


COMMENTARY

Inhale, exhale: Probing the inside-out mechanism of nicotine addiction using novel fluorescent sensors

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Scientific discoveries that impact human health motivate many of us and also provide a rationale for public support. Nicotine use disorder (NUD) remains a significant public health burden affecting over 20% of adults in the United States and over 30% of those with other mental health disorders (Chou et al., 2016). Tobacco use is the primary component of NUD and is the leading cause of preventable morbidity and mortality in the United States, being responsible for approximately one in five of such deaths (more than 480,000 deaths annually; Camenga and Klein, 2016; Centers for Disease Control and Prevention, 2019). In spite of intensive government public information campaigns that have highlighted the health risks associated with nicotine use over the past five decades, a fraction of the population continues to suffer NUD. Moreover, the emergence of alternative nicotine delivery products, for example electronic nicotine delivery systems like vaporizers or electronic cigarettes, provide fresh challenges to reducing NUD (U.S. Department of Health and Human Services, 2014).

Why does NUD persist in the face of widespread distribution of overwhelming evidence of its dangers? Clearly, the addictive properties of nicotine are a primary obstacle to reducing its use (Davis et al., 1988). An understanding of the molecular mechanism of nicotine dependence could form the basis for developing new and improved NUD therapeutics. The immediate effects of nicotine administration that users seek are mediated by a variety of nicotinic acetylcholine receptor (nAChR) subunit combinations in the plasma membrane of neurons involved in reward circuitry (Koob and Volkow, 2016). In addition, nicotine can act on nicotinic receptors positioned at intracellular locations, which affect cell function by an “inside-out” mechanism. The Lester laboratory at Caltech and their collaborators at Janelia Farm have worked to unravel the components of inside-out nicotinic receptor biology and its role in disease mechanisms including Parkinson’s disease and NUD. In this issue of the *Journal of General Physiology*, Shivange et al. describe the development and characterization of a novel optical nicotine sensor

iNicSnFR, which also detects varenicline (Chantix)—a drug that acts on nicotinic receptors and is used as a smoking cessation aid. iNicSnFR affords a means to answer key questions concerning the levels and timing of nicotine and drug exposure at intracellular and extracellular sites, as well as providing a basis for mechanistic investigations of NUD and a possible path to improved therapeutics.

nAChRs and inside-out pharmacology

nAChRs are among the most intensively studied ion channel receptors due to their important roles in neuromuscular transmission and signaling in both the central and peripheral nervous system. nAChR signaling has been most thoroughly studied at the neuromuscular junction (Sine, 2012), which has in turn led to much of the broader mechanistic understanding of synaptic transmission (e.g., Katz, 1999). The earliest concept of a receptor molecule originated, in part, from studies of effects of nicotine and curare on this synapse (Langley, 1914). The following century or more of work has led to an impressive accumulation of mechanistic understanding, which has largely been driven by preceding technical advances including intracellular recordings (Fatt and Katz, 1951), electron microscopy (De Robertis and Bennett, 1954; Palade and Palay, 1954), voltage clamp recording (Magleby and Stevens, 1972), fluctuation analysis (Katz and Miledi, 1972; Anderson and Stevens, 1973), single channel recording (Neher and Sakmann, 1976) and cryo-EM (Walsh et al., 2018).

Nicotine interacts with nAChRs via two different mechanisms (Fig. 1). In the canonical “outside-in” pathway, nicotine binds to cell surface nAChRs and rapidly elicits channel opening, membrane depolarization, and intracellular calcium signaling. Nicotine can also lead to nAChR desensitization, as, unlike acetylcholine, it is not degraded in milliseconds by extracellular acetylcholinesterase enzymes. This outside-in pathway mediates the acute effects of nicotine in the central nervous system and also suppression of nicotine withdrawal symptoms (Miwa

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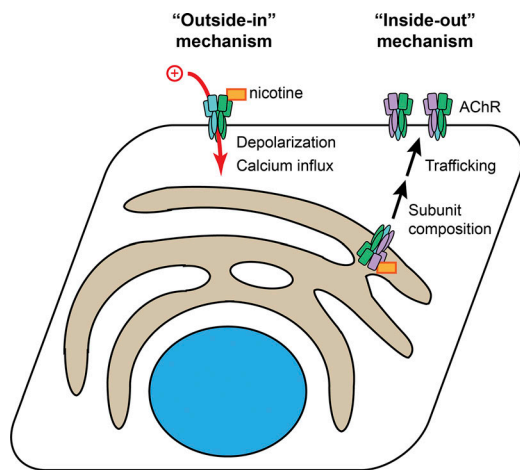


Figure 1. **Outside-in and inside-out mechanisms of nicotine signaling.** In the outside-in mechanism, nicotine binds directly to nAChR channels in the plasma membrane, causing membrane depolarization, calcium influx, and eventual channel desensitization. In the inside-out mechanism, nicotine travels inside the cell and stabilizes particular nAChR subunit combinations in the ER. This leads to increased channel trafficking to the plasma membrane and a modified distribution of subunits, which modifies endogenous acetylcholine signaling.

et al., 2011). In contrast, the inside-out pathway of nicotine signaling results from the membrane permeability of nicotine, which, unlike acetylcholine, can pass through cell membranes in its neutral form and bind to intracellular nAChRs. Although the consequences of inside-out signaling by nicotine are not fully elucidated, nicotine binding to nAChRs in the endoplasmic reticulum (ER) is known to stabilize the interface between $\alpha 4$ and $\beta 2$ nAChR subunits, thereby chaperoning channels to the plasma membrane to increase surface expression and modify subunit composition (Lester et al., 2009). This likely leads to sustained enhancement of outside-in signaling for both acetylcholine and nicotine. The paper by Shivange et al. (2019) provides the first direct measurements of the time course and concentration of nicotine in the ER following its transient extracellular application.

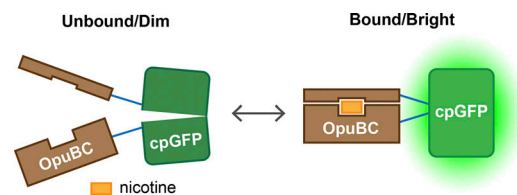


Figure 2. **iNicSnFR fluorescent nicotine sensor mechanism.** The engineered nicotine binding protein OpuBC is fused to a circularly permuted GFP protein. In the absence of nicotine, the GFP chromophore is perturbed and sensor fluorescence is dim. Upon nicotine binding, the GFP conformation is restored and the sensor becomes bright.

Intensity-based nicotine-sensing fluorescent reporters (iNicSnFRs): A fluorescent nicotine sensor

Determining the relative importance of outside-in and inside-out nicotine signaling was previously impossible because the nicotine concentration inside the ER was unknown. To address this knowledge gap, Shivange et al. (2019) developed a novel set of engineered protein-based fluorescent sensors called iNicSnFRs. Because the protein-based sensors can be targeted to different subcellular locations, they can provide detailed information about nicotine concentration and dynamics in different organelles.

The fluorescent sensor design followed that of the glutamate sensor iGluSnFR (Marvin et al., 2013). Pieces of a nicotine-binding protein are fused with short peptide linkers to a circularly permuted GFP (cpGFP; Fig. 2). The N and C terminals of the cpGFP are fused together, and the protein is instead cut in the middle of the β -barrel that protects the GFP fluorophore. If these new N- and C-ends are pulled apart (no nicotine bound), the fluorophore is perturbed and goes dark. When nicotine binds, however, the native GFP conformation is restored, and the GFP becomes bright. The nicotine binding protein was derived from OpuBC, a choline/betaine periplasmic binding protein from *T. spX513*. Nicotine binding and iNicSnFR sensitivity was engineered into the sensor by directed evolution: ~12,000 mutants were tested over multiple iterations, with a focus on the

Table 1. **Summary of inside-out and outside-in signaling during smoking**

	Outside-in: Activated nAChRs in the plasma membrane	Inside-out: Chaperoned nAChRs in the ER
Nominal cellular effects	Na ⁺ and Ca ²⁺ influx, increased neuronal excitability, calcium signaling, nAChR desensitization	Stabilization of $\alpha 4\beta 2$ subunit combinations, increased nAChR in the plasma membrane, biased subunit composition
EC ₅₀ for effect	0.3 – 4 μ M ^a (Buisson et al., 1996; Gopalakrishnan et al., 1997; Pacheco et al., 2001; Kuryatov et al., 2005)	30 nM (Kuryatov et al., 2005)
Fraction average activation for a typical smoking habit ^b	0.1	0.7
Hypothesized organismal effects	Sense of well-being, cognitive boost, appetite suppression, increased tolerance of stressful stimuli, suppression of withdrawal (Miwa et al., 2011; Naudé et al., 2015; Nees, 2015; Picciotto et al., 2015)	Early events of nicotine dependence (Govind et al., 2009; Henderson and Lester, 2015)
Timescale of organismal effects	Minutes	Days to weeks

^aDependent on subunit composition and assay.

^b1 cigarette/hour for 12 h leads to ~100 nM sustained nicotine concentration.

linkers and residues in the binding pocket. The final version, iNicSnFR3a, was equipped with an epitope tag for purification and immunostaining. The resulting half maximal effective concentration (EC_{50}) for nicotine was 19 μ M with subsecond on and off rates at physiologically relevant nicotine concentrations (Shivange et al., 2019). iNicSnFR3a can therefore detect 1 μ M nicotine, which is a typical value found in the blood and cerebrospinal fluid during smoking (Benowitz et al., 1991).

The authors targeted expression of the sensor to two different cellular compartments, the ER and plasma membrane, in order to enable direct measurement of nicotine in each. Imaging and colabeling of the ER with DsRed2-ER revealed that the sensors (iNicSnFR3a_ER and iNicSnFR3a_PM) show the expected localization patterns. Functional validation was performed using nicotine pulse-chase experiments. Robust fluorescence changes were detected at concentrations as low as 250 nM nicotine in both cellular regions. Further validation of the compartmentalization of the sensors was provided using membrane impermeant ligands: iNicSnFR3a_ER did not detect membrane impermeable molecules N'MeNic and ACh but did detect nicotine, whereas iNicSnFR3a_PM detected all ligands. Observations for HEK293, SH-SY5Y, and human iPS cell-derived dopaminergic neurons tracked observations in HeLa cells, showing the generality of the approach. One caveat of the sensors is their strong pH sensitivity, which constrains their utility in acidic organelles.

Findings in the paper

The primary finding of the publication is that the nicotine concentration in the ER rapidly (<10 s) equilibrates with the extracellular nicotine concentration (within twofold). Thus, the outside-in and inside-out mechanisms experience approximately equal nicotine exposure throughout the day. Because the inside-out mechanism (EC_{50} ~30 nM) is activated at much lower concentrations than the outside-in mechanism (EC_{50} 0.3–4 μ M), it is plausible that the inside-out mechanism dominates in some conditions. The authors used simulations of nicotine pharmacokinetics/pharmacodynamics to show that the concentration of nicotine in the blood/cerebrospinal fluid, and thus also the ER, is greater than the EC_{50} for chaperoning during the 1-h period between cigarettes for a typical smoker. In other words, the residual nicotine that remains in the body between cigarettes, the rapid entry of nicotine to the ER, and the achieved nicotine concentrations at the ER lend themselves to the inside-out mechanism of up-regulation of nAChRs as a potential culprit for addictive behavior. Table 1 summarizes the outside-in and inside-out mechanisms in light of these new data.

The authors offer some preliminary insights into the mechanism of action of varenicline (Chantix), the prescription medication used as a smoking cessation agent. iNicSnFR3a is, fortuitously, also sensitive to varenicline concentration. Much like nicotine, varenicline also rapidly equilibrates in the ER and could therefore activate the inside-out molecular chaperoning, which could be responsible for the efficacy and/or side effects of the drug.

Conclusions

The authors have built a powerful new tool for studying nicotine dynamics in cells, which has enabled them to strengthen the

case for the inside-out mechanism as a critical factor in nicotine addiction. In addition, the authors demonstrate how to develop a fluorescent sensor for a drug through protein engineering, which could help elucidate key mechanisms underlying addiction or therapeutic function.

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