorescence of SEP. An SEP tag fused with the C-terminus of a nAChR subunit is exposed to the pH on the luminal side of organelles during assembly and transport, and exposed to the extracellular pH upon insertion in the plasma membrane. In this way, the presence or absence of fluorescence is utilized to distinguish subcellular localization of receptors for direct monitoring of nAChR trafficking in real time. Receptors located in the endoplasmic reticulum, at $\text{pH} > 6$, will fluoresce at all times, while nAChRs in low $\text{pH} < 6$ trafficking vesicles will not. Upon insertion into the plasma membrane, fluorescence depends on the extracellular pH. Exploiting these pH differences allows the discrimination of nAChR localization to the plasma membrane compared to those receptors located in the endoplasmic reticulum.

A considerable number of specific fluorescence microscopy techniques have been developed for many diverse applications. For example, total internal reflection fluorescence (TIRF) limits excitation to a narrow region close to the plasma membrane, extending approximately 150 nm from the surface of a glass substrate supporting the cell and providing a high axial resolution. TIRF takes advantage of the total reflection of incident radiation when an interface of two media with different indices of refraction are encountered, such as a cell on glass, at a so-called critical angle. An evanescent wave is formed at the interface and decays exponentially as a function of distance from the interface of formation. Incident radiation is totally internally reflected, so the evanescent wave is solely responsible for fluorophore excitation, leading to an increased signal-to-noise ratio as background fluorescence is decreased [60]. In vivo imaging of cells or neurons expressing nAChRs using TIRF enables visualization of nAChRs within 200 nm from the glass interface, corresponding to those localized on the plasma membrane or nearby peripheral endoplasmic reticulum. Combining this technique with nAChRs expressing SEP allows a combination of pH manipulation and evanescent wave excitation to resolve single-vesicle insertion events and subcellular localization of receptors within the field of view. Changes in these physiological properties after exposure to a pharmacological agent can be studied to observe changes in trafficking rates, distribution between endoplasmic reticulum and plasma membrane, and expression levels on the plasma membrane.

2.1 Equipment, Materials, and Setup

Figure 2 illustrates experimental procedures as described in the following steps:

1. A fluorophore is incorporated into the gene coding the relevant nAChR subunit using PCR amplification. An SEP tag is fused to the C-terminal end of the nAChR subunit so that the fluorophore interacts with the luminal side of organelles in the secretory pathways.
2. Cells or neurons are grown on 35 mm glass-bottom dishes so that the cell-glass interface serves as the TIRF interface to form an evanescent field for excitation of fluorescently labeled nAChRs.
3. To determine the localization of nAChR-SEP on the plasma membrane, the extracellular pH is manually adjusted using an extracellular solution. The extracellular solution contains 150 mM NaCl, 4 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose adjusted to pH 5.4 or 7.4, resulting in dark or fluorescing membrane SEP.
4. An inverted fluorescence microscope capable of TIRF is required for these studies. Fluorophores are excited through an objective with a laser, such as a 488 nm DPSS laser ($\sim 1.00 \text{ W/cm}^2$) for SEP. To achieve TIRF, a high numerical aperture objective ($\text{NA} > 1.42$) is required, typically a 60 $\times$ or 100 $\times$ oil immersion objective. The critical angle is reached by focusing the beam of incident radiation on the back aperture of the objective lens, which can be accomplished manually or using a stepper motor to translate the beam laterally across the objective. Emission is detected by an electron-multiplying charge-coupled device. An automated stage capable

of position memory is also convenient to image identical cells at both pH 7.4 and pH 5.4.

5. A stage-top incubator can be incorporated into the system to maintain a physiological temperature. The stage-top incubator can be used in conjunction with humidity and CO₂ control, if necessary. Regular culture media can then be used, provided that the background fluorescence of this media is not high. Imaging medium, such as Leibovitz's L-15, is preferred since pH is CO₂ independent and background fluorescence is minimized.
6. Fluorescence intensity is determined using image processing software such as ImageJ (<http://imagej.nih.gov/ij/>). After background subtraction, an intensity-based threshold and region of interest are manually selected for cell- or neuron-based studies.

2.2 Superecliptic pHluorin Methodology

1. Mouse neuroblastoma 2a (N2a) cells or neurons are cultured using standard techniques. Cells are plated on poly-d-lysine-coated 35 mm glass-bottom dishes (90,000 cells/dish).
2. Cells not expressing a fluorescent protein are transfected in opti-MEM prior to imaging. Opti-MEM is a modified Eagle's minimum essential media, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, l-glutamine, trace elements, and growth factors. For transfection of nAChRs, 500 ng of each subunit plasmid construct is mixed in 250 μ L opti-MEM and combined with 250 μ L opti-MEM with 2 μ L Lipofectamine-2000 transfection reagent that has been incubated for 5 min. The Lipofectamine-2000 and plasmid combination is incubated at room temperature for 25 min and then added to pre-plated cells. The transfection mix remains on the cells for 24 h before being replaced by growth media for an additional 24 h.
3. To investigate the effect of a drug on trafficking and expression of nAChR, the drug is added to the cell's growth medium. Addition of drug can be at the time of transfection, and replenished during the 24-h recovery period.
4. Before imaging, growth medium is replaced with extracellular solution at pH 7.4. Images are collected using TIRF microscopy, with stage positions memorized. At pH 7.4, nAChRs located on the plasma membrane and endoplasmic reticulum are visible. Then, the extracellular solution at a pH of 7.4 is replaced with an identical solution at pH 5.4. Images are again collected for the same cells at the low pH which eliminates fluorescence from nAChRs on the plasma membrane. Thus, only nAChR in the endoplasmic reticulum is detected.
5. Real-time movies can also be collected to resolve single-vesicle insertion events when cells are in the pH 7.4 extracellular solution. Due to the endogenous low pH of transport vesicles, SEP is in a dark state during

trafficking from the endoplasmic reticulum to plasma membrane. When a vesicle fuses with the membrane and is exposed to pH 7.4, SEP fluorescence is reestablished. As the vesicle fuses with the plasma membrane and senses the higher pH, the result is a burst of fluorescence. Insertion events are captured by monitoring the fluorescence over a period of time (1000 frames) while continuously capturing images at a high frame rate (100–200 ms).

2.3 Obtainable Results

The pH sensitivity of SEP enables intracellular nAChRs in the endoplasmic reticulum to be distinguished from nAChRs residing on the plasma membrane. SEP undergoes 488 nm excitation at neutral pH, but does not excite under acidic conditions of $\text{pH} < 6$. When the extracellular pH is 7.4, fluorescence is measured for nAChRs residing in the endoplasmic reticulum ($\text{pH} > 7$) and on the plasma membrane ($\text{pH} = \text{extracellular solution} = 7.4$), but not for those in the lower pH secretory vesicles or Golgi ($\text{pH} < 6$) [23, 61]. Once the low pH trafficking vesicle fuses with the higher pH plasma membrane, the fluorophores on nAChRs within these vesicles will turn on, allowing quantification of nAChR trafficking.

2.3.1 Single-Vesicle Trafficking—Insertion of single transport vesicles into the plasma membrane can be measured when cells expressing SEP are imaged in an extracellular solution of pH 7.4. In vivo images are acquired at a frame rate of 200 ms over a period of time while exposed to 488 nm excitation. SEP neither photobleaches nor fluoresces in the low pH of a transport vesicle, but regains fluorescence upon insertion into plasma membrane. Insertion events are visualized as a burst of fluorescence at the plasma membrane lasting at least three frames (600 ms), corresponding to transient full fusion of the transport vesicle into the plasma membrane. An insertion event in an N2a cell expressing $\alpha 3\text{-sep}/\beta 4\text{-wt}$ is shown in Fig. 3. This corresponds to the arrival of a transport vesicle carrying $\alpha 3\text{-sep}/\beta 4\text{-wt}$ nAChRs (Fig. 3b2), followed by exocytosis of vesicle cargo (Fig. 3b3, b4) and diffusion of nAChRs across the plasma membrane (Fig. 3b5, b6). Frequency and intensity of each insertion event per cell (Fig. 3a) can provide information regarding the number of nAChR transport vesicles trafficking to the plasma membrane as well as relative number of nAChRs that each transport vesicle contains. Changes in trafficking as a result of exposure to a pharmacological agent, such as nicotine or cotinine, can be measured by a change in the rate of arrival of transport vesicles containing these receptors [19, 20, 23].

2.3.2 Relative

2.3.2.1 Expression Levels of nAChRs on Plasma Membrane: Emission from SEP labeled receptors on the plasma membrane is regulated by the pH of the extracellular solution, which can be varied to turn the fluorescence on or off. When the pH of the extracellular solution is 7.4, receptors from both the peripheral endoplasmic reticulum and plasma membrane fluoresce. The extracellular solution can then be exchanged with an otherwise identical solution of pH 5.4, causing nAChRs on the plasma membrane to transition into an off state, meaning all fluorescence is from the endoplasmic reticulum [20, 60, 62]. Fluorescence intensity of a cell is collected at both pH 7.4 and 5.4. The relative number of receptors on the plasma membrane is then calculated mathematically by subtracting the

integrated density of the fluorescence at pH 5.4 from that at pH 7.4, as shown in Fig. 4. The subtracted value corresponds to fluorophores located on the plasma membrane. Upregulation of nAChRs is evident by an increase in the number of receptors on the plasma membrane.

2.3.3 Subcellular Localization of nAChRs—Subcellular distribution of nAChRs expressing SEP within the plasma membrane region of the cell can also be measured. Changes in the relative number of nAChRs localized in the plasma membrane compared to the endoplasmic reticulum can be attributed to changes in trafficking of nAChRs. Once the fluorescence contribution from nAChRs on the plasma membrane is established, this value can be divided by the total intensity at pH 7.4 to obtain a percentage of visible receptors on the plasma membrane (% plasma membrane). Changes in subcellular localization of nAChRs as a response to drug are quantified by changes in this distribution between peripheral endoplasmic reticulum and plasma membrane [19, 20, 23, 27]. Differences in this distribution of nAChR between the endoplasmic reticulum and plasma membrane as a result of drug exposure correspond to drug-induced changes in trafficking of that nAChR subtype. Increases in the percentage of nAChRs on the plasma membrane suggest that nAChR trafficking towards the plasma membrane is increased.

3 Conclusions

Exposure to nicotine from tobacco and other nicotine-delivery products alters the trafficking and assembly of nAChRs, leading to upregulation of $\alpha 4\beta 2$ receptors on the neuronal plasma membrane, which is believed to contribute to nicotine addiction and tobacco dependence. To obtain a greater understanding of intracellular receptor dynamics, the pH-sensitive fluorescent protein, superecliptic pHluorin, can be used to differentiate between intracellular nAChRs and those expressed on the plasma membrane to quantify changes resulting from nicotine exposure. Using the methods described in this chapter, the properties and stoichiometry of individual nicotinic receptors located on the plasma membrane can be studied, and the pharmacological effects of potential therapeutics can be evaluated to determine their ability to counter the effects of nicotine on these membrane-resident proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Cartoon depicting trafficking of superecliptic pHluorin-labeled nAChRs through the secretory pathway. The fluorophore is fluorescent in the endoplasmic reticulum and at the plasma membrane, but is dark in the Golgi and in trafficking vesicles. Changing the pH of the extracellular solution (ECS) can be used to turn the fluorescence on ($pH > 7$) and off ($pH < 5.5$). TIRF-based fluorescence imaging is then used to quantify expression in the peripheral endoplasmic reticulum and on the plasma membrane

Fig. 2. Schematic of the experimental procedures. (1) A fluorescent construct containing superecliptic pHluorin is incorporated on the C-terminus of the receptor in order to orient it on the extracellular portion of the subunit. (2) Cells are transfected with the plasmid for the superecliptic pHluorin-labeled nAChR. (3) During imaging, the solution is exchanged in order to turn on the plasma membrane fluorescence (pH 7.4) and to turn off the fluorescence (pH 5.4). (4) A typical objective-style TIRF illumination is set up on an inverted microscope

Fig. 3.

An example of an N2a cell expressing $\alpha 3$ -sep/ $\beta 4$ -wt (**a**) and an insertion event in this cell (**b**). An insertion event corresponds to the arrival of a nAChR-SEP carrying vesicle at the plasma membrane. **b1** is the frame immediately preceding an insertion, followed by vesicle arrival (**b2**), fusion with the plasma membrane (**b3**, **b4**), and diffusion of newly inserted nAChRs across the plasma membrane (**b5**, **b6**)

Fig. 4.

An N2a cell expressing $\alpha 3$ -sep/ $\beta 4$ -wt at pH 7.4 (**a**) and pH 5.4 (**b**). In **A**, nAChR on the plasma membrane (PM) and endoplasmic reticulum (ER) are both fluorescent. In **b**, plasma membrane fluorescence is lost at pH 5.4, so only endoplasmic reticulum receptors fluoresce. The difference between pH 7.4 and pH 5.4 is the fluorescence from nAChRs on the plasma membrane (**c**)