HHS Public Access

Author manuscript

Pharmacol Res. Author manuscript; available in PMC 2018 August 03.

Published in final edited form as:

Pharmacol Res. 2014 May ; 83: 20–29. doi:10.1016/j.phrs.2014.02.005.

Pharmacological chaperoning of nAChRs: A therapeutic target for Parkinson's disease

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Abstract

Chronic exposure to nicotine results in an upregulation of neuronal nicotinic acetylcholine receptors (nAChRs) at the cellular plasma membrane. nAChR upregulation occurs via nicotine-mediated pharmacological receptor chaperoning and is thought to contribute to the addictive properties of tobacco as well as relapse following smoking cessation. At the subcellular level, pharmacological chaperoning by nicotine and nicotinic ligands causes profound changes in the structure and function of the endoplasmic reticulum (ER), ER exit sites, the Golgi apparatus and secretory vesicles of cells. Chaperoning-induced changes in cell physiology exert an overall inhibitory effect on the ER stress/unfolded protein response. Cell autonomous factors such as the repertoire of nAChR subtypes expressed by neurons and the pharmacological properties of nicotinic ligands (full or partial agonist versus competitive antagonist) govern the efficiency of receptor chaperoning and upregulation. Together, these findings are beginning to pave the way for developing pharmacological chaperones to treat Parkinson's disease and nicotine addiction.

Keywords

Pharmacological chaperone; Chaperoning; Nicotine; nAChR; Tobacco; Neuroprotection; Parkinson's disease; Neurodegeneration; Unfolded protein response; Dopaminergic; Endoplasmic reticulum stress; FRET; TIRF; Confocal; ER exit sites; Golgi; Ligand; COPII; COPI

1. Introduction

Pharmacological chaperoning has emerged as a potential strategy to treat diseases cystic fibrosis [1–3], Gaucher's disease [4,5], nephrogenic diabetes insipidus [6], retinitis pigmentosa [7,8] and some cancers resulting from mutations in p53 [9]. Notably, the treatment of transthyretin familial amyloid polyneuropathy with the pharmacological chaperone, tafamadis has been successful in a phase II/III clinical trial [10–13]. In these

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Conflict of interest

The authors declare no competing financial interests.

cases, ligand-mediated chaperoning corrects receptor mislocalization and/or prevents mutant proteins from forming toxic intracellular aggregates [14,15]. Pharmacological chaperoning has been employed to treat diseases associated with mutations in single genes [15], but the treatment of complex multifactorial disorders such as Parkinson's disease (PD) or nicotine addiction with pharmacological chaperones remains challenging and will first require a mechanistic understanding of the cellular processes involved in chaperoning.

Here, we review our understanding of the cellular mechanisms by which nicotine and nicotinic ligands chaperone neuronal nicotinic acetylcholine receptors (nAChRs) and describe one way in which nAChR chaperoning can exert a neuroprotective effect in Parkinson's disease (PD).

2. Chronic nicotine exposure upregulates nAChRs via pharmacological chaperoning

nAChR upregulation is defined as an increase in intracellular and/or plasma membrane receptors and likely underlies aspects of addiction to tobacco as well as relapse following smoking cessation. Since its discovery in the early 1980s [16–18], nAChR upregulation has become one of the best-studied consequences of chronic exposure to nicotine [19,20].

[^{3}H]nicotine binding and PET imaging in tobacco users demonstrate upregulated nAChRs [21–28], suggesting a role in nicotine dependence. Because nAChRs upregulate *in vitro*, *in vivo* [16,17,22,25,29–32] and across a range of species (mice, rats, monkeys and humans), the cellular processes governing upregulation are likely to be cell autonomous and evolutionarily conserved. The process of upregulation involves post-translational rather than transcriptional changes in nAChR expression because nicotine exposure does not alter the mRNA levels of nAChR subunits [33]. Proposed mechanisms for upregulation include a decreased degradation of receptors [34], desensitization of surface receptors [35], nicotine acting as a maturational enhancer [36], a novel slow stabilizer [37] and a pharmacological chaperone [30,31,38,39]. Although the mechanisms for upregulation have been described in separate reports over a period of a decade or so, they are in fact part of the intracellular machinery that governs protein folding, transport and turnover. Thus, together these studies converge on the idea that the major cellular mechanism for nAChR upregulation is pharmacological chaperoning of intracellular receptors by nicotine and that upregulation is a complex process that involves changes at several levels of intracellular trafficking such as subunit assembly in the endoplasmic reticulum (ER), export of assembled receptors from the ER, anterograde and retrograde vesicle transport and insertion of receptors into the plasma membrane [19,20].

Nicotine freely permeates the cellular plasma membrane and accumulates within intracellular organelles. As a result, nanomolar concentrations of nicotine are sufficient to alter the intracellular assembly and trafficking of nAChRs. The effects of nicotine on nAChR upregulation occur at nanomolar nicotine concentrations (~100–200nM) that are equivalent to the steady state nicotine concentration observed in chronic smokers [40–42]. Nanomolar concentrations of nicotine minimally activate surface nAChRs [39], indicating that nAChR upregulation is independent of second messenger signaling cascades triggered by channel

activation and Ca^{2+} influx. Therefore, a likely mechanism for nAChR upregulation is pharmacological chaperoning, which is an *intracellular* process involving selective changes in receptor number, stoichiometry, trafficking between subcellular compartments and the ER associated degradation of receptors [20]. In agreement with this hypothesis, high-resolution quantitative methods (summarized in Fig. 1) developed to study intracellular nAChR biology reveal specific cellular processes that are selectively engaged by the cell during pharmacological chaperoning and nAChR upregulation. The next sections describe high-resolution imaging techniques and their contribution to our understanding of the pharmacological chaperoning of nAChRs.

2.1. Nicotine alters the stoichiometry of nAChRs in the endoplasmic reticulum: Förster's resonance energy transfer

Förster's resonance energy transfer (FRET) microscopy is an invaluable tool to study nanometer scale interactions of proteins within multimeric complexes [43]. FRET is based on the idea that following excitation of a fluorophore, energy can dissipate via the non-radiative dipole coupling of the excited fluorophore with a nearby non-excited fluorophore. Because FRET is inversely proportional to the 6th power of distance between fluorophores, occurrence of FRET indicates that the two proteins or molecules undergoing FRET are separated by only a few nanometers.

We developed a broadly applicable pixel-resolved FRET method to study receptor stoichiometry [44]. Our FRET studies show that cells expressing α4β2 nAChRs assemble pentameric receptors in two stoichiometries: $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. These two nAChR stoichiometries are present in the endoplasmic reticulum (ER), the Golgi apparatus and in the plasma membrane (PM) [31,39,44]. Following nicotine treatment, receptors in the ER and Golgi primarily show a $(\alpha4)_2(\beta2)_3$ stoichiometry, indicating that nicotine stabilizes $(\alpha4)_2(\beta2)_3$ receptors in the ER, prior to reaching the Golgi [31]. Thus, the process of nAChR upregulation is triggered within the ER and at a very early stage in the cellular secretory pathway.

2.2. Pharmacological chaperoning of nAChRs out of the ER: quantification of ER exit sites with confocal microscopy

Following the stabilization of $(\alpha4)_2(\beta2)_3$ receptors in the ER, nicotine chaperones receptors from the ER to the plasma membrane. Export of receptors from the ER can be measured by quantification of specialized ER structures called ER exit sites (ERES), which concentrate cargo ready for export from the ER to the Golgi [31,39]. Visualization of ERES is achieved by tagging Sec24D, a component of COPII ERES vesicles with a fluorescent reporter tag and imaging with confocal microscopy.

In the absence of nicotine, the density of ERES observed in cells expressing nAChRs is directly proportional to the number of functional receptors at the cell surface [45], indicating that ERES density is a measure of active nAChR export from the ER and that the rate limiting step in nAChR trafficking through the secretory pathway is receptor export from the ER. Nicotine exposure causes a two-fold increase in the density of ERES, suggesting active nAChR chaperoning from the ER to the Golgi [31,39]. Because increases in the density of

ERES are a direct measure of pharmacological chaperoning, this method can be broadly applied to measure the chaperoning efficacy of most pharmacological ligands. The process of ER export of nAChRs is critically dependent on the presence of an LXM (X = any amino acid) motif in the intracellular M3–M4 loop of nAChR subunits [31]. The LXM motif binds to Sec24D, which is an integral component of the COPII ERES vesicles. Thus, the presence or absence of LXM motifs in nAChR subunits partially determines the chaperoning efficiency of nicotine for particular nAChR subtypes, a phenomenon that has been observed in mouse as well as human nAChR subunits [31,46].

In addition to quantifying the ER exit of nAChRs with fluorescently tagged COPII vesicles, COPI vesicles tagged to fluorescent proteins can be used to quantify the retrograde transport of nAChRs from the Golgi apparatus back to the ER. COPI vesicles are involved in the trafficking of proteins from the Golgi apparatus to the PM (anterograde transport) [47–49], from the Golgi to the ER (retrograde transport) [49–51] as well as trafficking of proteins within the Golgi apparatus (intra-Golgi transport) [49,52]. Our recent study suggests that COPI-mediated retrograde transport of nAChRs from the Golgi to the ER appears to be specifically engaged during pharmacological chaperoning by nicotine and not under basal trafficking conditions [30]. These results have opened an exciting new avenue in which specific targeting of the COPI machinery can allow more precise manipulation of pharmacological chaperoning and nAChR upregulation in cells.

2.3. Pharmacological chaperoning alters near-PM nAChR dynamics and cellular architecture: total internal reflection fluorescence (TIRF) microscopy

Total internal reflection fluorescence (TIRF) microscopy illuminates ~200nm of the z-axis at the cellular periphery, thus allowing the visualization of molecules at the cellular plasma membrane (PM). Factors such as cell structure (polarized versus non-polarized cells), the type of protein labeling (quantum dots versus genetically encoded fluorescent tags) and the subcellular localization of labeled proteins can significantly alter the subcellular compartments visualized using TIRF. With TIRF imaging of fluorescent protein (FP)-tagged nicotinic receptors expressed in mouse neuroblastoma (Neuro-2a) cells, one visualizes multiple peripheral cellular compartments within the footprint of cells. In our studies using FP-tagged nAChRs, the subcellular structures visualized using TIRF include the peripheral ER, *trans* Golgi network (TGN)and the PM [53].

Pharmacological chaperoning by nicotine and nicotinic ligands increases the number of receptors at the PM by ~2-fold and significantly alters the architecture of the ER and *trans* Golgi network (TGN) [31,39]. Nicotine increases the density of peripheral ER and causes an elaboration of the TGN morphology, specifically an increase in the number and size of TGN vesicles [39]. Although the precise mechanism by which this occurs is not understood, it is clear that pharmacological chaperoning can profoundly alter the structure and function of entire organelles in the cellular secretory pathway.

TIRF microscopy has also been used in combination with nAChR subunits tagged to a pH sensitive fluorophore (superecliptic pHluorin or SEP). This allows the visualization of nAChR insertion events into the PM [30,32]. Studies with SEP-tagged nAChRs show that

nicotine increases the insertion of vesicles containing nAChRs into the PM [30,32], indicating that nAChR upregulation is not due to a reduced turnover of receptors at the PM.

2.4. Nicotine chaperones nAChRs with a $(\alpha4)_2(\beta2)_3$ stoichiometry to the plasma membrane: single molecule imaging using zero-mode waveguides (ZMWs)

Nicotine causes an intracellular redistribution of stoichiometry to $(\alpha4)_2(\beta2)_3$ during the assembly of oligomeric receptors [31,39], however, a major challenge in determining shifts in receptor stoichiometry is to measure the stoichiometry of receptors at the plasma membrane (PM). One of the most effective means of accomplishing this is the use of single molecule spectroscopy to directly count fluorescently labeled subunits. While this has been accomplished in non-physiological expression systems such as oocytes, single molecule applications in live cells are hindered by poor spatial resolution and limited sensitivity due to cellular autofluorescence. Additionally, receptors tend to diffuse along the cell surface, which further complicates the isolation of individual receptors. A novel solution to observe single receptors on the cell membrane is to integrate live cells with zero mode waveguides (ZMWs). ZMWs consist of nanometer scale holes in thin metal films that can be used to isolate individual molecules from high concentration solutions. The application of ZMWs to live cells allows for the isolation of receptors by creating nanoscale observation ‘chambers’ on the plasma membrane. This imparts two primary advantages: (1) only molecules in the limited observation volume will be excited, suppressing background fluorescence, and (2) the nano-observation volume isolates membrane receptors for long periods of time allowing dynamics to be extracted. By transfecting cells with green fluorescent protein (eGFP) conjugated subunits, the receptors assemble such that each subunit is fluorescently labeled. By isolating a single receptor in the bottom of the nanoscale well and illuminating continuously, the eGFP tagged subunits can be bleached sequentially. This allows the bleaching steps of individual eGFP molecules to be counted as subunits and allows for the extraction of the stoichiometry of receptors. Measuring the stoichiometry at the plasma membrane allows us to observe the downstream effects of pharmacological chaperoning on the assembly of heteromeric nAChRs. This technique has been applied to determine the influence of nicotine on the stoichiometry of α4β2 nicotinic receptors [54]. It is clear that nicotine causes a PM upregulation of receptors with a $(\alpha4)_2(\beta2)_3$ stoichiometry, while a partial nAChR agonist, cytisine, results in the insertion of $(\alpha4)_3(\beta2)_2$ receptors at the PM [54]. The observed differences in stoichiometry indicate that chaperoning efficiency depends on the type of chemical chaperone and the subtypes of receptors that are being chaperoned. These factors are discussed below.

3. Factors influencing nAChR upregulation

nAChR upregulation and pharmacological chaperoning display tiers of selectivity at the level of brain regions and circuits, cell types (dopaminergic versus GABAergic neurons), subcellular organelles, receptor subtypes and the type of chaperone. Although each of the above factors can significantly influence nAChR chaperoning, we will focus on two cell autonomous factors: (i) nAChR subtypes and (ii) pharmacological properties of the chaperone.

3.1. nAChR subtypes

nAChRs are a non-homogenous population of ion channels consisting of α (α2 to α6) and β (β2 to β4) subunits arranged as heteromeric or homomeric receptor pentamers around a central non-specific cation conducting pore [55]. α7 and α4β2* nAChR subtypes (* indicates that other uncharacterized subunits may be present in the pentamer) are abundantly expressed throughout the CNS, while other nAChRs such as α6β2*, α3β4*, and α2β2 receptors show a more restricted localization pattern [55,56].

3.1.1. nAChR subtypes possess different ligand binding properties—nAChRs possess functional and ligand binding properties that are unique to the specific subtype. Thus, nicotine-induced chaperoning and upregulation can vary to a great extent due to differences in the ligand binding affinity of receptor subtypes. For example, high affinity α4β2* and α6β2β3* (* denotes an uncharacterized subunit in the receptor pentamer) nAChRs bind to nicotine with nanomolar affinity and therefore upregulate more readily at smoking-relevant nicotine concentrations (100–200 nM) [30,57] than the lower affinity a7 or α3β4 receptors that show an EC_{50} for nicotine upregulation in the micromolar range [58,59].

Conflicting reports for α6* nAChRs show upregulation, downregulation, or no change in response to chronic nicotine [60–62]. These discrepancies likely arise due to the presence or absence of accessory β3 subunits in α6β2* pentamers. β3 subunits can dramatically increase α6β2* receptor sensitivity to nicotine, resulting in upregulation at nanomolar concentrations [30]. Differential upregulation of nAChRs therefore appears to depend on the affinity of a particular receptor subtype to nicotine.

3.1.2. nAChR subtypes and trafficking motifs—The large intracellular loop between the receptor M3 and M4 transmembrane segments contains motifs that govern receptor trafficking out of the ER [30,31,46,63,64]. The loop also includes specialized sorting motifs that export receptors to either somatodendritic or axonal compartments of neurons [63]. The chaperoning and consequent upregulation of receptors is critically dependent on the specific combination of M3-M4 trafficking motifs present within a given receptor subtype [31,32,45,46]. LXM motifs (where X is any amino acid) in the M3-M4 loop of α4, α3 and β4 subunits govern the rate of ER exit of nAChRs, while RXRR motifs in the β2 subunits result in the ER retention of nAChRs [31]. α4β4 and α3β4 receptors contain LXM motifs on all five subunits do not upregulate significantly [31,58], presumably because these receptors already exit the ER with maximal efficiency in the absence of nicotine and nicotine chaperoning cannot significantly increase receptor trafficking through the secretory pathway. On the other hand, α4β2 nAChRs contain RXRR motifs in the β2 subunits that retain receptors in the ER [31]. In this case, nicotine binds to and chaperones nAChRs out of the ER, resulting in upregulation, while a majority of the receptors remain in the ER in the absence of nicotine.

A recently described trilysine motif (KKK) in the mouse β3 nAChR subunit binds to COPI vesicles and mediates the retrograde trafficking of nAChRs from the Golgi to the ER [30]. This process is exclusively engaged during nicotine-mediated upregulation of β3* receptors

and not during the basal trafficking of receptors in the absence of nicotine. Interestingly, COPI mediated retrograde transport is also essential for the upregulation of α4β2 receptors and does not occur during nAChR trafficking in the absence of nicotine. Thus, pharmacological chaperoning likely involves the repeated cycling of receptors between the ER to the Golgi, which allows chaperones to induce the most stable conformation of nAChRs prior to the forward trafficking of receptors to the PM.

Based on these studies, it is clear that specific trafficking signals within receptor subtypes play a pivotal role in nAChR chaperoning. We will therefore consider known effects of chaperoning on nAChR subtypes. Fig. 2 summarizes the effect of chaperoning by nicotinic ligands on known nAChR subtypes.

3.1.3. α4β2* nAChRs—Among the known CNS nAChR subtypes, α4β2* receptors are best characterized and most widely distributed across the CNS. It is clear that long-term nicotine administration in cell lines, cultured neurons, rodents, and humans results in the chaperoning of α4β2 nAChRs [16,17,22,25,29–32]. Chaperoning of α4β2* nAChRs occurs in cortex, midbrain, and hypothalamus, but not in thalamus or cerebellum [29,33,57,65–67]. α4β2* nAChR populations have recently found to be more complex and may exist with the addition of α5, α6, and/or β3 nAChR subunits [68,69]. Two populations of α4β2* nAChRs exist in the striatum: α4β2 and α4α5β2 subtypes [69]. The presence of an accessory α5 nAChR subunit alters chaperoning such that while α4β2 nAChRs are chaperoned by chronic nicotine, α4α5β2 nAChRs fail to undergo chaperoning [70,71]. This may be a consequence of subtle changes in the ligand binding affinity of α4α5β2 nAChRs when compared to α4β2 receptors [72,73].

Peng et al. [34] showed that one mechanism that may explain the increase of α4β2* nAChRs on the PM is a nicotine-induced decrease in the turnover of PM nAChRs. Despite this, others have shown that there is no change in the turnover of α4β2* nAChRs on the PM [37,74]. Several reports now point to nicotine-induced subunit maturation and assembly of α4β2* nAChRs in the ER as a primary mechanism for nAChR upregulation [19,31,36]. α4β2* nAChRs assemble rather inefficiently and it is likely that nicotine increases the assembly and stability of pentamers in the ER, thereby allowing mature and stable pentamers to efficiently traffic through the secretory pathway.

3.1.4. α6* nAChRs—Characterizing the chaperoning of α6* nAChRs has been more tedious than α4β2 nAChRs, mainly due to the poor expression of α6* nAChRs in heterologous systems. Many studies in mice suggest that α6p2* nAChRs are not chaperoned by nicotine [66,71,75–77], but other studies have observed chaperoning of α6* nAChRs following chronic nicotine treatment [30,60]. Yet other, assays in cultured cells expressing α6* nAChRs, show that both α6β2 and α6β2β3 nAChRs are chaperoned following chronic nicotine treatment [61,62]. Perez et al. [60] showed that α6β2* nAChRs that did not contain α4 nAChR subunits are chaperoned by nicotine, while α6α4β2* nAChRs do not undergo chaperoning. These discrepancies likely arise due to differences in expression systems, assays and/or exposure paradigms to the chaperoning ligand.

We have shown that *in vitro*, the upregulation of α6β2β3 nAChRs occurs via an increased insertion of receptors into the PM [30]. Using a pH sensitive eGFP analog (supercliptic pHluorin [SEP]) we found that there is an increase in the number of α6* nAChRs inserted on the PM following chronic nicotine treatment. We also found that the fold increase in insertion to the PM is directly proportional to the increase in nAChR density on the PM. Therefore it is possible that the upregulation of α6* nAChRs is principally due to an increased insertion of new receptors rather than a change in the stability or turnover of pre-existing receptors at the PM. Furthermore, we found that both α6β2* and α6β4* nAChRs are chaperoned by nicotine and α6β2* nAChRs chaperone only in the presence of β3 subunits, while α6β4 nAChRs are chaperoned with and without the β3 nAChR subunit [30]. As described earlier, chaperoning in this case, appears to be dependent on the presence of a trilysine motif in the M3-M4 loop of p3 subunits. The trilysine motif binds to COPI components and mediates the retrograde transport of receptors from the ER to the Golgi [30].

3.1.5. α3* nAChRs—α3* (α3β4, α3α5β4) nAChRs are primarily located in the peripheral nervous system. In the CNS, α3* nAChRs are found in the thalamus, hypothalamus, locus coeruleus, and habenula [78]. α3β4 nAChRs generally do not upregulate easily and require much higher concentrations than that found in a smoker's brain [58]. This is likely due to the fact that β4* nAChRs are exported from the ER to the PM very efficiently [31,32]. It is known that α3 and β4 nAChR subunits have an ER export motif [31,46], but lack an ER retention motif found in β2 nAChR subunits [31]. Therefore α3β4 nAChRs may be expressed on the PM at densities where no further upregulation is possible.

In vitro, α3β2 nAChRs have been shown to upregulate in response to chronic nicotine [61]. This was shown to occur at nicotine concentrations much higher than that typically found in a smoker's brain (>1 μM). The fact that α3β2 nAChRs upregulate while α3β4 receptors are not upregulated by nicotine agrees with the idea that the LXM ER export signals in β4 nAChR subunits produce a 'chaperone' like effect in the absence of nicotine. In contrast, the β2 nAChR subunit is normally retained in the ER because of the presence of an ER retention motif in the M3–M4 loop and is therefore available to bind to and be chaperoned by nicotine and nicotinic ligands.

3.1.6. Chaperoning of α7 nAChRs—α7 nAChRs are another subtype found distributed in the CNS. α7 nAChRs are found in the spinal cord, amygdala, olfactory region, cortex, hippocampus, cerebellum, and hypothalamus [78]. Typically, mRNA levels of nAChRs are unaffected by chronic nicotine and have led to the understanding that nAChR upregulation occurs through posttranscriptional mechanisms [19,20,79]. Despite this, Lam et al. [80] showed that chronic nicotine treatment increased mRNA levels of α7 nAChRs. This increase by transcriptional mechanisms was mediated through the S1-GATA2 pathway. More recently, upregulation of α7 nAChRs (and their mRNA levels) has been shown to occur through recruitment of Sp1-GATA4 or Sp1-GATA6 [81]. This upregulation occurs at concentrations of nicotine (100 nM) that are physiologically relevant to human smokers.

3.2. Factors influencing upregulation: chaperone-specific properties

An efficient pharmacological chaperone must: (i) be able to access the compartment in which the target protein exists, which in the case of nAChRs is the ER and (ii) display a high affinity for binding to the target protein.

3.2.1. Chaperone accumulation in intracellular organelles—Nicotine is an exemplar pharmacological chaperone that, in the free base form, easily penetrates the plasma membrane. Positively charged quaternary ammonium compounds such as the agonist, acetylcholine (ACh) or peptides such as α-Conotoxin MII and α-bungarotoxin cannot permeate the plasma membrane and will not reach intracellular nAChR targets, which makes these molecules inefficient pharmacological chaperones.

3.2.2. Chaperone affinity—Within seconds of inhaling cigarette smoke, nicotine rapidly enters the bloodstream, passes the blood brain barrier and achieves micromolar concentrations in the brain, however, in chronic smokers, the steady state concentration of nicotine is ~100–200nM [40–42]. These chronic concentrations of nicotine are well within the range required to upregulate high-sensitivity α4β2* and α6β2β3* nAChRs. Unlike acetylcholine, nanomolar nicotine concentrations are not rapidly cleared from cells, allowing the drug to interact with intracellular nAChRs over a period of minutes, hours and even days. Because of this prolonged exposure to nAChRs, nicotine can bind to and stabilize multiple states of nAChRs, including the open, closed and desensitized receptor conformations, but a deeply desensitized nAChR conformation is likely favored. Because of a restricted binding capability, nAChR antagonists such as dihydro-beta-erythroidine (D^E) and non-competitive antagonists such as the open channel blocker, mecamylamine will stabilize only a small subset of open, closed or desensitized receptor conformations. As a result, these compounds chaperone nAChRs at ~10–100-fold greater concentrations than nicotine [34,82].

The efficiency of pharmacological chaperoning of nAChRs is therefore determined by a combination of the ability of chaperones to accumulate in intracellular compartments and binding kinetics to nAChRs. Generally, membrane-permeable agonists and partial agonists are likely to be more efficient nAChR chaperones than competitive or non-competitive antagonists.

4. Pharmacological chaperoning of nAChRs as a therapeutic strategy for Parkinson’s disease

We described nicotine as a pharmacological chaperone of nAChRs and detailed the influence of receptor-specific and chaperone-specific factors on pharmacological chaperoning. We will now explain how pharmacological chaperoning by nicotine can serve a neuroprotective function in Parkinson’s disease (PD).

4.1. Tobacco and nicotine prevent Parkinson’s disease

Epidemiological studies over the past 50 years show a strong inverse correlation between a person’s history of tobacco use and his/her risk for PD [83–86]. The neuroprotective effects of tobacco are independent of genetic background, because in retrospective studies with

identical twins that are discordant for smoking, PD occurs in the non-smoking twin [84]. The neuroprotective effect of tobacco persists in studies utilizing age-matched controls of nontobacco users [85], ruling out spurious effects due to early mortality following the use of tobacco.

Nicotine, the active addictive ingredient of tobacco likely mediates neuroprotection because: (i) nicotine exposure prevents neuronal cell death *in vivo* [86–89], (ii) clinical trials with nicotine patches show an attenuation of PD symptoms [90–93], (iii) nicotine binds to nAChRs at smoking-relevant concentrations (~100–200 nM) and nAChRs are abundantly expressed in the dopaminergic (DA) neurons of the substantia nigra pars compacta (SNc) that are lost in PD [68] and (iv) nicotine neuroprotection is lost in nAChR knockout mice [89]. Thus, it is likely that the neuroproective effects of tobacco specifically proceed via interaction(s) between nicotine and nAChRs.

4.2. “Outside-in” versus “inside-out” mechanisms of neuroprotection

Although the neuroprotective effects of nicotine are well documented, the molecular mechanism(s) by which this occurs remain unclear. Some studies hypothesize an “outside-in” mechanism for neuroprotection in which nicotine activates surface nAChRs, resulting in Ca^{2+} influx and transcriptional changes [94–97].

Nicotine can also exert neuroprotection via “inside-out” pharmacology [19,98]. In this model, nicotine concentrates within intracellular compartments and chaperones nAChRs out of the endoplasmic reticulum (ER) [20,98]. Nicotine-induced nAChR chaperoning can alter the physiology of the ER and the ER stress/unfolded protein response (UPR), resulting in transcriptional changes and neuroprotection [39,98]. Although not yet systematically studied, it may well be that both mechanisms act in concert to exert a net neuroprotective effect on the cell, but we will focus on the “inside-out” hypothesis of nicotine neuroprotection.

4.3. The ER stress response and neurodegeneration

The ER stress/unfolded protein response (UPR) is a multi-armed signaling cascade triggered by the presence of misfolded proteins, excess Ca^{2+} or oxidative stress in the ER. Following activation by these stressors, the three ER resident sensors, activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) initiate complex cell signaling cascades culminating in transcriptional changes within the nucleus. A detailed description of the UPR is outside the scope of this review and is described elsewhere [99–101]. Importantly, the UPR has emerged as a major causative factor in the development of several neurodegenerative disorders, including Parkinson’s disease (PD). The dopaminergic (DA) neurons that are lost in PD are under continuous ER stress due to the presence of reactive oxygen species from toxic metabolites of dopamine and cyclical Ca^{2+} influx. Indeed, ER stress markers are activated in the SNc of PD patients and generally during neurodegeneration indicating that the ER stress/unfolded protein response is a causative factor for neurodegeneration and PD [102–106].

4.4. Pharmacological chaperoning inhibits the ER stress response

Our studies show that nicotine inhibits the PERK and ATF6 ER stress pathways via pharmacological chaperoning [39] and that inhibition of ER stress occurs in cultured dopaminergic neurons expressing native nAChRs (unpublished). Because pharmacological chaperoning involves profound changes in cellular physiology such as an increased export of cargo from the ER and increases in the size and/or elaboration of subcellular organelles such as the ER and Golgi one can hypothesize that nicotine generally increases the efficiency by which dopaminergic neurons export cargo from the ER via ER exit sites, thus reducing the protein burden and increasing efficiency. In addition, an increased ER size due to chaperoning can enable more efficient Ca^{2+} buffering, thereby improving the overall health of dopaminergic neurons and preventing their death. It may well be that techniques used to measure chaperoning can become useful drug discovery tools for identifying neuroprotective compounds against PD.

4.5. Genetic mutations in PD and pharmacological chaperoning

Studies show that the overexpression of α-synuclein [107], mutations in leucine rich repeat kinase 2 (LRRK2) [108] or mutations in the α-synuclein gene [109–112] can result in PD. In these cases, dopaminergic cell death occurs due to a dysfunction in the cellular trafficking machinery and processing of α-synuclein. Specifically, dysfunction in the export or endosomal/lysosomal processing of α-synuclein have been implicated in PD pathogenesis [113]. Pharmacological chaperoning of nAChRs can potentially affect α-synuclein trafficking by: (i) increasing the formation of ER export sites, which can counter the lack of forward trafficking of α-synuclein and attenuate the disease process, (ii) increasing the formation of COPI-containing vesicles, which can influence the number as well as the efficiency by which early and late endosomes are formed in dopaminergic neurons and (iii) influencing the biology of lysosomes in dopaminergic neurons by increasing the number of nicotine-bound nAChRs that are resistant to degradation and therefore possess a longer intracellular half life.

Another important area of research is the role of mitochondrial dysfunction in PD. Mutations in mitochondrial proteins such as Parkin may account for as much as 5% of PD cases [114]. These mutations likely result in mitochondrial dysfunction, mitophagy and the consequent depletion of energy stores in dopaminergic neurons, thus causing cell death and PD [115,116]. Research indicates that the ER stress response is accompanied by a similar response in the mitochondria known as the mitochondrial stress or mitochondrial unfolded protein response [117]. One can speculate that the inhibitory effect of nAChR chaperoning on ER stress can directly or indirectly influence mitochondrial stress and dysfunction via as yet undiscovered mechanisms. Further research is required to understand the interplay between the mitochondrial the ER stress response in the context of neurodegeneration.

4.6. Can pharmacological chaperoning be broadly applied to neurodegeneration?

Since ER stress plays a role in the pathogenesis of several neurodegenerative diseases, including Parkinson’s diease (PD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS), Huntington’s disease (HD) and prion-related degeneration [102], it is tempting to

speculate that inhibition of the ER stress response by nicotine can be neuroprotective in the broader context of neurodegeneration.

If nicotine inhibits ER stress via pharmacological chaperoning of nAChRs, one might expect a nicotine-mediated neuroprotective effect on any CNS neuron with natively expressed nAChRs. Conceptually, when one applies the inhibition of ER stress by nicotine-induced pharmacological chaperoning as a general treatment strategy for neurodegeneration, factors such as the subtype of expressed nAChRs, the absolute number of nAChRs expressed per neuron and the context of neuronal degeneration (genetic versus environmental) likely play a decisive role on whether or not nicotine is neuroprotective. It may well be that the SNc dopaminergic neurons degenerating in PD possess a combination of nAChR subtypes and native receptor expression levels that are readily chaperoned by nicotine. Clearly, more work needs to be done to understand if and how nicotine can exert a neuroprotective effect in other forms of neurodegeneration.

5. Perspectives and future directions

nAChR chaperoning is a complex process involving changes at almost every step of the cellular secretory pathway. Cell autonomous factors such as expressed nAChR subtypes and the properties of the ligand can exert a profound influence on the pharmacological chaperoning of nAChRs.

Although high-resolution imaging techniques have shed light on the cellular mechanism of pharmacological chaperoning by nicotine, several questions remain to be answered: (i) a major future challenge is to study pharmacological chaperoning *in vivo*. This is an important step in order to understand the physiological relevance of chaperoning for nicotine addiction and in the context of neuroprotection against PD. *In vivo* studies will require the development of imaging techniques and biological tools, including transgenic mice to study nAChR chaperoning in areas of the brain that are currently difficult to access using standard methods. (ii) Understanding the molecular mechanisms by which chaperoning reduces ER stress in dopaminergic neurons is critical for the development of therapeutics against PD and possibly other neurodegenerative disorders.

Acknowledgements

Supported by grants from the Tobacco-Related Disease Research Program (TRDRP 18FT-0066), the Michael J Fox Foundation (MJFF), U.S. National Institutes of Health, Louis and Janet Fletcher.

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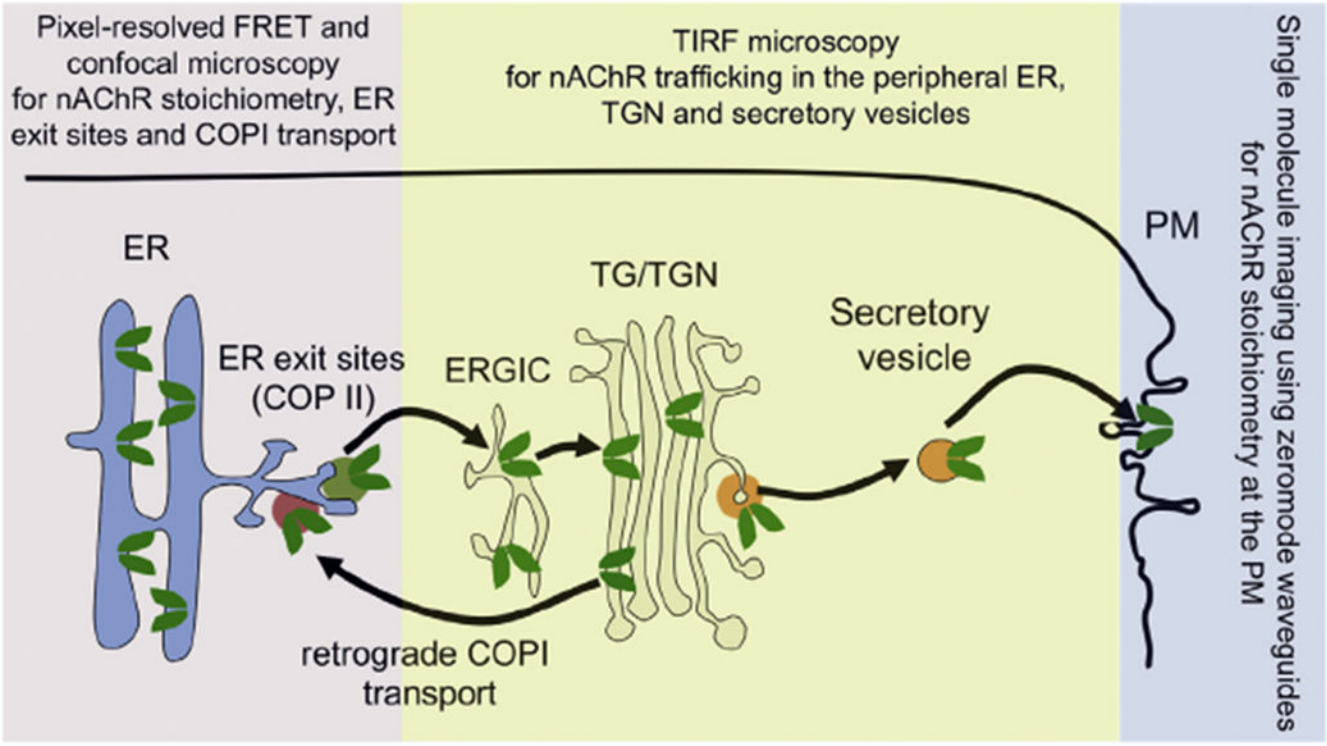

Fig. 1.

Methods to study pharmacological chaperoning of nAChRs. A schematic of nAChR trafficking in cells is shown. Pentameric receptors (green) assemble in the endoplasmic reticulum (ER) and concentrate in ER exit sites (dark green vesicle). Receptors then traffic to the *trans* Golgi network (TGN) via the ER to Golgi intermediate compartment (ERGIC). During chaperoning, ligand bound nAChRs return from the Golgi to the ER via COPI vesicles (orange-red) and are then cycled back to the Golgi via COPII vesicles. Some ligand bound receptors enter secretory vesicles bound to the plasma membrane (PM) from the TGN (yellow vesicle), resulting in upregulation at the PM. Also depicted are the methods that have been developed to study nAChR trafficking at each stage of the cellular secretory pathway. FRET and confocal microscopy allow high-resolution measurement of nAChR assembly and trafficking between the ER and Golgi. As explained in the text, under certain conditions, TIRF microscopy can be used to study nAChRs in the peripheral ER, TGN and upregulation at the PM. Single molecule imaging using zeromode waveguides allows measurement of nAChR stoichiometry of isolated receptors at the PM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

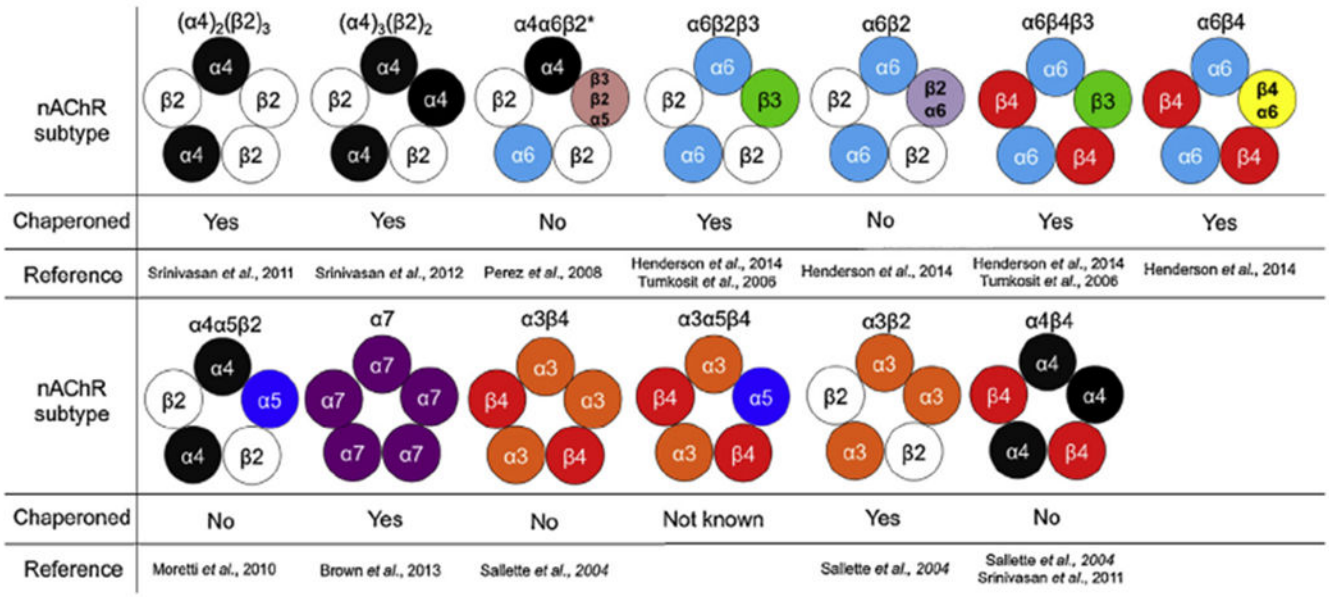

Fig. 2.

Pharmacological chaperoning of nAChR subtypes. The nAChR subtypes known to assemble in various brain regions is shown along with whether or not the particular subtype undergoes pharmacological chaperoning.