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Multidimensional Intersection of Nicotine, Gene Expression and Behavior

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ABSTRACT

The cholinergic system plays a crucial role in nervous system function with important effects on developmental processes, cognition, attention, motivation, reward, learning and memory. Nicotine, the reinforcing component of tobacco and e-cigarettes, directly acts on the cholinergic system by targeting nicotinic acetylcholine receptors (nAChRs) in the brain. Activation of nAChRs leads to a multitude of immediate and long-lasting effects in specific cellular populations, thereby affecting the addictive properties of the drug. In addition to the direct actions of nicotine in binding to and opening nAChRs, the subsequent activation of circuits and downstream signaling cascades leads to a wide range of changes in gene expression, which can subsequently alter further behavioral expression. In this review, we provide an overview of the actions of nicotine that lead to changes in gene expression and further highlight evidence supporting how these changes can often be bidirectional, thereby inducing subsequent changes in behaviors associated with further drug intake.
The cholinergic system exerts widespread actions in multiple brain regions to regulate developmental processes, cognition, attention, motivation, reward, sleep, learning and memory [1; 2; 3; 4; 5; 6]. Deficits in cholinergic signaling are found to result in a broad range of negative impacts on cognitive processes, such as that found with Alzheimer’s disease, Parkinson’s disease, and other cognitive disorders [6; 7]. In the central nervous system, the endogenous neurotransmitter, acetylcholine, is released from axon terminals in all main subdivisions of the brain [2; 8]. Acetylcholine acts on two main receptor subclasses, nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs). The downstream effects of receptor activation not only modulate immediate behavioral and cognitive effects, but also lead to downstream effects on gene expression.

Nicotine is an alkaloid derived from the tobacco plant and when consumed, acts as a full agonist on the nAChRs [9; 10]. Tobacco cigarettes and e-cigarettes contain high levels of nicotine, which infer a high addiction liability through actions on nAChRs in the reward-related systems in the brain. Activation of nAChRs by nicotine leads to both similar and different actions as that found with acetylcholine, depending on the method of administration, dose, and frequency of exposure [2; 10; 11; 12; 13]. Moreover, in addition to changes in the brain, it should also be noted that long-term effects of nicotine are found in other organ systems, such as the lungs [14; 15; 16; 17; 18; 19], although such peripheral actions of nicotine will not be reviewed further herein. In this review, we provide an overview of nicotine’s brain region and cell type specific actions on gene expression, and we further explore the bidirectional relationship between nicotine consumption and changes in gene expression.

**Nicotine’s Actions on Cholinergic Signaling Mechanisms**

After release from the presynaptic terminal, acetylcholine binds to either mAChRs or nAChRs, depending on the receptor localization across cell types in the brain. Both receptors are involved in the autonomic nervous system, nociception, cognition, and learning and memory processes [9;
The nAChRs are ionotropic, allowing for the influx of Na\(^+\) and Ca\(^+\) and efflux of K\(^+\) during the receptor’s open conformational state following ligand binding [23; 24]. In contrast, activation of the metabotropic mAChRs leads to G-protein mediated second-messenger signaling cascades [25]. The mAChR exhibits five different subtypes (M1-M5) [26]. M2 and M4 subtypes couple with Gi/Go proteins, whereas M1, M3, and M5 subtypes couple with Gq proteins [27]. While nicotine acts directly on the nAChRs, but not the mAChRs, it appears that both receptor classes may modulate processes underlying drug addiction. For instance, a recent human GWAS study found that allelic variation in the M2 gene, CHRM2, is associated with nicotine dependence in women [28]. Interestingly, activation of M2 mAChRs mediates aspects of pain perception, which has been shown to be dysregulated in individuals with nicotine dependence [29; 30]. These findings suggest a possible indirect link between nicotine, pain regulation and mAChR signaling. Alternatively, nicotine-mediated changes in cholinergic levels (e.g., via nAChR localization on presynaptic terminals that modulate acetylcholine release) could impact endogenous volume transmission of acetylcholine within discrete brain areas, leading to altered activity at dendritic mAChRs [31].

**Nicotinic Acetylcholine Receptors**

In the brain, nAChRs are expressed on both neuronal and non-neuronal cells. These receptors are membrane bound proteins from the Cys-loop family of the pentameric ligand-gated ion channels superfamily, which includes the serotonin 5-hydroxytryptamine type 3 receptor, L-aminobutyric acid type A receptor, and glycine receptor [32]. As noted above, neuronal nAChRs can be expressed at either postsynaptic membranes or presynaptic terminals, and thus, nAChR activation can exert broad actions on multiple systems via modulation of neurotransmitter release from the synaptic terminal [32; 33; 34]. Heteromeric nAChRs are composed of a combination of \(\alpha\) and \(\beta\) subunits, which can include \(\alpha_2-\alpha_7\) and \(\beta_2-\beta_4\), and \(\alpha_7\) nAChR subunits can also combine to form a homomeric receptor [33]. The different subunits co-assemble to generate a wide variety of receptor subtypes with diverse patterns of expression across cell populations, and the specific
combinations of α and β subunits can result in differences in ligand binding, Ca\(^{2+}\) permeability and desensitization kinetics [35; 36; 37; 38; 39]. For example, in the ventral tegmental area (VTA), a variety of different nAChR subtypes have been proposed, which includes α4α5(β2)₂, (α4)₂(β2)₃, (α4)₂(β2)₃, and α7 [40]. Moreover, based on the specific localization within brain circuits, the resulting effects of nAChR activation may lead to differing effects on behavior, which can be evidenced with nicotine self-administration; providing access across a range of doses leads to an inverted U-shaped dose response curve that is indicative of both the reinforcing and aversive actions of the drug at the different doses [41; 42]. Specifically, when nicotine doses increase from low to moderate, animals increase their responding to obtain more drug administrations, but in contrast, when doses increase from moderate to high, a decrease in responding is evidenced that is indicative of both aversion and satiation. Interestingly, the aversive properties of nicotine at higher doses are also evidenced by an increase in brain reward thresholds above baseline levels, as assessed with intracranial self-stimulation [42; 43; 44]. The reinforcing and aversive properties of nicotine have been shown to involve opposing brain pathways, which are discussed in the following sections.

**Nicotine’s Actions on Brain Circuits Underlying Addiction**

The reinforcing effects of nicotine are mediated by activation of the VTA, leading to increased dopaminergic signaling in the nucleus accumbens (NAcc) [45; 46; 47; 48]. The α4 and β2 nAChR subunits are expressed on the majority of dopaminergic and GABAergic neurons in the VTA and have been shown to modulate nicotine-evoked currents and reinforcing properties of the drug [49; 50; 51]. For instance, knockout of α4, α6 or β2 nAChR subunits in mice results in a failure to self-administer nicotine, which can be rescued by re-expression of these nAChR subunits in the VTA [50; 52]. Given that the α4, α6 and β2 nAChRs can combine together to form a functional nAChR subtype, it has been postulated that nicotine’s actions on the α4α6β2 nAChRs in the VTA-NAcc circuit control the reinforcing properties of the drug [46; 50; 52; 53]. In contrast, the α7 nAChR subtype appears to modulate the fine tuning of nicotine-mediated dopamine response with lower
levels of expression on VTA neurons, as well as via localization on presynaptic glutamatergic terminals in the VTA [54; 55; 56]. Neurons in the VTA receive input from brain regions including the prefrontal cortex (PFC), amygdala, bed nucleus of the stria terminalis, tegmental nuclei, and local GABAergic interneurons [57; 58], indicating multiple levels of modulation for integrative processing with the putative localization of nAChRs on axon terminals from some of these circuits. Cholinergic interneurons in the NAcc also play an important role in nicotine seeking behavior [59]. Of note, chronic administration of nicotine enhances the functional connectivity between the cortex and NAcc during cue presentation with reward learning and has also been shown to increase NMDA signaling in the PFC-NAcc circuit [60; 61], thereby providing evidence of behavioral and circuit level changes in reward related processing with nicotine use.

In opposition to the mesolimbic behavioral effects, the medial habenulo-interpeduncular (MHb-IPN) pathway plays an integral role in the aversive actions of nicotine and symptomology associated with nicotine withdrawal [42; 62; 63; 64; 65; 66; 67; 68]. This pathway densely expresses several nicotinic subunits, including α3, α4, α5, β2 and β4 [67; 69; 70; 71; 72; 73]. Of significance, in humans, polymorphisms of the CHRNA5-CHRNA3-CHRN4 gene cluster encoding for the α3, β4, and α5 nAChR subunits has been repeatedly associated with nicotine dependence [75; 76; 77; 78]. These nAChR subunits in the MHb-IPN pathway appear to modulate the aversive properties of nicotine, particularly at higher doses. Both constitutive knockout of the α5 nAChR subunit in mice and MHb-specific knockdown of the α5 nAChR gene in the rat leads to an increase in nicotine self-administration at higher doses [42; 43]. In the knockout mice, α5 nAChR subunit re-expression in the MHb was sufficient to restore the behavioral phenotype, with no significant difference found in nicotine self-administration behavior compared to wildtype controls [42]. In contrast, mice with overexpression of the β4 nAChR gene exhibit a strong aversion to nicotine [62]. Finally, knockdown of the α3 nAChR subunit in the MHb or IPN increases nicotine intake at higher doses, a behavioral effect also found with infusion of the α3β4 antagonist, a-conotoxin AuIB, into the IPN [79]. Interestingly, the α3β4-containing nAChR has further been
implicated in the physical signs of nicotine withdrawal [80], which may involve altered GABAergic signaling in the IPN [81]. Finally, inhibition of these IPN GABAergic projections to the laterodorsal tegmentum has been shown to attenuate nicotine conditioned place aversion [82]. Therefore, given the multiple actions of nicotine on varying brain circuits, the downstream effects on gene expression can lead to a persistent impact on processes underlying nicotine use and abuse.

**Nicotinic Signaling and Gene Expression Along the Drug Use Trajectory**

Long-term changes in gene expression are thought to lead to behavioral habits and underlying processes that characterize the state of drug dependence, which can occur following consumption of nicotine-containing products including tobacco cigarettes and e-cigarettes/vapes (Figure 1). While the effects of nicotine on gene expression may be directly attributed to activation of nAChRs on the postsynaptic membrane, it is important to recognize that nAChRs can act as heteroreceptors to regulate the release of many neurotransmitters, in addition to acetylcholine, from the presynaptic terminal. Thus, nicotine’s effects on gene expression may be both direct on the nAChR-expressing cell, as well as indirect with involvement of multiple neurotransmitter systems. In the following paragraphs, we discuss evidence supporting an interaction among nicotinic signaling, gene expression and behavior, which may underlie various facets of drug use and abuse. These findings are further summarized in Table 1.

**Acute Nicotine Exposure**

Experimenter-administered nicotine allows for precise dosing when one is interested in examining acute drug effects across experimental conditions, thereby elucidating findings particularly relevant to initial drug use. Acute nicotine exposure has been shown to increase the mRNA and protein expression of c-fos, an immediate early gene and marker of cellular activation, in various regions throughout the brain. For instance, nicotine-mediated increases in c-fos mRNA and/or protein expression have been documented in the VTA, MHb and IPN, particularly at higher doses [42; 83; 84; 85; 86]. Since the VTA, MHb, and IPN have important roles in nicotine reinforcement
and aversion, activation of these brain regions likely contributes to the drive to further consume the drug [42; 43; 49; 50; 51; 62]. These activational effects are generally attributed to the direct actions of nicotine on the nAChRs, since pretreatment with the general nAChR antagonist, mecamylamine, can prevent such c-fos expression [85]. Given the regulatory regions in the c-fos gene, it is likely that the increased Ca\(^{2+}\) influx induced with nAChR opening leads to activation of second messenger systems, such as MAP- and CAM-kinases, thereby inducing c-fos gene transcription [87].

In addition to reinforcement and aversion-related brain regions, nicotine-induced c-fos mRNA and/or protein expression has been evidenced in the hippocampus, substantia nigra, nucleus of the tractus solitarius, striatum, amygdala and cortex, all of which appear to play a role in different aspects of drug dependence [81; 85; 88; 89; 90]. Therefore, the extensive changes in c-fos gene expression provide insight into the broad actions of nicotine on neural signaling. Interestingly, repeated dosing appears to elicit nAChR desensitization in these actions, as a reduction in the expression of c-fos mRNA is found in hippocampus and cortex after a second nicotine dose [85]; desensitization of nAChRs has been proposed to contribute to conditioned drug reward through salience of environmental cues associated with nicotine consumption [40]. Moreover, while the c-fos protein has been shown to induce activation of multiple gene targets, one should note that: (1) expression may not change in cells exhibiting a net inhibitory state, (2) the protein may form a dimer with JunB to induce an inhibitory effect, and (3) the level of c-fos protein induced may not reach a sufficient threshold level to drive transcriptional activity of all the target genes within a cell [87; 91]. Furthermore, while examining the acute actions of nicotine may provide some initial insights, chronic exposure conditions provide arguably more translational relevance for the changes in gene expression that likely occur in human smokers, which could alter neural function to potentially propagate the dependence state across time.

**Chronic Nicotine Exposure**

Longer-term exposure to nicotine has been demonstrated to exert changes in gene expression
across cell types and brain regions. In recently abstinent smokers, increased β2-containing nAChRs are found in cortex and striatum [92]. Data derived from animal models provides further evidence of increased nicotine-mediated expression for a subset of nAChR subunits in a region-specific manner [92; 93; 94; 95; 96]. For instance, chronic nicotine exposure leads to upregulation of the α6 and β3 nAChR subunits in the substantia nigra pars compacta [97] and α7 nAChR subunits in the substantia nigra and VTA [98]. In addition, the effects on nAChR expression involves differences in the assembly of the subunits to generate functional receptors at the membrane. Specifically, the α4 and β2 nAChR subunits can combine together to form different isoforms with either two α4 subunits (α42β23) or three α4 subunits (α43β22). Chronic exposure to nicotine preferentially upregulates α42β23 nAChRs in the cortex, but not the thalamus [99], thereby demonstrating increased expression of the higher sensitivity α42β23 isoform following nicotine exposure in a region-specific manner. Additionally, the α4 and β2 subunits can also combine with the α5 subunit to form the α42β2α5 subtype, but the expression of this subtype does not change with nicotine exposure [100]. Further specificity is found for cell type specific patterns. Nicotine-mediated upregulation of α4-containing nAChRs occurs in the VTA and substantia nigra, but the increased expression is specific to GABAergic, not dopaminergic, neurons [94]. With regard to the MHb-IPN circuit, α5 nAChR subunit expressing neurons in the IPN upregulate expression of the Nos1 and Sst genes following repeated nicotine administration, leading to increased nitric oxide and somatostatin neurotransmitter release, respectively [101]. Since blocking nitric oxide signaling also reduces nicotine preference [101], this further demonstrates a potential feedback mechanism for bidirectional effects between nicotine seeking behavior and gene expression. Additionally, somatostatin has been shown to inhibit glutamate release in the IPN [81], suggesting that upregulation of somatostatin could further serve to inhibit activity of the aversion-related MHb-IPN circuit. Interestingly, sex differences have also been found in the IPN expression of acetylcholine and nAChRs following nicotine treatment. Specifically, females exhibit a greater increase in acetylcholine and α5 nAChR subunit mRNA levels, whereas males show an increase α7 and α2 nAChR subunit transcripts, in the IPN [102]. It is also important to note that changes
in nAChR subunit mRNA are not always associated with the level of protein expression at the membrane, and inversely, changes in nAChR membrane expression are not always a product of changes in nAChR gene expression. Indeed, membrane nAChR upregulation can be modulated by post-translational modifications or trafficking. For example, nicotine has been shown to upregulate receptors containing the $\alpha 4$ and $\beta 2$ nAChR subunits via several mechanisms, including phosphorylation and chaperoning in the endoplasmic reticulum, which could lead to increased receptor insertion at the cell surface [103; 104; 105; 106]. Since $\alpha 4$ and $\beta 2$ nAChR subunits in the VTA-NAcc circuit are necessary for nicotine reinforcement, the net effect of an upregulation in $\alpha 4\beta 2$-containing nAChRs could be enhanced reinforcing effects of the drug at various doses [49; 50; 51].

Next, chronic nicotine exposure may lead to the modulation of membrane nAChRs through effects on other genes expressed in the cell. In a recent study, an innovative proteomics approach was taken to immunoprecipitate protein-protein interactions of $\beta 2$-containing nAChRs and subsequently characterize the associated proteins [107]. It was found that chronic nicotine exposure increases expression of specific nAChR interacting proteins, including Na/K ATPases, syntaxins, SNAP25, and synaptotagmin, in the cortex of both mice and human smokers [107], thereby revealing the impact of nicotine on the expression of intracellular factors involved in nAChR regulation. In addition to nicotine, other constituents in tobacco and e-cigarette products may independently or synergistically act with nicotine to alter neuronal function. For instance, menthol is a common additive found both in tobacco cigarettes and e-cigarette vape solutions. When menthol and nicotine are co-administered, a significant upregulation of $\alpha 4\alpha 6\beta 2$ nAChRs is found in the VTA, substantia nigra, and hypothalamus, as compared to nicotine alone [108; 109], which is positively correlated with dopaminergic neuron excitability and increased drug reward [108]. Therefore, a bidirectional relationship is present between behavior and gene expression, in which drug consumption causes upregulation of nAChRs, which can subsequently affect neural responsiveness in brain regions that mediate aversion or reinforcement signaling that regulate
later drug intake.

A variety of other genes have also been identified to be differentially regulated by nicotine exposure. In the VTA, increased mRNA expression of the delta opioid receptor and GluA1 AMPA receptor are found with nicotine [110; 111]; both of these receptors have been implicated in nicotine self-administration behavior [42; 112; 113]. Further, decreased density of the metabotropic glutamate receptor 5 (mGluR5) has been documented in human smokers [114]. These findings were confirmed in rats following 250 days of nicotine exposure, in which decreased mGluR5 was found in the striatum, hippocampus, thalamus and midbrain, and these changes in density were also associated with decreased exploratory behavior [115]. Abstinence following nicotine exposure restored mGluR5 expression [115]. However, another study examining nicotine-mediated changes in mGluR5 transcripts resulted in discrepant findings [116]. Repeated nicotine injections have also been shown to induce expression of CREB in the NAcc, which appears to be essential for Pavlovian conditioning to nicotine associated cues [117]. Finally, nicotine can alter the expression of miRNAs in the brain, which may lead to subsequent effects on protein expression for multiple target genes. For instance, our recent study found that nicotine self-administration leads to an increase in mir204 and transthyretin in the choroid plexus of rats, both of which are released into the cerebrospinal fluid as circulating signaling factors [118; 119; 120]. Of note, both transthyretin and mir-204 have been implicated in regulating cell survival and may be involved in pathological states, such as Alzheimer’s disease and/or Schizophrenia [119; 121; 122; 123; 124]. Moreover, in females, but not males, nicotine self-administration was also found to induce an upregulation of mir199a and mir214 in the PFC, leading to a downregulation in protein expression of the target mRNA SIRT1 [125]. Therefore, nicotine appears to induce actions on multiple genes across brain regions in a sex-dependent manner.

*Nicotine Withdrawal and Relapse*
Nicotine withdrawal is characterized by several adverse symptoms in both human and rodent models, including increased anxiety and cognitive deficits [126; 127]. Differential gene expression occurring during the withdrawal state may mediate symptomology. As noted above, the MHb-IPN circuit has been implicated in nicotine withdrawal. In the IPN, nicotine abstinence is associated with increased gene expression of the α4, α5 and β2 nAChR subunits [102] and decreased expression of the Pfn2 gene [128]. Interestingly, knockdown of Pfn2 in the IPN, but not in the VTA, results in an increased anxiety-related phenotype [128], supporting a role in withdrawal-induced anxiety. The hippocampus has also been implicated in regulating anxiety-associated behaviors. During nicotine withdrawal, an increase in neuregulin 3 appears to mediate the synaptic plasticity that underlies anxiety-associated symptomology [129; 130]. Further, in the ventral hippocampus, the transcription factor CREB contributes to anxiety-associated behaviors during the withdrawal state [131]. Abstinence from drugs of abuse also activate stress-related signaling that involves corticotropin releasing factor (CRF) and the associated CRF₁ and CRF₂ receptors [132]. Following chronic nicotine, CRF levels are upregulated in the VTA, striatum, PFC, and hippocampus [133; 134; 135], but downregulated in the paraventricular nucleus [136]. Further, CRF₁ and CRF₂ receptors are found to be increased in the hippocampus [135], and hippocampal CRF signaling has been implicated in anxiety-associated behaviors [137]. Taken together, these findings demonstrate that a variety of gene expression changes during the withdrawal state may contribute to an anxiety- and/or stress-related behavioral effects.

Deficits in cognitive function are also found with nicotine withdrawal. For instance, mice treated chronically with both moderate and high doses of nicotine exhibit impaired cognitive flexibility during a set-shifting task, and these deficits were associated with increased Bdnf gene expression in the medial PFC but decreased Bdnf in the dorsal striatum [138]. Increased expression of nAChRs and CREB in the dorsal hippocampus has also been associated with learning deficits during nicotine withdrawal [131; 139], which also involves changes in other genes associated with long term potentiation for new contextual learning events, but not expression of previously
acquired contextual memory [140]. Finally, it is important to recognize the emerging importance of glial signaling in brain function. During nicotine withdrawal, proinflammatory effects have been associated with anxiogenic behaviors [141]. In the NAcc, nicotine withdrawal induces a change in microglial morphology, decreases astrocyte GFAP expression, and increases expression of Nox2, tumor necrosis factor-α, and interleukin-1β [141; 142]. Thus, gene expression changes during abstinence from nicotine may contribute to various behavioral effects associated with the withdrawal state.

High relapse rates and are often found when one attempts to quit the tobacco/nicotine smoking habit, and thus, ascertaining a better understanding of the neurobiological components modulating drug relapse may reveal new targets for therapeutic intervention. Drug relapse can involve the persistent memories of nicotine reward, aversive effects of withdrawal, and nicotine-associated conditioned stimuli [143; 144]. Increased nicotine seeking behavior after a prolonged period of abstinence is correlated with higher levels of c-fos protein expression in the amygdala, orbitofrontal cortex, medial prefrontal cortex, and NAcc [145]. Further, glutamatergic plasticity in the NAcc, including alterations in expression of the AMPA receptor and cholinergic interneuron function, has been shown to mediate cue-induced reinstatement [59; 146; 147]. In addition, neuronal sub-populations within the basolateral amygdala appear to encode reward-associated memories, as selective inhibition of these neurons prevents nicotine seeking in both the conditioned place preference and incubation of craving paradigms [148]. These findings support the notion that changes in gene expression may contribute to the likelihood of relapse-associated behaviors during abstinence.

Finally, weight gain following cessation may also contribute to high relapse rates, and studies examining the intersection of nicotine and feeding have found that nicotine induces c-fos protein expression in pro-opiomelanocortin and neuropeptide Y neurons of the arcuate nucleus, a brain region shown to mediate satiety [149; 150; 151; 152]. Withdrawal from nicotine increases body
weight, neuropeptide Y and agouti-related protein expression in the hypothalamus, and these proteins have been implicated in regulating appetite and metabolism [151]. Together, these studies highlight a role for nicotine-related gene expression changes in body weight regulation, which may contribute to the propensity for drug use and relapse.

**Further Considerations for Nicotine-Mediated Changes in Gene Expression**

Nicotine’s actions across the drug use trajectory may be modulated by a number of factors, in addition to nAChR receptor subtype expression and intracellular signaling cascades. In the following sections, we discuss additional considerations that can mediate nicotine’s actions at the various stages of drug use. First, we highlight the effects of nicotine across early developmental stages, and then we discuss the role of endogenous allosteric modulators that can play a key role in nicotine’s ability to induce a nAChR conformational change and/or modulate membrane expression [153]. Thereafter, we examine emerging evidence correlating changes in the epigenetic landscape with nicotine use.

*Developmental Effects of Nicotine on Gene Expression*

While the above studies have focused on changes in gene expression occurring in the adult, it is important to consider the impact of nicotine during earlier developmental stages when the brain is highly susceptible to environmental influences. Maternal smoking has been associated with adverse consequences for the fetus, which includes altered development of cholinergic systems and low birth weight [154]. Importantly, the potential negative impact on the fetus is not restricted to high levels of smoking behavior, as second-hand smoke exposure has also been correlated with impaired cognitive development [155]. Studies of post-mortem tissue document altered gene expression in the prefrontal cortex of fetuses from smoking mothers, including increased expression of genes involved in neurodevelopment (*CNTN4, EPHA8* and *GABRA4*) and decreased expression of genes involved in cell adhesion (*NRCAM*) and calcium signaling (*KCNN2*) [156]. However, for the most part, long-term behavioral outcomes in humans remain
inconclusive due to potentially confounding factors, such as differential environmental, nutritional, and genetic influences among subjects. Thus, rodent models are essential to better elucidate the effects of developmental nicotine exposure on gene expression and behavior. A study from Mao and colleagues confirmed nicotine-induced dysregulation within the cholinergic system, in which early prenatal nicotine exposure led to decreased expression of choline acetyltransferase, vesicular acetylcholine transporter, and choline transporter in the forebrain and hindbrain of rats [157]. In addition, sidestream cigarette smoke exposure in dams, a model of second-hand exposure, resulted in dysregulation of numerous genes in the mouse hippocampus, including upregulated serotonin transporter gene SLC6A4 and synaptic nuclear envelope gene SYNE1 [158]. Importantly, sex-dependent changes have also been found with in utero nicotine exposure. The axon myelination associated genes, MObP, PLP1 and Gje1, were significantly upregulated in male rat PFC, but downregulated in female rat PFC, following chronic gestational nicotine exposure [159]. Maternal cigarette smoke exposure also resulted in increased expression of inflammatory signaling factors, including IL-1β, IL-6 and toll like receptor 4, in adult male mice, although females were not examined in this study [160]. Consistent with these findings, the impact of nicotine on immune signaling molecules has been further demonstrated with in vitro studies. In an α4 and β2 expressing neuroblastoma cell line, nicotine application downregulated gene expression of the inflammatory proteins IL-1β and IL-6 and increased expression of the endoplasmic reticulum proteins CRELD2, PDIA6 and HERPUD1 [161]. Interestingly, knockout of the CRELD2 protein increased α4 and β2 nAChR subunit expression, suggesting a bidirectional effect on gene regulation in this in vitro system [161]. Finally, a recent study has demonstrated that prenatal nicotine exposure increases expression of Nur71 in the VTA, leading to a greater number of dopaminergic neurons and enhanced nicotine preference in adulthood [162].

In addition to in utero developmental exposure, adolescence is a sensitive period for consequential effects of nicotine on the brain, which also involved sex-specific outcomes [163]. Following nicotine exposure in adolescence, an upregulation in expression of the nAChR subunits
α5, α6, and β2, as well as genes associated with synaptic plasticity (Dnm1), neuron density (Ghr), and dendrite elongation (Map), is found in the VTA in adulthood [96; 164; 165]. Interestingly, many of these changes in gene expression are specific to nicotine exposure in adolescence, but not adulthood [164]. Other brain regions, including the hippocampus, NAcc and PFC, exhibit nicotine-mediated changes in gene expression following adolescent exposure, with differential effects based on the specific stage of adolescence [166]. Adolescent nicotine exposure also induces an upregulation in DeltaFosB in the NAcc [167], a transcription factor associated with neural changes underlying substance abuse [168]. Additional genes identified are involved in synaptic plasticity, including Arc and Dendrin [169; 170], suggesting that such long-term changes with adolescent nicotine exposure can mediate a variety of effects in adulthood underlying various affective, cognitive and drug use behaviors [165; 167; 171; 172; 173]. These studies demonstrate that nicotine consumption during adolescence can induce long-term changes in brain circuitry persisting into adulthood.

**Endogenous Modulators of Nicotinic Receptors**

Recently, endogenous allosteric modulators have been found to associate with nAChRs to alter the ability of ligands, including nicotine or acetylcholine, to induce an open conformational state of the receptor [174]. By modulating nAChR activity at the membrane, the consequential significance of the allosteric modulator on gene expression can be both short- or long-lived via modulation of Ca²⁺ influx for subsequent second messenger signaling. In addition to effects on membrane-localized nAChRs, endogenous modulators may also affect the trafficking of nAChR subunits to the membrane via association in the endoplasmic reticulum [175; 176]. Moreover, different endogenous modulators have been found to preferentially interact with specific nAChR subtypes and to be expressed in a region- and cell-specific manner, allowing for a potential high specificity in their effects [175; 177; 178]. For instance, RIC-3, the resistance inhibitors of cholinesterase 3, has been shown to increase α4 and β2 nAChR subunit protein expression, but not the assembly of these subunits into the α4β2 nAChR subtype [179]. Whereas initial studies
suggested that RIC-3 does alter assembly and cell surface trafficking of \( \alpha^7 \) nAChRs, but not \( \alpha^7 \) protein expression, it was subsequently demonstrated that RIC-3 can affect \( \alpha^7 \) nAChR expression but in a ratio dependent manner [179; 180; 181]. Specifically, RIC-3 decreases \( \alpha^7 \) nAChR expression, but \textit{only} at low ratios of RIC-3 to \( \alpha^7 \) nAChR subunits [180; 181]. In addition to the independent actions of allosteric modulators, two different allosteric modulators may work together to exert effects on nAChRs. For example, NACHO is a small, multi-pass transmembrane protein enriched in the neuronal endoplasmic reticulum that acts as a chaperone to mediate assembly and surface expression of \( \alpha^7 \) nAChR subunits [182]. When localized together, RIC-3 can interact with NACHO to differentially regulate expression of the \( \alpha^7 \) nAChR subunit [182].

Prototoxins are another class of allosteric modulators, which are classified into the lymphocyte antigen-6 (Ly-6)/urokinase plasminogen activating receptor (u-PAR) superfamily. Similar to RIC-3 and NACHO, prototoxins can associate with and differentially modulate nAChR expression and function through interactions in the endoplasmic reticulum, cytoplasm and membrane surface [183]. The identified protoxins include lynx1, lynx2 and lypd6, which can associate with nAChRs by anchoring adjacent to the receptor on the membrane via a GPI link [184; 185; 186]. Lynx proteins act as a “molecular brakepad” of the cholinergic system, negatively modulating the nAChR to reduce its activity in the presence of an agonist [187]. Association of lynx proteins with nAChRs decreases nicotine- and acetylcholine-induced currents and increases desensitization [188]. Prototoxins can modulate cholinergic activity in a biologically applicable, spatially specific, and nAChR subtype specific manner. For example, lynx1 has been shown to interact with the extracellular subunit interface of the nAChR, thereby altering receptor stoichiometry, nAChR assembly, and cell surface expression levels, leading to altered ligand-mediated cellular currents [176]. Lynx1 can alter the function of \( \alpha^6\beta^2\), \( \alpha^4\beta^2\), \( \alpha^3\beta^4\) and \( \alpha^7\)-containing nAChRs, as demonstrated in cell culture systems [177; 188]. Lynx1 is highly expressed in brain regions implicated in nicotine dependence with localization in glutamatergic, GABAergic, and dopaminergic neurons [189; 190; 191]. Moreover, in dopaminergic neurons of the substantia nigra
pars compacta, deletion of lynx1 reduces the function of the α6-containing nAChRs [192], indicating lynx1 modulation of α6-containing nAChRs occurs in these cellular populations. In contrast, lynx2 has been shown to interact with α4β2 nAChRs, to modulate nicotine’s effects on glutamatergic signaling in the prefrontal cortex, and to mitigate anxiety-related behaviors [183; 184; 186; 191]. Thus, allosteric modulators can act in conjunction with nicotine (or acetylcholine) to modulate protein expression in a cell type- and brain region-specific manner, but further studies in vivo will be necessary to more clearly delineate the relative importance of this interaction on subsequent behavior related to nicotine dependence.

**Epigenetic Regulation**

Epigenetic modifications can serve to increase or decrease gene promoter accessibility for transcriptional activation. Interestingly, increased methylation in the promoter region of the monoamine oxidase A gene, the enzyme that metabolizes serotonin and norepinephrine, has been linked with increased vulnerability for nicotine dependence in women [193], and differences in DNA methylation can serve as a predictor of smoking behavior in humans [194]. An elegant study in mice demonstrated that nicotine increases expression of the Ash2l/Mef2c complex during cortical development, which subsequently leads to changes in histone methylation in the promoter region of glutamatergic synaptic genes and representative changes in dendritic spine number and branching [195]. In rats, chronic nicotine exposure has also been associated with a decrease in methylation of several genes in the medial prefrontal cortex, orbitofrontal cortex, and NAcc [196; 197]. In the prefrontal cortex, nicotine self-administration was correlated with decreased histone methylation at the H3K27me3 and H3K9me2 marks in the brain derived neurotrophic factor gene (BDNF) and cyclin-dependent kinase 5 gene, but in contrast, withdrawal from nicotine elicited a decrease in H3K14 acetylation at the BDNF promoter [197], demonstrating different changes across stages of drug use. Following nicotine abstinence, histone deacetylase inhibitor administration results in an attenuation of cue-induced reinstatement [198], suggesting a further role for epigenetic factors in relapse-associated behaviors. Thus, altered gene expression due to
changes in the epigenetic landscape may contribute to the development and/or maintenance of nicotine dependence, withdrawal effects, or vulnerability to relapse.

Finally, it is worthwhile to note that in utero exposure to nicotine has also been associated with epigenetic changes. Because of the longevity of these changes, the effects of such regulation may lead to detrimental outcomes persisting through adolescence and into adulthood. For example, differential methylation patterns have been documented in blood samples following exposure to prenatal maternal smoking, with associations noted at CpG sites in the genes MYO1G, FRMD4A, CYP1A1, CNTNAP2, ARL4C, AHRR, TIFAB, MDM4, AX748264, DRD1, FTO [199]. Of note, some epigenetic changes associated with maternal tobacco exposure have been replicated in other studies in humans, can persist across the lifespan, and have been linked to schizophrenia-associated symptomology [199; 200; 201; 202]. However, several caveats must be considered for human studies based on numerous potentially confounding factors, such as comorbidity of drug use, socioeconomic status, nutritional deficits, stress, etc., and as such, it is important to validate such findings in a more controlled model system. Further, epigenetic changes detected in blood samples may not relate to the processes occurring in the brain. Thus, additional studies have begun to validate and extend these findings with studies in rodents. For instance, nicotine administration to pregnant dams results in hypomethylated DNA in the fetal cortex [203], supporting the notion of brain-relevant epigenetic changes with nicotine exposure. Moreover, nicotine exposure in male mice, prior to copulation, leads to their offspring exhibiting methylation changes in hippocampal genes related to neural development and plasticity, which is correlated with increased fear conditioning and decreased nicotine reinforcement [204]. Thus, these studies suggest that nicotine can induce epigenetic changes in early development through direct actions in utero in pregnant females, or by altering gene expression in male sperm prior to fertilization. However, future studies are necessary to more specifically delineate the causative effects of such changes in the epigenetic landscape.
Conclusions

The impact of nicotine on cholinergic function and gene expression has been shown to modulate multiple downstream effects that can contribute to different facets of nicotine dependence. The relationship between nicotine seeking behavior and gene expression is cell type-specific, sex-specific, developmental, and bidirectional. Moreover, nicotine’s actions on the cholinergic system can affect cognition, feeding, learning and memory, attention, and anxiety- and depression-associated symptoms, in addition to nicotine reinforcement and seeking behavior, and as such, the implications of nicotine use on gene expression is extensive and multifaceted [5; 9; 152; 205]. Thus, future efforts to more fully characterize the changes in gene expression occurring in specific cell types and brain regions at various stages along the drug use trajectory will be necessary to ascertain a comprehensive understanding of the intersectional and bidirectional contributions that regulate the relationship between gene expression and behavior. Through these analysis, novel gene targets may be identified as a foundation for more efficacious therapeutic development efforts.
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**Figure Legend**

**Figure 1.** Schematic illustrating the consequences of inhalation of nicotine from tobacco/e-cigarette products to induce changes in gene expression. After entry into the brain, nicotine binds to and activates nicotinic acetylcholine receptors (nAChRs) located on the cellular membrane, thereby inducing an open conformational state permitting the influx of Ca\(^{2+}\) and Na\(^+\) ions and efflux of K\(^+\) ions. Subsequent changes in gene expression occur through modulation of downstream signaling cascades. Created with BioRender.com
Table 1. Examples of gene expression changes with nicotine exposure.

| Nicotine Exposure | Brain Region(s) | Genes         | Functional Implications                                                                 | Citation(s)        |
|-------------------|-----------------|---------------|----------------------------------------------------------------------------------------|--------------------|
| Acute Increase    | AMG, Cortex, Hipp, IPN, MHb, NTS, SN, STR, VTA | Fos           | Fos proto-oncogene; involved in neuronal activation; activation of these brain regions may be sufficient to alter nicotine intake | [42; 43; 49; 50; 51; 62; 81; 83; 84; 85; 86; 88; 89; 90; 136; 150; 152] |
|                   | Forebrain       | Arc           | Activity-regulated cytoskeleton associated protein; involved in neuronal activation and plasticity; may increase reinforcing effects of nicotine | [169; 173]        |
|                   | PFC             | Ddn           | Dendrin; involved in learning and memory; adolescent-specific changes following acute nicotine | [164; 170; 206]   |
|                   | VTA             | Gria1         | Glutamate ionotropic receptor AMPA subunit; involved in glutamatergic cell signaling; may enhance reinforcing response to nicotine | [50; 52; 111; 207] |
| In vitro          | IL1B, IL6, CRELD2, PDIA6, HERPUD1 | Interleukin 1 beta and Interleukin; involved in inflammatory response Cysteine rich with EGF like domains 2; may be involved in transport of nAChRs Protein disulfide isomerase family A member 6; involved in cell proliferation Homocysteine inducible ER protein with ubiquitin like domain 1; involved in stress response in the endoplasmic reticulum | [161; 208; 209]   |
| Decrease          | IPN             | Pfn2          | Prolifin 2; involved in extracellular signaling                                           | [128]              |
| Sub-Chronic       | Hipp            | Chhr1, Chhr2  | Corticotropin releasing hormone receptor 1 and 2; involved in the physiological stress response | [135]              |
|                   | Hiph, PFC, STR  | Crh           | Corticotropin releasing hormone; involved in the physiological stress response            | [135]              |
|                   | NAcc            | Creb1, Fosb   | CAMP responsive element binding protein 1; involved in stimulation of the cAMP pathway  | [117; 167; 210; 211] |
|                   | SNc             | Chrma6, Chrmb3 | α6 nAChR subunit and β3 nAChR subunit; involved in forming nAChRs on which nicotine directly binds | [90; 97]          |
|                   | VTA             | Oprd1         | Opioid receptor delta 1; involved in opioid dependence and aspects of nicotine action     | [110; 212]        |
| Decrease          | Hipp, cortex    | Fos           | Fos proto-oncogene; involved in neuronal activation; pattern of nicotine dosing indicates nAChR desensitization may contribute to conditioned drug reward | [40; 85]          |
| Chronic Increase  | AMG, OFC, mPFC, NAcc | Fos          | Fos proto-oncogene; involved in neuronal activation; activation of these brain regions may be sufficient to alter nicotine intake | [42; 43; 49; 50; 51; 62; 81; 83; 84; 85; 86; 88; 89; 90; 136; 150; 152] |
|                   | Cortex, SN, VTA(GABAAergic neurons) | Chrmα4        | α4 nAChR subunit; forms subtypes of nAChRs on which nicotine directly binds               | [45; 46; 47; 48; 99; 102; 111; 207] |
|                   | Cortex, IPN (male-specific), IPN (female-specific) | Chrb2         | β2 nAChR subunit; forms subtypes of nAChRs on which nicotine directly binds               | [92; 99; 102]     |
| Structure   | Gene(s) | Description                                                                                                                                                                                                 | References          |
|-------------|---------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| Choroid plexus | MiR204, Ttr | MicroRNA 204; involved in regulating non-coding RNAs, anti-apoptotic signaling, linked to Schizophrenia in GWAS study Transferrin; involved in thyroid hormone and retinol transport, Aβ clearance in brain, linked to Alzheimer’s disease and Schizophrenia in GWAS study | [118; 119; 121; 122; 123] |
| Hipp | Nrg3, Creb1 | Neuregulin 3; involved in intracellular signaling, nicotine-related anxiety symptomology CAMP responsive element binding protein; involved in stimulation of the CAMP pathway, nicotine-mediated responses and withdrawal symptoms | [129; 130; 131] |
| Hypothalamus | Npy, Agp | Neuropeptide Y and Agouti related neuropeptide; involved in food intake and weight regulation | [149] |
| IPN | Nos1, Sst | Nitric oxide synthase 1 and Somatostatin; upregulation in α5 containing neurons contributes to signaling for nicotine withdrawal and aversion | [42; 62; 63; 64; 65; 66; 67; 68; 101] |
| IPN (male-specific), SN, VTA | Chrna7 | α7 nAChR subunit; forms homomeric subtype of nAChRs on which nicotine directly binds; involved in reward-related behaviors | [97; 98; 213] |
| IPN (Female-specific) | Chrna5 | α5 nAChR subunit; forms subtypes of nAChRs on which nicotine directly binds; contributes to nicotine withdrawal and aversion phenotypes | [101; 102] |
| NAcc | Nox2, Il1b, Tnf | NADPH oxidase 2; involved in microglia morphology Interleukin 1 beta; involved in the inflammatory response Tumor necrosis factor; involved in cell proliferation | [141; 142] |
| PFC | MiR199a, MiR214, Bdnf | microRNA 199a and 214; implicated in cell proliferation with cancer Brain derived neurotrophic factor; involved in nerve growth, learning and memory, and cellular signaling | [125; 138; 214; 215] |
| SN | Chrna6, Chrnb3 | α6 nAChR subunit and β3 nAChR subunit; forms subtypes of nAChRs on which nicotine directly binds; correlated with increased nicotine self-administration | [97; 216; 217] |
| VTA | Chrna5, Chrna6, Chrnb2, Dnm1, Ghr, Map | α5 nAChR subunit, α6 nAChR subunit; β2 nAChR subunit; forms subtypes of nAChRs on which nicotine directly binds; may enhance reinforcing response to nicotine Dynamin; involved in cellular membranes Growth hormone receptor; involved in cellular growth Mitogen-Activated Protein; involved in cell proliferation | [97; 108; 112; 164] |
| Decrease | Dorsal striatum | Bdnf | Brain derived neurotrophic factor; involved in nerve growth, learning and memory and cellular signaling | [135] |
| PFC | Sirt1 | Sirtuin1; involved in epigenetic gene slicing | [125] |
Table 1. Examples of gene expression changes with nicotine exposure. Acute: one injection; Sub-chronic: <10 injections/exposures; Chronic: >10 injections/exposures; AMG: Amygdala; Hipp: Hippocampus; IPN: Interpeduncular nucleus; MHb: Medial habenula; mPFC: Medial prefrontal cortex; NAcc: Nucleus accumbens; NTS: Nucleus of the tractus solitarius; OFC: Orbitofrontal cortex; PFC: Prefrontal cortex; PVN: Paraventricular nucleus; SN: Substantia nigra; SNC: Substantia nigra pars compacta; STR: Striatum; VTA: Ventral tegmental area.

| Region          | Increase                | Genes | Description                                                                 | Reference(s) |
|-----------------|-------------------------|-------|-----------------------------------------------------------------------------|--------------|
| Whole brain     | IliB, Il6, Tlr4         | Interleukin 1 beta and Interleukin 6; involved in inflammatory response | [160]        |
| Prenatal Increase homogenization | Il6, Tlr4              | Toll-like receptor 4; involved in the immune response                     |              |
| Hipp            | Slc6A4, Syn1            | Serotonin transporter; involved in serotonergic signaling; may contribute to nicotine withdrawal symptoms | [158; 218]  |
| PFC             | CNTN4, EPHA8, GABRA4, Mobb*, Plp1*, Gje1* (*male-specific) | Contactin 4; involved in neuronal plasticity; EPH receptor 8; involved in axonal projections | [156; 159; 219; 220; 221] |
| VTA             | Nurr1                   | Nuclear receptor subfamily 4 Group A member 2; involved in dopaminergic signaling | [162; 222]  |
| Decrease forebrain, hindbrain | Slc18A3, Slc5a7, Chat | Vesicular acetylcholine transporter, High affinity choline transporter, and Choline acetyltransferase; involved in cholinergic signaling | [157]        |
| PFC             | NRCAM; Mobb*, Plp1*, Gje1* (*female-specific) | Neuronal cell adhesion molecule; promotes directional axonal growth; Myelin associated oligodendrocyte basic protein and Proteolipid protein 1; both involved in forming the myelin surrounding nerve fibers; Gap junction protein epsilon 1; component of gap junctions for intercellular signaling | [156; 159; 223] |