The AAPS Journal, Vol. 11, No. 1, March 2009 (© 2009)
DOI: 10.1208/s12248-009-9090-7

Mini-Review

Theme: NIDA Symposium: Drugs of Abuse: Cutting-edge Research Technologies
Guest Editors: Rao Rapaka, Thomas Aigner, Joni Rutter, and David Shurtleff

Nicotine is a Selective Pharmacological Chaperone of Acetylcholine Receptor Number and Stoichiometry. Implications for Drug Discovery

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Received 31 December 2008; accepted 7 February 2009; published online 12 March 2009

Abstract. The acronym SePhaChARNS, for "selective pharmacological chaperoning of acetylcholine receptor number and *s*toichiometry," is introduced. We hypothesize that SePhaChARNS underlies classical observations that chronic exposure to nicotine causes "upregulation" of nicotinic receptors (nAChRs). If the hypothesis is proven, (1) SePhaChARNS is the molecular mechanism of the first step in neuroadaptation to chronic nicotine; and (2) nicotine addiction is partially a disease of excessive chaperoning. The chaperone is a pharmacological one, nicotine; and the chaperoned molecules are α4β2* nAChRs. SePhaChARNS may also underlie two inadvertent therapeutic effects of tobacco use: (1) the inverse correlation between tobacco use and Parkinson's disease; and (2) the suppression of seizures by nicotine in autosomal dominant nocturnal frontal lobe epilepsy. SePhaChARNS arises from the thermodynamics of pharmacological chaperoning: ligand binding, especially at subunit interfaces, stabilizes AChRs during assembly and maturation, and this stabilization is most pronounced for the highest-affinity subunit compositions, stoichiometries, and functional states of receptors. Several chemical and pharmacokinetic characteristics render exogenous nicotine a more potent pharmacological chaperone than endogenous acetylcholine. SePhaChARNS is modified by desensitized states of nAChRs, by acid trapping of nicotine in organelles, and by other aspects of proteostasis. SePhaChARNS is selective at the cellular, and possibly subcellular, levels because of variations in the detailed nAChR subunit composition, as well as in expression of auxiliary proteins such as lynx. One important implication of the SePhaChARNS hypothesis is that therapeutically relevant nicotinic receptor drugs could be discovered by studying events in intracellular compartments rather than exclusively at the surface membrane.

KEY WORDS: ADNFLE; dopamine; GABA; proteostasis; upregulation.

TRANSLATIONAL RELEVANCE OF SePhaChARNS

A Disease: Nicotine Addiction

Pioneering results showed that when rodents are exposed to chronic nicotine, the level of [^{3}H]-nicotine binding increases (1,2), probably denoting increased receptor numbers ("N" in SePhaChARNS). We now know that α4β2* receptors (* = IUPHAR nomenclature, "other subunits may be present") are selectively upregulated (a partial reason for the initial "Se" in SePhaChARNS) but that other subtypes are also upregulated (3); that the upregulated receptors are probably active rather than desensitized (4,5); and that the extent of upregulation is region- and cell-selective (5) (major reasons for the initial "Se"). Human smokers also show increased levels of nAChRs in functional magnetic resonance imaging and post mortem measurements (6–9).

Some investigators have thought that upregulation was an epiphenomenon in nicotine addiction. But increasing evidence suggests that upregulation of high-sensitivity nAChRs is, at least partially, the basis for several key biological results of chronic nicotine administration: tolerance, locomotor sensitization, and cognitive sensitization (5,10–12).

We give two examples. The first example is in the midbrain. Chronic nicotine exposure cell-selectively upregulates receptor numbers ("Se" and "R" and "N"). Upregulation occurs in the GABAergic neurons of the ventral tegmental area (VTA), but not in the somata of the dopaminergic neurons which are postsynaptic targets of, and

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inhibited by, the GABAergic neurons. As a result, chronic nicotine increases both the baseline firing rate and the excitatory effect of nicotine in GABAergic neurons, while decreasing baseline firing rate and attenuating the excitatory effect of nicotine in dopaminergic neurons (5) (C. Xiao and H. Lester, unpublished). These data suggest that alterations in dopaminergic signaling due to chronic nicotine are due to receptor upregulation within GABAergic VTA neurons, and that increases in inhibition could provide an explanation for tolerance to the chronic effects of nicotine (5). These hypotheses involve reasoning at the level of a circuit rather than individual neurons.

The second example is in the hippocampus. Chronic exposure to nicotine increases α4* receptors on glutamatergic axons of the medial perforant path, which consists of projections from the entorhinal cortex to the dentate gyrus. In hippocampal slices from chronically treated animals, acute exposure to nicotine during tetanic stimuli lowers the threshold for induction of long-term potentiation in the medial perforant path. However, no such augmentation was observed when either the chronic nicotine exposure was omitted, or the acute exposure during the tetanus was omitted, or both (5). Thus, the upregulated presynaptic α4* receptors in this pathway are also functional. This instance of cell selective ("Se") numerical upregulation ("N") of functional receptors provides a plausible explanation for another effect of chronic nicotine: sensitization of cognitive function in forebrain. This phenomenon probably does not require a circuit but arises from a change in presynaptic properties.

In the sections below, we place these examples of cell-selective upregulation in a broader context. We hypothesize that nicotine addiction is in part a disease of excessive chaperoning. The chaperone is a pharmacological one, nicotine, and the chaperoned molecules are α4β2* nAChRs. Nicotine addiction seems to involve more brain areas, and consequently more behavioral processes, than cocaine or morphine addiction. SePhaChARNS probably does not explain withdrawal (13,14), and SePhaChARNS may not explain the heavy smoking in schizophrenia; the latter may involve Ca^{2+}-dependent signalling (see below).

An Inadvertent Therapy: Tobacco and Parkinson's Disease

We also hypothesize that SePhaChARNS underlies the strong inverse correlation between smoking (probably via nicotine itself) and Parkinson's disease (15–20). Nicotine protects rodent DA neurons agonist toxin-induced cell loss (21–24), but these neuroprotective effects are absent in α4 nAChR knockout (KO) mice (22).

Our data show cell-selective upregulated nAChR numbers in substantia nigra that strongly resemble the data in VTA. Chronic exposure to nicotine produces new functional α4β2* receptors on all substantia nigra pars reticulata (SNr) GABAergic neurons, but not on their postsynaptic targets, the DA neurons of substantia nigra pars compacta (SNc). This upregulation produces higher GABAergic SNr neuron firing rate, even in the absence of nicotine (5). In consequence, SNc DA neurons from chronic nicotine-treated mice have a lower baseline firing rate and a lower incidence of burst firing. These effects vanish in α4 knockout mice (C. Xiao and H. Lester, unpublished. These alterations, which we postulate arise from SePhaChARNS, could protect SNc neurons from excessive activation during any of the several inherited or acquired metabolic conditions that tend to depolarize DA neurons.

Subcellular Selectivity ("Se" in SePhaChARNS)

In recent experiments (C. Xiao and H. Lester, unpublished), we have confirmed and extended observations that chronic nicotine upregulates nAChR sensitivity in GABAergic neurons of mouse SNr. We find increased sensitivity both in the somata and in the synaptic terminals; the latter is assessed by recordings on the downstream target, SNc dopaminergic neurons.

We also examined nAChR sensitivity at the striatal axon terminals of dopaminergic neurons in the dorsal striatum, the termination of the nigrostriatal DA pathway. This sensitivity is detected indirectly by reduced glutamate release from the corticostriatal afferents, recorded in medium spiny neurons. Our experiments reveal that chronic nicotine upregulates the function of α4β2* nAChRs on dopaminergic terminals. These effects on dopaminergic terminals extend and confirm other reports that chronic nicotine increases nicotine-induced dopamine release (25,26) and upregulates receptors in striatum (5). Combined with the non-effect of chronic nicotine on somatodendritic α4β2* nAChRs in SNc dopaminergic neurons, the data suggest a subcellular selectivity of nAChR upregulation, in addition to previously defined selectivity in nAChR subtypes, brain regions, and neuronal cell types.

Evidently, chronic nicotine selectively upregulates α4β2* nAChRs in components of the nigrostriatal dopaminergic pathway. Although the complete circuit description is not available, these effects of chronic nicotine may amplify inhibition to SNc dopaminergic neurons and temper the release of glutamate in the dorsal striatum. This would be an additional mechanism to reduce the risk of excitotoxicity and would counteract the hyperactivity of striatal glutamate synapses resulting from dopamine denervation.

Another Inadvertent Therapy: Tobacco and ADNFLE

We also hypothesize that SePhaChARNS underlies the inadvertent therapeutic effect of tobacco usage in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), which has at least six missense alleles in the α4 or β2 subunits (27–29).

In experiments conducted since ADNFLE was mapped in 1995, heterologous expression shows that ADNFLE receptors have a gain of function. Some data show that the mutant receptors have lower EC_{50} values than WT receptors (30). Other data show that the mutant receptors are less sensitive than WT receptors to decreased external Ca^{2+} (31,32).

Our recent experiments show that five ADNFLE receptors have an additional common abnormality. The intracellular pool of mutant receptors is shifted toward the $(\alpha4)_3(\beta2)_2$ stoichiometry. Treatment for 24 h with nicotine reverses this abnormality, bringing the stoichiometry to approximately WT levels (C. Son, F. Moss, B. N. Cohen, H. A. Lester, submitted). The community does not yet understand the

pathophysiology of ADNFLE; it may well involve selective trafficking of one stoichiometry. However, the *in vitro* effect of nicotine brings partial rationalization to its *in vivo* effect and suggests new clues about the pathophysiology.

NICOTINE *VS* ACETYLCHOLINE

Protection against herbivores (33,34) is presumably the selective advantage that production of nicotine confers on the tobacco plant. Similar advantages may be conferred by cocaine and morphine. Some herbivores have adapted to the presence of nicotine (33,34). Because nicotine was introduced to most continents, and to most human societies, <600 years ago, there has been little no selective pressure on the mammalian genome associated with nicotine. Humans have, however, been unusually able, among species, to titrate their intake of neurotoxins, such as nicotine, to achieve pleasurable sensations while avoiding acute toxicity.

Nicotine Remains Near Neurons for Many Minutes; ACh Does Not

Nicotine is metabolized only by liver enzymes and disappears from blood with a half-life of ~120 min (35). On the other hand, ACh is hydrolyzed by acetylcholinesterase, which has a turnover rate of ~10^4/s (36). Therefore, most simulations of synaptic transmission conclude that ACh remains near receptors for <1 ms (37,38). As a result, nicotine participates in two processes than are much less accessible to ACh. First, nicotine can desensitize receptors; second, nicotine can permeate into cells, as described in the next section.

Nicotine Permeates Membranes Readily; ACh Does Not

A strong aspect of the SePhaChARNS idea is that nicotine acts at an intracellular locus, not at the surface membrane. Nicotine is quite effective as a smoked drug because it permeates at least six membranes, in the lungs and capillary walls, within a few seconds of inhalation. This implies that nicotine can also enter the endoplasmic reticulum and other organelles.

The log *P* value for uncharged drug is an important concept in its membrane permeation. Table I presents log *P* values computed by the algorithm at the Pubchem Web site. All drugs in this list may be assumed to have p*K*a values between 7.4 and 8. Therefore, most of the drug is nonionized in the blood and CSF. Therefore a log *P* value more positive than −1 implies reasonable membrane permeability, and consequently, the physical presence, at least, to interact with intracellular receptors.

Table I. Log *P* Values for Various Amphiphilic Nicotine Drugs

	X log *P*
Dihydro-β˜-erythroidine	−0.5
Cytisine	−0.3
A-85380	0.5
Varenicline	0.9
Nicotine	1.1
ABT-594	1.2
Epibatidine	1.7
Galanthamine	1.8
Amantadine	2.3
Mecamylamine	2.7
Lobeline	3.9

From the PubMed Web site

Quaternary amines remain ionized and therefore have no defined log *P*. However, in experiments with clonal cell lines, ACh, and other quaternary amine agonists do penetrate into intracellular organelles within ~3 h, albeit much more slowly than nicotine (39). This was shown by partial protection against the binding of [^{3}H]epibatidine, which is roughly as permeant as nicotine itself. Thus, receptor upregulation by nicotine cannot be taken as proof that upregulation occurs primarily because of interactions at surface receptors. If ACh survived for several hours near neurons (which is not the case), it too would produce SePhaChARNS.

Quaternary amines with substantial hydrophobic substituents also display detectable membrane permeability; this is revealed in the literature on blockers of voltage-gated ion channels. When externally applied to squid giant axons, C_{12}-tetraethylammonium (TEA) is a more effective K channel blocker at the internal TEA binding site than C_9-TEA, which was attributed to their relative lipid permeabilities (40). Internal block by tetrapentylammonium after external application to *Xenopus* oocytes persists after washout, suggesting that once it accumulates inside the cell, it is trapped there for some time (41). Similarly, N-beta-phenylethyl lidocaine blocks Na channels for at least 30 min after application to neuronal GH3 cells and persists for 3–6 h *in vivo* (36,42). There is a large body of related work suggesting that other quaternary ammonium derivatives of local anesthetics can penetrate membranes as well (43).

Nicotine Acts More Strongly on Neuronal than Muscle AChRs

The EC_{50} for nicotine at $(\alpha4)_2(\beta2)_3$ neuronal receptors is ~1 μM; at $(\alpha1)_2\beta1\gamma\delta$ muscle receptors, ~ 400 μM. This difference arises because nicotine makes a cation-π interaction with a Trp residue in loop B of the α4 subunit, and a hydrogen bond with the backbone carbonyl group of the peptide bond between this residue and the adjacent downstream residue (44). Nicotine makes neither interaction with the muscle receptor (45,46).

On the other hand, ACh makes the cation-π bond equally strongly with the $(\alpha4)_2(\beta2)_3$ neuronal receptor and with $(\alpha1)_2\beta1\gamma\delta$ muscle receptors. These differences show how nicotine can activate and upregulate $(\alpha4)_2(\beta2)_3$ receptors but have minimal effect on muscle receptors.

MECHANISTIC DETAILS OF SePhaChARNS

The "Pharmacological Chaperone" Concept ("PhaCh" in SePhaChARNS)

Evidence is accumulating to support Lindstrom's proposal that nicotine acts as pharmacological chaperone for nascent nAChRs (39). Upregulation induced in animal

models and transfected cells by chronic nicotine is certainly not transcriptional and is probably post-translational (47). Selective nicotine binding to α4* nAChRs is sufficient for α4* receptor upregulation when α4* receptors are heterologously expressed in mammalian cell lines, as well as in almost pure cultures of GABAergic ventral midbrain neurons (48). We extended classical genetic concepts to state that upregulation displays "cell autonomy". This means that upregulation occurs in the very cell where nicotine acts on the appropriate receptors (48). Upregulation does not require synaptic transmission or other diffusible extracellular signals.

Experiments with cultures from α4-hypersensitive mice also show that upregulation occurs (a) at chronic nicotine concentrations that activate only α4* receptors, (b) in homotypic GABAergic cultures lacking their typical presynaptic partners, and (c) when tested with ACh concentrations that activate only the α4* receptors (48,49). We summarized these findings with the term "receptor autonomy" because activation of no other nicotinic receptors appears necessary (48).

Importantly for these concepts, upregulation is also observed in clonal cell lines transfected with nAChRs. Descriptions that may be essentially equivalent to SePhaChARNS are the statements that nicotine acts as a "maturational enhancer" or a "novel slow stabilizer" for α4β2* receptors (39,50–52). Additional stabilization of chaperoned receptors (48) could also arise from mechanisms regulating receptor turnover (39) or from proteins governing assembly and trafficking (50,52–57).

Characteristics of Pharmacological Nicotinic Chaperones

A clear aspect of SePhaChARNS is that the basic molecular interaction that causes upregulation takes place within the endoplasmic reticulum rather than at the cell surface. Also one expects to detect upregulated receptors in the ER and Golgi before one detects them at the cell membrane. Experiments to test these ideas are under way in the Caltech lab. In previous studies on isolated cells, both agonists and antagonists can upregulate receptors (50,52,54,58,59). These observations agree well with the SePhaChARNS mechanism, which makes no explicit statements about agonists *vs* antagonists. Here are the requirements for an effective pharmacological chaperone for nAChRs. (1) The drug must penetrate to the lumen of the endoplasmic reticulum. (2) It must be present at concentrations greater than its equilibrium dissociation constant for the nAChR subtype (including the particular stoichiometry) that it will chaperone. (3) It should bind at one or more subunit interfaces in the pentameric complex, so that it can stabilize the assembled rather than unassembled receptor.

Determining an equilibrium binding constant is generally straightforward for competitive antagonists. Direct binding experiments generally give the same value as functional tests that employ Schild-type dose-ratio analyses. However, it is less straightforward to determine an equilibrium binding constant for an agonist from functional measurements. Electrophysiological analyses are often performed with millisecond pulses in order to eliminate the distorting effects of desensitization. This is very important for understanding activation; but to assess a drug's suitability as a pharmacological chaperone, equilibrium binding experiments in membrane fragments may actually be more appropriate because, by definition, such experiments allow the drug–receptor interaction to reach the highest-affinity states. As noted below, the high-affinity states are often desensitized. Nonetheless, binding to such states chaperones receptors. Thus, a dose–response experiment for desensitization, even after several hours, might yield the most appropriate binding value for an agonist within the SePhaChARNS context.

These considerations explain how nicotine exhibits an EC_{50} of <100 nM for upregulation in clonal cell lines transfected with α4 and β2 subunits (51). Even the lowest log *P* value, −0.4 for the competitive antagonist DHβE (Table 1), would allow it to penetrate eventually. These considerations explain how DHβE upregulates receptors.

Channel blockers are another class of nAChR drugs. They typically bind on the axis of the channel pore and therefore touch all subunits; if such binding stabilizes the assembled state of an nAChR, it would be a chaperoning influence. "Foot-in-the-door" open-channel blockers like QX-222 bind rather weakly at muscle nAChRs (tens of μM). At these concentrations, they strongly stabilize agonist binding (60) because they lock the channel into the open conformation, which in turn locks the agonist onto its binding site. Therefore upregulation might be enhanced when a foot-in-the-door blocker is applied simultaneously with nicotine at a high-sensitivity nAChR. In fact, neither hexamethonium (61) nor chlorisondamine (62), two well-studied channel blockers at nAChRs, are foot-in-the door blockers at high-sensitivity α4β2 receptors; it is not surprising that the latter has no effect on nicotine-induced upregulation in animals (63). Mecamylamine, a frequently used nonspecific nAChR channel blocker, lacks foot-in-the-door properties at ganglionic nAChRs (64) and has not been evaluated for such properties at α4β2 receptors. Unsurprisingly, hexamethonium, mecamylamine, or decamethonium by themselves do not produce appreciable up-regulation (54,65). Thus, upregulation by the combination of nicotine plus a foot-in-the-door blocker remains only a theoretical possibility at present.

Nicotinic receptors are upregulated by quaternary ammonium agonists including simple tetramethylammonium and carbachol. In one interpretation, surface interactions alone may provide a sufficient thermodynamic driving force; however, as discussed above, it is more likely that the quaternary drugs have sufficient membrane permeability to allow intracellular actions at the rather high concentrations (0.1–1 mM) that produce upregulation (50,65,66).

Desensitization

It is often asked whether upregulation is linked more to activation or to desensitization. The discussion has become more subtle recently (67). We suggest a further reformulation. First, desensitization is somewhat more prevalent for cation channels (e.g., nAChRs) than for anion channels (e.g., GABA and glycine receptors). The selective advantage of this desensitization presumably derives from the fact that prolonged activation of cation channels, but not of anion channels in mature neurons, would be excitotoxic. Such prolonged activation might occur if ACh remains in the vicinity of receptors; for instance, acetylcholinesterase may be

partially lacking at some development stages. Note, however, we have argued above that nicotine itself is not likely to have played a role in this selective pressure.

Nicotine's characteristics described previously, however, allow it to interact with receptors, both intracellular and surface-exposed, for many minutes. In the absence of energy input, any reversible chemical interaction eventually reaches its state of lowest free energy, if the kinetic barriers are low enough to allow access to these states on the time scale of nicotine's presence. This is equal to the state of tightest binding. This is, of course, the fundamental mechanism by which SePhaChARNS allows nicotine to select for high-affinity binding states. Whether the high-affinity states have open or closed channels would matter little. However, because agonists open the channel, they allow access to many more conformational states than do antagonists; and if these new states have high affinity, then SePhaChARNS is strengthened, whether or not the new conformational states have open channels. In summary, desensitization is one set of conformational states that increase the thermodynamic driving force for SePhaChARNS (48).

Changes in Subunit Stoichiometry (final "S" in SePhaChARNS)

The SePhaChARNS hypothesis states that the selective changes in nAChR numbers occur neither because of changed receptor mRNA levels, nor because of altered receptor trafficking (39,47,68,69), but instead because nicotine acts as a pharmacological chaperone in the endoplasmic reticulum to stabilize certain stoichiometries of the receptor. The $\alpha4\beta2$ receptors exist in a mixed population whose nicotine EC_{50} values differ by 50- to 100-fold (70–73) (~1 and ~100 μM). Several studies suggest that the two forms differ in subunit composition: the high- and low-sensitivity forms would consist of the $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ pentamers, respectively (74–76). The upregulated receptors are apparently shifted toward the high nicotine affinity, high-sensitivity $(\alpha4)_2(\beta2)_3$ stoichiometry, at the expense of the lower-sensitivity $(\alpha4)_3(\beta2)_2$ stoichiometry. The thermodynamic reasoning implies that micromolar nicotine concentrations preferentially stabilize and therefore preferentially up-regulate the high- but not the low-sensitivity form of the pentamer.

Acid Trapping

Because nicotine is a weak base and was available as a radiotracer rather early, it was employed in the classic experiments that showed the acidic pH of organelles (77,78). Nicotine accumulates within cells, so that the apparent "intracellular volume" occupied by nicotine is six- to eightfold greater than the total intracellular volume measured, for instance, by inulin exclusion. It is now understood that nicotine actually concentrates by many more fold, in the small volume occupied by acidic organelles.

The progression of pH along the secretory pathway is fairly well understood for some cell types (79) (although we caution that the values have not been determined for neurons). The first steps in assembly and synthesis of nAChRs occur in the endoplasmic reticulum (ER) itself, which is roughly neutral. Therefore the so-called "acid-trapping" mechanism described here is not likely to amplify the pharmacological chaperoning effect of nicotine. The pH decreases to 6.7–6.0 from the *cis* to *trans* Golgi, then to 6 to 5 within the granules of the constitutive and regulated secretory pathway. A pH of 6.5, roughly 0.5 pH unit lower than the cytoplasmic pH, implies a concentration of nicotine threefold higher in the organelle than in the cytoplasm; at pH 5.5, the ratio is 30. This acid trapping would not occur for quaternary amines.

Acid trapping produces concentrations of nicotine many times higher than the smoked extracellular concentration, within the vesicles of the secretory pathway. We do not know the functional state of receptors within this pathway. However, if their assembly and function is approximately normal, then it is a strong conclusion that, in the presence of smoked nicotine, receptors are primarily in a desensitized state before they reach the cell surface. A similar state would apply to receptors endocytosed into the acidic vesicles of the endocytic pathway. We emphasize that the high concentration of nicotine in the organelles is not speculation; the data have been in the literature since 1971 (77).

Thus, we expect that intracellular receptors are, indeed, mostly desensitized when a person smokes. When receptors do reach the surface; however, they are immediately exposed to the extracellular concentration of nicotine. Presumably, they recover from desensitization within a matter of minutes. In summary, we believe that acid trapping does cause massive desensitization of receptors in the exocytic pathway; it does not strongly affect pharmacological chaperoning within the ER; and it does not strongly affect the function of surface receptors.

The positron emission tomography (PET) ligand, 2-[F^{18}] fluoro-A-85380, binds well to $\alpha4\beta2$* and to α6* nAChRs. A-85380 is nearly as membrane-permeant as nicotine itself (Table 1) and may accumulate, like nicotine itself, in acidic organelles. Once in organelles, A-85380 would compete with nicotine at nAChR binding sites that face the lumen of these organelles. Therefore, PET studies that interpret A-85380 binding may report receptor saturation by nicotine in intracellular as well as surface membrane compartments. Reports that surprisingly low concentrations of nicotine saturate $\alpha4\beta2$ receptors, and for surprisingly long periods, may partially measure the effects of much higher nicotine concentrations at receptors in such organelles (80,81).

SePhaChARNS is a Special Case of Proteostasis

A chemical chaperone is a special case of a more general class of molecules called "proteostasis regulators". Proteostasis pathways in various cells act on newly translated proteins that may be unfolded or partially folded. Chaperoning and enzyme-assisted folding produces correctly functioning proteins, and small-molecule chaperones may stabilize this process. On the other hand, misfolding and aggregation lead to biological degradation. Chemical chaperones, chaperone proteins, and cochaperone proteins help their substrate proteins to traverse local energy barriers so that they find the most stable, fully folded states. Thus, in one view, stabilization of high-affinity nicotinic receptors by chronic nicotine is a thermodynamic necessity (48). Signaling pathways control the

transcription and activity of chaperone proteins. It is a key concept that proteostasis pathways vary among cell types, in accord with the "selective" aspect of SePhaChARNS (82).

There are many proposals, and some early successes, in using small chemical chaperones to favor the correctly folded state of crucial proteins. In the ion channel field, one relevant example is the use of small-molecule proteostasis regulators to stabilize the misfolding-prone ΔF508 mutant of the cystic fibrosis transmembrane conductance regulator (83).

In the context of small-molecule proteostasis regulators, nicotine is unusual in two respects. First, although most proteostasis diseases arise from insufficient chaperoning, nicotine addiction would be a disease of excess chaperoning. On the other hand, the inadvertent therapeutic effects of nicotine on Parkinson's disease exemplify beneficial chaperoning, which is a common concept for neurodegenerative diseases like Parkinson's but perhaps more rare for epilepsies like ADNFLE. Second, we hypothesize that nicotine not only stabilizes correct folding, but also the assembled, multimeric state of the nAChR protein. These concepts' special relevance to nAChR is summarized by Wiseman *et al.*'s explanation that large, slow-folding, multimembrane-spanning proteins are especially sensitive to the kinetic constraints imposed by proteostasis machinery (84).

ALTERNATIVE MECHANISMS FOR UPREGULATION

Phosphorylation, Sorting, Endocytosis, and Exocytosis

SePhaChARNS differs in at least two ways from mechanisms that apparently regulate opioid receptor number in opioid tolerance and dependence (85), and that regulate AMPA receptor number in long-term potentiation. First, SePhaChARNS does not explicitly depend on direct phosphorylation (86) or other covalent modification of the nAChR. However, it is quite likely that the covalent modifications during endoplasmic reticulum-associated degradation (ERAD) and ER exit do amplify the kinetically based effects of SePhaChARNS (48).

Second, SePhaChARNS does not explicitly include nicotine-evoked changes in endocytosis or exocytosis. Exocytosis is required for upregulation, presumably because exocytosis is downstream from SePhaChARNS (53). During prolonged exposure to nicotine, there is deceased receptor degradation (presumably via endocytosis), and this mechanism for upregulation must be considered as an adjunct to SePhaChARNS (8,39,66,87).

Signal Transduction Triggered by Ca^{2+} influx

Could nAChR upregulation be due to signal transduction mechanisms initiated by Ca^{2+} influx through the receptors? There are two strong counterarguments to the Ca^{2+} influx hypothesis. (a) The competitive antagonist DHβE also induces functional upregulation, but at higher concentrations consistent with the limited number of states available to an antagonist (48). (b) Human α4 harboring a Ser247Phe mutation (at the M2 6′ position) renders the channel nonfunctional in HEK tsA201 cells, and yet chronic nicotine induces robust upregulation of the receptor in this clonal cell line (39). Despite these arguments against signal transduction mechanisms evoked by Ca^{2+} influx through α4β2 nAChRs, Ca^{2+} influx is likely to play an important role in sequelae to activation of the α7 nAChR, which is highly Ca^{2+} permeable.

ORIGINS OF CELLULAR AND SUBCELLULAR SELECTIVITY

Selectivity Introduced by Accessory Subunits: α5 as an Example

Although many neuronal cell types express α4β2* receptors, cell types vary substantially in their repertoire of additional expressed subunits (88). Among the additional subunits: dopaminergic neurons express α6 and β3; layer VI cortical pyramidal cells express α5. Medial habenula expresses a wealth of additional subunits (89). Apparently, interneurons express the fewest additional subunits.

The SePhaChARNS mechanism depends strongly on the subunit repertoire because the subunit composition presumably controls the affinity for nicotine, the extent of interaction with the ERAD machinery, the extent to which desensitization makes many additional states available, and the extent and stability of additional stoichiometries. At present, we believe that α4β2 receptors, in the absence of additional subunits, may be more amenable to SePhaChARNS than other subtypes, for the constellation of reasons just given; but this impression may simply derive from our more complete knowledge of this subtype.

The α5 subunit, which co-assembles with α4β2, is of intense present interest. The first high-density genome-wide association and large-scale candidate gene study of nicotine dependence used a case-control sample of unrelated individuals to identify common genetic variants that contribute to the transition from cigarette smoking to the development of nicotine dependence (90,91). The most compelling evidence for risk variants contributing to smoking behavior was in the *CHRNA5/A3/B4* gene cluster, which encodes the α5, α3, and β4 subunits. There were several independent associations. The most thoroughly investigated and replicated finding to date is the association with rs16969968, in *CHRNA5* (91). This association has been replicated either directly or using highly correlated SNPs by several groups (92–97). This SNP results in Asp398Asn substitution in the α5 subunit, in the M3-M4 cytoplasmic loop.

A second signal in the *CHRNA5/A3/B4* cluster is tagged by SNP rs578776; it is strongly associated with nicotine dependence and has a low correlation with rs16969968 (r^2= 0.18), indicating that it represents a statistically independent signal (90,91). This second association with nicotine dependence has been replicated in additional studies using the same SNP or a highly correlated SNP (92,95,96,98). Expression studies have demonstrated that *CHRNA5* mRNA levels show high interindividual variability in both frontal cortex and lymphocytes (99). More than 40% of this variability is explained by *cis*-acting variation within the *CHRNA5* locus. Unpublished haplotype data suggest that the nicotine dependence association tagged by rs578776 may reflect these differences in *CHRNA5* mRNA levels (A. M. Goate, J. Wang, L. Bierut, unpublished data).

The replicated nicotine dependence loci tagged by rs16969968 (96,100–102) and rs578776 (101,103) have also been reported to influence lung cancer risk in several genome-wide association studies. This is an exciting convergence of findings for nicotine dependence risk loci and lung cancer risk loci. The lung cancer reports differed in their interpretation of the association with lung cancer: some indicate that the association could be explained through an indirect effect on risk for smoking (96), while others report an increased risk of lung cancer even in nonsmokers, suggesting the possibility of a direct effect on lung cancer vulnerability (101,104).

The α5 subunit is expressed widely. In rodent brain which has been extensively studied, it is found in some, but not all, α4β2* receptors in cortex, thalamus, striatum, hippocampus, medial habenula, olfactory tubercle, hypothalamus, and midbrain (dopaminergic neurons only) (105,106). In peripheral ganglia such as the superior cervical ganglion, it is probably found exclusively in some, but not all, α3β4* receptors (107). Bronchial epithelium, squamous cell lung carcinoma, non-small-cell lung cancers, and BEP2D cells express α5 subunits (108–110); but their partner subunits and functional states are not known. Knockout mice for the α5 subunit display a reduction in the somatic but not affective signs of nicotine withdrawal, probably arising from autonomic ganglia and other peripheral α3* nAChRs (14). Other studies on α5 knockout mice show changes in both presynaptic and somatodendritic nAChR responses (111).

Recent data indicate that the α5 subunit modifies upregulation of α4β2* receptors (112) and Lindstrom speculates that the effect proceeds at the level of chaperoning (113). It is possible that both of the major effects on nicotine dependence in the *CHRNA5/A3/B4* region are effects on the α5 subunit. Low levels of normal α5 are apparently associated with lower risk for nicotine dependence than higher levels. The rs16969968, coding-region mutation, in the M3–M4 cytoplasmic loop, could interact with ERAD machinery to alter chaperoning. The noncoding mutations, such as rs578776, could affect the level of expression of the α5 protein, therefore affect the proportion of α4β2* receptors that are α4β2α5* receptors, and thus also affect chaperoning. Thus, this accessory subunit may partially govern the cellular or subcellular selectivity in the SePhaChARNS hypothesis.

Selectivity Introduced by Other Proteins: Lynx as an Example

nAChRs behave in a distinctly different way when complexed with lynx proteins. Lynx prototoxin molecules are expressed in brain areas heavily involved in nicotinic function: cerebral cortex, hippocampus, VTA, and amygdala. Lynx proteins bind with some selectivity to nAChR subtypes; for instance, lynx1 binds to α4β2, α7, and α4β4 nAChRs and not to the glutamate receptor δ2. In the presence of lynx1, α4β2 nAChRs are up to 20-fold less sensitive to ACh in a *Xenopus* oocyte expression system, desensitize more rapidly, and recover more slowly from desensitization (114). The dose–response relations of nAChR-lynx complexes agree better with *in vivo* agonist responses than with nAChRs expressed alone *in vitro* (115). Indeed, lynx1 KO mice display decreased ACh EC_{50} values, closer to EC_{50} values of the receptor expressed in oocytes alone (116). These data indicate that lynx molecules can account for much of the differences between *in vivo* and *in vitro* nAChR functioning. Furthermore, they suggest that lynx proteins oppose some of the effects of chronic nicotine exposure, and have a desensitizing influence on nAChRs.

The importance of lynx modulators in supporting proper nAChR function is further supported by experiments conducted on mice deficient in the lynx1 gene. Lynx1KO mice demonstrate cognitive enhancements and neurological changes (116), features associated with hyperactive nAChRs, including improved learning, increased intracellular Ca^{2+} in response to nicotine treatment, less receptor desensitization, and heightened nicotine sensitivity. Aging lynx1 KO mice undergo a striatal degeneration which is exacerbated by nicotine and ameliorated by crossing lynx1KO mice with those lacking α7 nAChRs, indicating that both α7 and α4β2-type nAChRs may be important targets of lynx1 genes (116). We conclude that lynx1 modulates nAChR function *in vivo*. We also conclude that lynx1 plays a critical role in maintaining a balance between the beneficial effects of short-term nAChR activation (117) and the potentially devastating degenerative excitotoxic effects of chronic overactivation of nAChRs (118,119).

Other nAChR modulators in the lynx gene superfamily are linked to human disease outside the CNS. For example, SLURP-1 is mutated in Mal de Meleda (120). SLURP1 differs from lynx1 in that SLURP1 is expressed in the periphery, not in neurons, and is not GPI anchored, but is secreted out of the cell (121,122). Nicotine stimulates proliferation of lymphocytes and keratinocytes via nAChRs. SLURPs enhance this process (123) as well as oral and lung cancer cell proliferation (124). In lung, lynx1 is co-expressed with nAChRs (125).

Because lynx has structural homology to the snake venom neurotoxins such as α-bungarotoxin, several investigators suggest that lynx binds to a homologous site on nAChRs: the agonist-binding site at an intersubunit interface. Lynx, a glycophosphoinositide-linked (GPI-linked) protein, is topologically well placed to bind in such fashion. A modest and obvious extension of this idea is that lynx may bind to non-agonist interfaces, such as the α4–α4 interface in $(\alpha4)_3(\beta2)_2$ receptors, or the allosterically important α4–α5 interface if the complex includes an α5 subunit (113,126). Another modest extension of the interfacial binding idea engages with contemporary research on the delicate proteostasis during assembly of multisubunit membrane proteins, especially in the endoplasmic reticulum (see next section). In other words, we think it is possible that a key lynx–nAChR interaction occurs during receptor biosynthesis and maturation, in addition to acute nicotine–receptor interactions at the surface membrane.

Conclusions: Implications for Drug Discovery

The most important implication of the SePhaChARNS hypothesis is that the medically relevant manipulations of α4β2* nAChRs take place in the ER, not at the surface membrane. Thus, one may best discover drugs that affect SePhaChARNS by studying events in the ER. This review shows that nicotinic drugs are expected to be effective pharmacological chaperones by acting in the ER. It is also possible that SePhaChARNS could be manipulated by drugs that modify protein chaperones. Fluorescence-based analyses

of nAChR assembly, trafficking, and stoichiometry are under way in the Caltech lab (73,127–129). In principle, such analyses may be suitable for drug discovery relevant to the SePhaChARNS process.

ACKNOWLEDGMENTS

Relevant work in the authors' laboratories is supported by grants from NINDS (NS11756, NS34407), NIDA (DA17279, DA19375), NCI (CA089392), NIA (AG033954), NIAAA (AA08401), the Michael J. Fox Foundation, the California Tobacco-Related Disease Research Program, the Croll Autism Research Foundation (1004564-01-CEN5300443), and Targacept, Inc. Postdoctoral fellowships were awarded to C. S. from Philip Morris USA/International, to X. C. from the California Tobacco-Related Disease Research Program, and to R. P. from the Ford Foundation and APA-DPN.

Conflict of Interest Statement Drs. Goate and Wang are listed as inventors on patent application US 2007/0258898 held by Perlegen Sciences Inc., covering the use of certain SNPs, including rs16969968 in diagnosing, prognosing, and treating addiction. Dr. Miwa acts as a consultant to Ophidion, Inc.

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