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Nicotine Withdrawal

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Abstract

An aversive abstinence syndrome manifests 4–24 h following cessation of chronic use of nicotine-containing products. Symptoms peak on approximately the 3rd day and taper off over the course of the following 3–4 weeks. While the severity of withdrawal symptoms is largely determined by how nicotine is consumed, certain short nucleotide polymorphisms (SNPs) have been shown to predispose individuals to consume larger amounts of nicotine more frequently—as well as to more severe symptoms of withdrawal when trying to quit. Additionally, rodent behavioral models and transgenic mouse models have revealed that specific nicotinic acetylcholine receptor (nAChR) subunits, cellular components, and neuronal circuits are critical to the expression of withdrawal symptoms. Consequently, by continuing to map neuronal circuits and nAChR subpopulations that underlie the nicotine withdrawal syndrome—and by continuing to enumerate genes that predispose carriers to nicotine addiction and exacerbated withdrawal symptoms—it will be possible to pursue personalized therapeutics that more effectively treat nicotine addiction.

Keywords

Nicotine withdrawal; SNP; Behavior; Medial habenula; Interpeduncular nucleus; Nicotinic subunits

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1 Withdrawal Syndrome in Humans

1.1 Symptoms

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) reports 7 primary symptoms associated with nicotine withdrawal: irritability/anger/frustration, anxiety, depressed mood, difficulty concentrating, increased appetite, insomnia, and restlessness (American Psychiatric Association 2013). The syndrome might also include constipation, dizziness, nightmares, nausea, and sore throat. For practical purposes, nicotine withdrawal symptoms are classified as affective, somatic, and cognitive. Affective symptoms include anxiety, anhedonia, depression, dysphoria, hyperalgesia, and irritability. Somatic manifestations include tremors, bradycardia, gastrointestinal discomfort, and increased appetite. Cognitive symptoms manifest as difficulty concentrating and impaired memory (Heishman et al. 2010). This constellation of symptoms reflects the brain-wide influence of cholinergic transmission. Studies characterizing the participation of specific nAChRs in signaling during particular manifestations of withdrawal are helping to reveal their underlying mechanisms (Paolini and De Biasi 2011).

1.2 Genetic Influences

Nicotine addiction is influenced by both genetic and environmental factors. Depending on the parameters used to define dependence, and the population considered, heritability contributes 50–75 % of the risk for dependence (Dokal et al. 1989; Hall et al. 2002; Lessov et al. 2004; Furberg et al. 2010; Nugent et al. 2014; also see chapters entitled Genetics of Smoking Behaviour and Pharmacogenetics of Nicotine and Associated Smoking Behaviors; volume 23). Risk factors for nicotine dependence can be identified using genetic methods such as linkage and candidate gene analyses, as well as genome-wide association studies (GWAS). Linkage analysis assesses the presence of a phenotype in large, high-risk families to map the location of diseasecausing loci in relation to a known genetic marker. Candidate gene analysis assesses the association between a particular gene allele (or alleles) potentially involved in the disease and the disease itself. GWAS, in contrast to candidate gene approaches, do not limit analyses to relationships between specific genes and a phenotype and aim to identify loci for novel susceptibility genes. An example of how such genetic approaches can be successfully utilized in nicotine research is the gene cluster on chromosome 15q25 that encodes the α5, α3, and β4 nAChR subunits. Gene variants within the cluster have been repeatedly shown to affect nicotine dependence and smoking quantity (Saccone et al. 2009). A non-synonymous nucleotide polymorphism (SNP), rs16969968, which substitutes an aspartic acid for an asparagine (D398N) in the CHRNA5 region of the cluster, has been associated with reduced receptivity to nAChR agonists in vitro, reduced neuronal calcium permeability, and more extensive nAChR desensitization (Jackson et al. 2010). Individuals who are homozygous for this SNP are more likely to progress to heavy smoking and nicotine dependence (Hartz et al. 2012), and it has been suggested that the reduction in agonist responsivity and increased desensitization may contribute to these increased propensities (Jackson et al. 2010). Apart from the missense rs16969968 variant, there are other SNPs within the CHRNA5-CHRNA3-CHRNB4 cluster that are relevant to nicotine addiction and withdrawal, and further biological characterizations are needed to establish the functional consequences of those mutations. Other nAChR genes have also

been shown to influence smoking quantity and nicotine dependence, such as common variants in the chromosome 8p11 region that contains the genes encoding the α6 and β3 nAChR subunits (Thorgeirsson et al. 2010). Overall, linkage analyses have highlighted 13 regions on 11 chromosomes that include genes with potential influences on nicotine dependence (Leeb and Tamse 1985; Li 2008). Genes involved in nicotine metabolism are also likely important to the nature of nicotine withdrawal. Cytochrome P450 2A6 (CYP2A6) is the enzyme mainly responsible for the conversion of nicotine to cotinine, which typically accounts for 70–80 % of nicotine metabolism (Hecht et al. 2000). In subjects of European descent, GWAS meta-analyses identified SNPs in the region of CYP2A6 associated with the number of cigarettes smoked per day, as well as other smoking behavior phenotypes (Thorgeirsson et al. 2010).

Genetic factors may also account for 29–53 % of the variance in withdrawal symptoms and approximately 50 % of the variance in quitting success (Xian et al. 2003, 2005; Pergadia et al. 2006). In a linkage analysis study, a sample of Australian and Finnish smokers was queried about withdrawal symptoms within the context of a smoking cessation attempt that they recalled well. The study revealed a linkage signal that meets genome-wide significance on chromosome 11p15 in the Finnish families (Pergadia et al. 2009). Four strong candidate genes lie within or near the peak area on chromosome 11: dopamine receptor 4 (DRD4), TPH tryptophan hydroxylase 1 (TPH), tyrosine hydroxylase (TH), and nAChR subunit 10 (CHRNA10). A second region identified by linkage on chromosome 11q23 includes hydroxytryptamine receptor 3A (HTR3A), hydroxytryptamine receptor 3B (HTR3B), dopamine D2 receptor gene (DRD2), ankyrin repeat and kinase domain containing 1(ANKK1), and ionotropic kainate glutamate receptor 4 gene (GRIK4). An earlier study reported that DRD2 TaqI-B polymorphisms influence abstinence and withdrawal symptoms (Robinson et al. 2007). Smokers carrying the DRD2 TaqI-B1 risk allele (B1/B1 or B1/B2) reported significantly more symptoms of daily smoking withdrawal compared to smokers homozygous for the TaqI-B2 allele (B2/B2). In addition, while withdrawal symptoms—measured 14 days pre-quit and 42 days post-quit—decreased significantly in Taql-B2 homozygous over time, smokers with the TaqI-B1 allele reported little improvement in self-reported withdrawal symptoms.

Several other pharmacogenetic studies, including a couple of genome-wide association study (GWAS) analyses, have examined genetic influences on smoking cessation and response to therapy (Leeb and Tamse 1985; Uhl et al. 2007; Furberg et al. 2010; Gold and Lerman 2012; King et al. 2012; Bloom et al. 2013; Chen et al. 2014). SNPs in CHRNB2 and the CHRNA5-CHRNA3-CHRNB4 cluster seem to influence smoking cessation, although the reported effects are not always reproducible (Conti et al. 2008; Baker et al. 2009; Perkins et al. 2009; Gold and Lerman 2012; Hartz et al. 2012). The influence of CHRNA5-CHRNA3-CHRNB4 haplotypes on tolerance, craving, and loss of control seems greatest in individuals who began smoking early in life, suggesting that the risk associated with those genes is greatest with early tobacco exposure (Baker et al. 2009). The rs8192475 variant in CHRNA3 was shown to predict withdrawal symptoms and craving after quitting (Sarginson et al. 2011). The major G allele was associated with stronger but relatively short-lived symptoms, while the minor A allele resulted in a relatively constant level of symptoms and craving during the acute phase of withdrawal. The rs8192475 α3 SNP leads to an R37H change in

the amino acid sequence that increases agonist sensitivity of heterologously expressed α3β4 nAChRs (Haller et al. 2012). Rare missense variants at conserved residues in CHRNB4 (T375I and T91I) are associated with reduced numbers of cigarettes per day and fewer signs of withdrawal (Haller et al. 2012). Similar to the α3 SNP rs8192475, expression of the two β4 mutations in HEK 293 cells leads to larger currents in response to ACh stimulation, which has been suggested to increase the aversive properties of nicotine.

Similar to what was found for phenotypes related to nicotine dependence, there are also associations of CYP2A6 enzyme activity and nicotine metabolic rates with smoking cessation and smoking cessation strategies (Lee et al. 2007; Chen et al. 2014). Individuals carrying the null activity allele, CYP2A6*2, are twice as likely to quit smoking as subjects without that allele (Gu et al. 2000). Conversely, smokers with high-activity alleles (CYP2A6*1/*1B) experience more severe withdrawal symptoms when trying to quit (Kubota et al. 2006).

Currently, the literature suggests that multiple genetic loci influence the severity of withdrawal symptoms and the ability to abstain from smoking. Such polygenic contributions overlap, at least in part, with those associated with vulnerability to nicotine dependence. Because the majority of published studies was conducted on relatively small samples, examined different cessation strategies, and did not always address withdrawal symptoms directly, additional work is needed to yield more robust and reproducible associations between genes and withdrawal phenotypes. Once putative genes are identified, it will be critical to assess the potential for genetic variations to alter biological function and, consequently, nicotine-related behaviors. Preclinical studies that address both behavior and molecular mechanisms can help to explain how the changes in DNA sequence are associated with the behavioral phenotypes observed in smokers. Rodents, and particularly mice, offer unique opportunities to explore and characterize the relationships between gene function and nicotine withdrawal. Given the difficulty of effectively characterizing gene products and circuits responsible for the symptomatology of nicotine withdrawal in humans, genetic manipulations can be carried out in mice to directly address the circuits and molecular mechanisms involved.

2 Withdrawal Syndrome in Mice

Many symptoms of nicotine withdrawal described in humans can be recapitulated in rodent models of addiction and withdrawal under experimental conditions (see chapter entitled The Role of Mesoaccumbens Dopamine in Nicotine Dependence; this volume). After at least a week of chronic nicotine administration in mice, cessation of nicotine exposure or administration of a nAChR antagonist induces affective, somatic, and cognitive signs of withdrawal (Malin et al. 1994; De Biasi and Salas 2008). The withdrawal syndrome exhibited can be alleviated by nicotine administration—akin to nicotine replacement strategies such as nicotine patches or gum—or by administration of pharmacological agents used as cessation aids in humans, such as bupropion or varenicline (Rennard and Daughton 2014). Because mice express neuroadaptations that result in the nicotine withdrawal syndrome, and because nAChR subunit knockout mouse lines are readily available,

researchers have been able to study the functional contributions of particular nAChR subunits to specific symptoms of withdrawal.

2.1 Behavioral Manifestations and Testing Paradigms

The nicotine withdrawal syndrome can be studied in mice by observing the frequencies of certain stereotypies, or by evaluating changes in behavior during withdrawal, relative to baseline behaviors exhibited by control mice naïve to nicotine (see chapter entitled Behavioral Mechanisms Underlying Nicotine Reinforcement; this volume). As in humans, the behavioral manifestations of nicotine withdrawal in mice can be categorized as somatic, affective, and cognitive.

Somatic symptoms in mice include excessive grooming, chewing, tremors, “wetdog” shakes, yawns, and teeth chattering (Paolini and De Biasi 2011). The occurrence of these symptoms tends to increase according to the severity of withdrawal. Affective symptoms are somewhat subtler in mice than somatic symptoms; their evaluation requires a series of behavioral assays to reveal changes in affect that are associated with withdrawal. Just as humans undergoing nicotine withdrawal experience anxiety, anhedonia, depression, and hyperalgesia, behavioral paradigms applied to mice expose analogous affective states. A challenge researchers face when evaluating rodent affect is that no single paradigm effectively isolates any one particular affective state. Each measure likely evokes multiple affective states, unintentionally recruiting off-target neuronal circuits that are out-side the scope of a given study. However, several behavioral paradigms applied as a set can help reveal relative differences in affect following experimental treatments. The emergence of anxiety-like symptoms following nicotine abstinence can be measured in the open field test (OFT) and the elevated plus maze (EPM), two of the most commonly employed paradigms in studies evaluating emotional states of rodents (Crawley et al. 1997). The OFT is conducted by placing a mouse or rat in a square arena and evaluating the ratio of time rodents spend in the center of the environment to time spent along its perimeter. As rodents experience increased anxiety, they tend to avoid the center and spend more time along the walls of the environment, a behavior termed thigmotaxis. As rodents undergo nicotine withdrawal, they tend to exhibit increased levels of thigmotaxis. The OFT also enables researchers to evaluate rearing behavior, overall locomotion, freezing, and changes in defecation during withdrawal. As a further source of experimental control, levels of ambient illumination can be adjusted to modulate baseline levels of anxiety. The EPM consists of two enclosed arms with walls running along their periphery, and two open arms with no walls that present an environment resembling elevated cliffs (Crawley et al. 1997). The arms are arranged in the shape of a plus sign, and anxious mice tend to make fewer entries into the open arms and spend less time in open arms. Rodents undergoing nicotine withdrawal exhibit both of these anxiety-associated behaviors, and anxiolytic drugs have been observed to increase open-arm entries (Crawley et al. 1997).

Conditioned place aversion (CPA) can be used to measure the dysphoric manifestations associated with withdrawal. The paradigm involves repeated pairing of distinct environmental cues with negative stimuli (i.e., withdrawal), such that when given a choice, mice avoid the withdrawal-paired cues relative to neutral cues. The time mice spend

avoiding environments paired with withdrawal serves as an indicator of the severity of aversion (Kenny and Markou 2001). The emergence of depression-like symptoms during withdrawal can be assessed with the forced swim test (FST), which assesses learned helplessness by monitoring passive coping strategies such as immobility (Cryan et al. 2002; Thanos et al. 2013). During withdrawal, immobility is increased, indicating a depression-like state.

Intracranial self-stimulation (ICSS) is used to investigate the "reward" circuitry and can be useful to evaluate anhedonia during withdrawal. Electrodes are implanted into the rodent brain targeted to the medial forebrain bundle, which includes the mesolimbic DA pathway associated with hedonia or reward. The rodent is first trained to perform the operant task of self-stimulation and then is allowed to self-administer small electrical stimulations to the targeted pathway. As rodents perform this operant conditioning, the stimulus intensity is experimentally adjusted to determine the baseline stimulation threshold at which self-stimulation is consistently achieved and retained. Following the establishment of this threshold, researchers can explore the effects various stimuli have on brain reward and hedonic signaling. A lowered threshold resulting from a treatment is suggested to represent increased reward signaling along the pathway, while a heightened threshold is interpreted as indicating a state of greater anhedonia (O'Dell and Khroyan 2009). Animals experiencing nicotine withdrawal display elevated thresholds for ICSS.

The effects of withdrawal on cognition, especially hippocampal-dependent learning and memory, can be examined with contextual fear conditioning (FC). Subjects must learn contextual information and form an association between the context and an aversive electric shock (Fanselow and Poulos 2005; Sigurdsson et al. 2007). Acute nicotine administration enhances contextual FC, while nicotine withdrawal impairs it (Davis and Gould 2008).

The five-choice serial reaction time task (5-CSRTT), also called operant signal detection, is a behavioral test used to characterize the effects of treatments on sustained and divided attention (Robbins 2002). The task consists of a maze that presents five holes, each of which may be paired with a reward. A brief flash of light inside one of the holes indicates the one that contains a reward, and rodents must poke the correct hole to receive the reward. This task quantifies the capability of rodents to maintain spatial attention that has been split among five locations over the course of many trials by dividing the proportion of correct nose-pokes (pokes into the lit hole) by total nose-pokes. While nicotine administration has been observed to generate cognitive enhancements, rats withdrawing from nicotine have been observed to exhibit attention deficits in the 5-CSRTT paradigm (Shoaib and Bizarro 2005), reflecting some of the symptoms that human smokers report while undergoing withdrawal.

While these rodent behaviors may reflect symptoms of the human withdrawal syndrome, the neuronal circuits recruited during experimental paradigms remain largely unknown. Differences in the behavioral outputs between animals undergoing withdrawal and control subjects can help reveal the circuits nicotine withdrawal impinges upon, as each behavioral test offers unique environmental stimuli and engages specific neuronal circuits (Wahlsten 2010). The integration of mice genetically modified to carry nAChR null [knockout (KO)]

or SNP-related mutations into behavioral research has introduced a finer level of resolution to the characterization of the neuronal basis of withdrawal. By examining how eliminating signaling contributions from specific receptor subunits affects behavioral outcomes, researchers can determine which receptor subunits are necessary for the expression of certain nicotine withdrawal symptoms. Additionally, the genetic differences that distinguish each inbred strain of mice have important consequences for behavior, as each strain is characterized by a unique repertoire of consistent behavioral traits (Crawley 1996, 2008; Krackow et al. 2010; Matsuo et al. 2010). As these differences can translate to increased or decreased detectability of behavioral changes induced by withdrawal, it is prudent to establish which strain is most sensitive to the hypothesized behavioral changes in question (Bailey et al. 2006; Crawley 2008; Lalonde and Strazielle 2008; Wahlsten 2010).

3 Receptors and nAChR Subunits Underlying Withdrawal Symptoms

3.1 Insight from nAChR Subunit KO Mice

Ultimately, the development of enhanced therapeutics that effectively promote smoking cessation with minimal side effects will result from the identification of particular receptor subunit compositions that are necessary for the expression of withdrawal symptoms. Mouse models carrying nAChR null mutations are helping to identify the nAChR subtypes responsible for the various symptoms of nicotine withdrawal.

In rodents, α2 mRNA levels are highest in the interpeduncular nucleus (IPN), with more restricted expression in scattered neurons within the amygdala, hippocampus, cortex, retina, spinal cord, and cerebellum (Lotfipour et al. 2013). Analyses of withdrawal symptoms in α2 KO mice have shown that the physical manifestations of nicotine abstinence are context-dependent, as α2 KO mice exhibit more somatic symptoms of withdrawal in novel environments (Lotfipour et al. 2013), but display no somatic signs of withdrawal in habituated environments (i.e., the home cage) (Salas et al. 2009). Additionally, the lack of α2 is sufficient to abolish somatic symptoms of precipitated nicotine withdrawal (Salas et al. 2009). In male mice, Chrna2 deletion also produces nicotine withdrawal-induced deficits of cued FC (Lotfipour et al. 2013). α2 KO mice also self-administer higher doses of nicotine than WT controls (Lotfipour et al. 2013). It should be noted that in a study of European Americans and African-Americans, CHRNA2 showed a strong association with the Fagerström Test for Nicotine Dependence (FTND) after correction for multiple testing (Wang et al. 2014). The rs2472553 SNP, which seems to have the strongest association with nicotine dependence, encodes a functional variant in the signal peptide, which leads to a threonine-to-isoleucine amino acid substitution at residue 22. In oocytes, the T22I mutation changes the sensitivity to nicotine of α2β4-containing nAChRs (Dash et al. 2014).

The α3 nAChR subunit is encoded by CHRNA3, one of the genes in the chromosome 15q25 cluster that has shown the most robust association with smoking behavior and nicotine dependence. α3 forms functional nAChR complexes with α5 and β4, the other two subunits encoded by CHRNA5-CHRNA3-CHRNB4. These subunits are densely expressed in the medial habenula (MHb) and IPN (Grady et al. 2009). Due to developmental abnormalities—including bladder enlargement and infection, urinary stones, and difficult urination—α3 KO mice survive birth, but exhibit severely impaired growth and perinatal mortality (Xu et al.

1999). This phenotype has rendered evaluation of the subunit's potential involvement in withdrawal symptomatology impractical in α3 null mice. However, pharmacological data suggest that receptors comprising the α3 and β4 nAChR subunits influence both nicotine reward and somatic manifestations of withdrawal (Jackson et al. 2013). AuIB, an α-conotoxin peptide that potently blocks α3β4 nAChRs (Luo et al. 1998), dose-dependently inhibits nicotine elicited reward as measured in the conditioned place preference paradigm. The α-conotoxin also reduces somatic signs of withdrawal and withdrawal-induced hyperalgesia, while it has no effect on the aversive motivational component of withdrawal as measured in the CPA paradigm (Jackson et al. 2013).

α5-containing nAChRs are expressed in various brain areas implicated in the key effects of nicotine, including ventral tegmental area (VTA), MHb, IPN, hippocampus, and cortex (Salas et al. 2003a). α5 KO mice do not display physical symptoms associated with both spontaneous and mecamylamine-precipitated nicotine withdrawal (Salas et al. 2009) nor withdrawal-induced hyperalgesia (Jackson et al. 2008). The MHb/IPN pathway plays a crucial role in mediating the physical manifestations of nicotine withdrawal and is the likely location of the effects of α5-containing nAChR on this phenotype (Salas et al. 2009). Acute nicotine application to MHb slices enhances the intrinsic excitability of MHb neurons (Dao et al. 2014). This dynamic depends on the presence of α5-containing nAChRs within the MHb and the release of neurokinins (Dao et al. 2014), as such enhancement of excitability was prevented by bath application of neurokinin 1 (NK1) or NK3 receptor antagonists. In addition, infusion of the same NK receptor antagonists into the MHb of mice chronically treated with nicotine precipitated somatic signs of nicotine withdrawal (Dao et al. 2014). Microinjection of neurokinin receptor antagonists into adjacent anatomical structures, including the lateral habenula (LHb), failed to elicit behavior resembling withdrawal. Similarly, microinjection of the NK1 and/or NK3 antagonists into the MHb of nicotine-naïve mice failed to generate somatic symptoms of withdrawal. It was concluded that interactions between cholinergic and neurokininergic systems contribute to the emergence of nicotine withdrawal symptoms (Dao et al. 2014). The α5 null mutation does not appear to influence affective symptoms such as withdrawal-induced CPA (Jackson et al. 2008). The same study reported that α5 KO mice do not display increased anxiety levels in the EPM during withdrawal (Jackson et al. 2008). However, interpretation of those data is not straightforward, as Chrna5 deletion reduces anxiety in the EPM in basal conditions independent of nicotine treatment (Gangitano et al. 2009). It has also been shown that α5-containing nAChRs do not regulate the reward-inhibiting effects induced by nicotine withdrawal in the ICSS paradigm (Fowler et al. 2013), suggesting that the receptors do not influence anhedonia.

The α6 subunit also appears to play a role in the nicotine withdrawal syndrome.

DA release in the NAcc following nicotine administration is regulated in part by α6-containing (α6*) nAChRs on DAergic terminals in the dorsal and ventral striatum (Exley et al. 2008) and DAergic somata in the VTA (Grady et al. 2007; Zhao-Shea et al. 2011). Intracerebral infusion of a selective antagonist of α6β2* nAChR blocks CPA and withdrawal-precipitated anxiety-like behavior in the EPM (Jackson et al. 2009). There was

no influence on the somatic symptoms of withdrawal, suggesting a selective role for α6 nAChR subunits in the affective manifestations of withdrawal (Jackson et al. 2009).

Unlike most other subunits, α7 nAChR subunits are capable of forming homomeric receptors that are broadly distributed in the brain. The hyperalgesia symptoms that emerge during mecamylamine-precipitated withdrawal are reduced in α7 KO mice (Grabus et al. 2005). In contrast to wild-type mice, α7 null mice failed to exhibit elevated ICSS thresholds during nicotine withdrawal between 3 and 6 h after their last nicotine exposure (Stoker et al. 2012). When the anhedonic affective state was evaluated between 8 and 100 h after nicotine withdrawal, ICSS thresholds were equally elevated in α7 WT and null littermates, indicating that a lack of α7 nAChR subunits delays, rather than abolishes, withdrawal symptoms. As for the physical signs of abstinence, α7 null mice exhibit significantly reduced withdrawal symptoms immediately after precipitation of withdrawal by mecamylamine injection (Salas et al. 2007). However, their physical signs are indistinguishable from those of WT mice when measured at later times, up to 48 h after withdrawal (Jackson et al. 2008; Stoker et al. 2012).

β2 KO mice exhibit levels of somatic signs of withdrawal and abstinence-induced hyperalgesia comparable to those of WT mice (Salas et al. 2004). However, the mutant mice do not exhibit anxiety-related behaviors normally associated with withdrawal from chronic nicotine exposure (Jackson et al. 2008). Overall, these results suggest participation of β2 nAChR subunits in the signaling responsible for affective, but not somatic, symptoms of nicotine withdrawal. α6β2* nAChRs are expressed in the VTA and ventral striatum and are associated with reward and addiction, but they are not expressed peripherally. Therefore α6β2* nAChRs may represent viable targets for the treatment of affective symptoms experienced during nicotine withdrawal. Indeed, a non-nicotine pharmaceutical currently FDA approved for the treatment of smoking cessation, varenicline, acts as a partial agonist at α6β2* nAChRs (Grady et al. 2010; Bordia et al. 2012).

While sequence variants associated with smoking behavior lie within the regions that harbor the CHRNB3-CHRNA6 genes on chromosome 8p11 (Thorgeirsson et al. 2010), no preclinical data are available on the effects of the β3 null mutation on nicotine-related behavior. However, it has been determined that β3 KO mice exhibit lower baseline levels of anxiety-related behavior in three different paradigms (Booker et al. 2007). As reported for the α5 KO mice (Gangitano et al. 2009), β3 KO mice have altered hypothalamic pituitary adrenal axis responses (Booker et al. 2007). Changes were also reported for locomotor activity and prepulse inhibition of acoustic startle, behaviors that are controlled, at least in part, by nigrostriatal and mesolimbic dopaminergic activity (Cui et al. 2003). As β3 mRNA is detected in the substantia nigra, VTA, and medial habenula (Cui et al. 2003), it is tempting to attribute the anxiolytic phenotype to MHb mechanisms and the locomotor phenotype to nigrostriatal mechanisms.

β4 KO mice exhibit no somatic signs of nicotine withdrawal or hyperalgesia following mecamylamine-induced withdrawal (Salas et al. 2004). Similar results were found for β4 KO mice undergoing spontaneous withdrawal from chronic nicotine administration (Stoker et al. 2012). In addition, β4 KO mice do not display anhedonia-like symptoms during

withdrawal, as identified by unchanged intracranial self-stimulation thresholds (Stoker et al. 2012). The reported phenotypes likely reflect an involvement of different subtypes of β4* nAChRs. α3β4* nAChRs are the most likely contributors to physical components of withdrawal given the high levels of expression of α3 and β4 mRNA in the MHb, and the fact that the α3β4-selective α-conotoxin AuIB blocks the emergence of somatic signs of withdrawal (Jackson et al. 2013). Because AuIB does not interfere with the affective symptomatology of withdrawal in the EPM and CPA paradigms, α6β4* nAChRs might be involved. Indeed, a transgenic mouse model that overexpresses β4 nAChR subunits exhibits altered nicotine consumption and CPA (Frahm et al. 2011), and there is a documented role for α6* nAChRs in withdrawal-induced CPA (Jackson et al. 2009).

4 Molecular Mechanisms Involved in the Nicotine Withdrawal Syndrome

4.1 nAChR Upregulation

Long-term exposure to nicotine leads to an increase, or upregulation, of nicotinebinding sites in the brain of smokers (Benwell et al. 1988) and rodents subjected to repeated nicotine administrations (Marks et al. 1983). The increased pool of nAChRs arising from chronic nicotine exposure may drive symptoms associated with the nicotine withdrawal syndrome (Turner et al. 2011; Gould et al. 2012) and might impact the ability to maintain abstinence in the clinical population (Staley et al. 2006). Using a radioligand with specificity for β2-containing nAChRs, which enabled the use of single-photon emission computed tomography (SPECT) (Staley et al. 2006), it was found that chronic smokers have more cortical, striatal, and cerebellar β2* nAChRs than non-smokers (Staley et al. 2006). Furthermore, β2* nAChRs in the anterior cingulate and frontal cortex were significantly correlated with the number of days following cessation of smoking. This was interpreted as a progressive increase of the number of β2* nAChRs in proportion to the number of consecutively abstinent days. In addition, a significant negative correlation was observed between β2* nAChR availability in the post-central gyrus or somatosensory cortex and the urge to relieve withdrawal symptoms by smoking (Staley et al. 2006). Similarly, in rodents, nAChR upregulation in the dorsal hippocampus was associated with withdrawal-related deficits in hippocampal learning (Gould et al. 2012; Portugal et al. 2012).

A variety of cellular processes influence receptor upregulation (Govind et al. 2009; Rezvani et al. 2009, 2010; Henderson et al. 2014). Receptors containing the β2 subunits are particularly sensitive to nicotine-induced upregulation. If β4 replaces β2 subunits in either α3* or α4* nAChRs, receptor upregulation is significantly reduced (Wang et al. 1998; Sallette et al. 2004). As replacement of β2 by β4 is sufficient to increase nAChR levels at the plasma membrane in the absence of nicotine, the proposed “chaperoning” function of nicotine might not be as effective (Srinivasan et al. 2011; Henderson et al. 2014). The presence of an accessory nAChR subunit in the receptor complex can also influence nicotine-induced upregulation. For example, the presence of α5 renders α4β2* nAChRs insensitive to nicotine-induced upregulation (Mao et al. 2008). Conversely, co-expression of β3 increases α6β2 and α6β4 receptor levels and enhances nicotine-induced upregulation of α6β2β3 receptors compared to α6β2 receptors (Tumkosit et al. 2006). The effects of β3 on receptor trafficking and upregulation may help explain why in the striatum α6-containing

receptors without β3 are downregulated by nicotine, while those containing β3 are unaffected (Perry et al. 2007).

4.2 Desensitization of nAChRs

While some combinations of nAChR subunits render receptor complexes more prone to upregulation than others, desensitization is a prominent mechanism that contributes to this upregulation (Fenster et al. 1999). As nicotine has a half-life in humans of 2 h or more, nicotine accumulates during a day of regular smoking to reach steady-state plasma concentrations typically ranging between 10 and 50 ng/mL (Graham et al. 2007). This long-lasting level of nicotine ensures that high-affinity nAChRs recurrently bind and unbind nicotine throughout the day (Picciotto et al. 2008). Consequently, receptors undergo transitions between different conformations in response to ligand binding and dissociation, and agonists will tend to stabilize particular conformations. Because desensitized nAChR conformations have a higher affinity for agonist, nAChRs will increasingly adopt desensitized conformations in response to chronic nicotine exposure (Quick and Lester 2002; Picciotto et al. 2008). It has been suggested that receptor desensitization may be a major contributor to the upregulation observed in chronic users of nicotine-containing products (Benowitz 2008). As the usage patterns of regular smokers result in persisting levels of circulating nicotine over the course of the day, nAChR desensitization occurs in response to ongoing occupancy of CNS nAChRs. For example, working with 11 tobacco-dependent individuals using a radiotracer developed to image α4β2* nAChRs with positronemission tomography (PET), it was found that regular smokers approach complete saturation of CNS α4β2* nAChRs throughout the day (Brody et al. 2006). After 2 days of abstaining from smoking, participants' cravings were reduced only once nAChRs were again nearly saturated. It is important to note that rates of desensitization are not necessarily equivalent across all nAChR subunit combinations. For example, inclusion of the α5 subunit into α4β2* nAChRs decreases the extent of desensitization (Bailey et al. 2010).

When considering the implications of receptor upregulation and desensitization for symptoms of nicotine withdrawal, kinetics characterizing the two dynamics may present critical processes by which withdrawal signaling occurs. In fact, nAChRs recover from desensitization on the scale of seconds to hours (Gentry and Lukas 2002). Additionally, studies have demonstrated that upregulation of nAChR expression can persist for several days following termination of chronic nicotine (Pietila et al. 1998; Staley et al. 2006). Accordingly, as the rates of desensitization and recovery from receptor upregulation during nicotine withdrawal are incongruent, a physiological landscape is established in which there are more receptors expressed than would be present in nicotine-naïve individuals, and these receptors are less responsive to agonist binding. When nicotine levels are low or absent, the nAChRs recover from desensitization, leading to potentially overactive cholinergic signaling. This process represents a major factor in the neurobiology of nicotine withdrawal (Dani and Heinemann 1996). Considering this dynamic, Gould and colleagues (Gould et al. 2012) have proposed a pharmacological strategy of treating hypersensitive nAChR systems with compounds that maintain receptor desensitization, or diminish cholinergic signaling while avoiding upregulation and activation, to alleviate the negative impact of withdrawal.

5 Anatomical Structures and Circuits Implicated in Nicotine Withdrawal

Given the diversity of symptoms manifested during nicotine withdrawal, a constellation of anatomical structures is likely involved in its etiology. Different anatomical structures in the CNS express distinct populations of nAChRs. The variety of nAChRs, combined with the specificity determined by afferent/efferent projections, produces the distinct neurochemistry and signaling that underlies the withdrawal syndrome. Evaluating changes in behavior relative to WT controls upon removal of particular nAChR subunits has been crucial to characterizing the contributions that each subunit makes to the withdrawal syndrome. If a withdrawal symptom exhibited by wild-type mice is absent in mice that lack functional expression of a specific nAChR subunit in a particular CNS structure, the specific subunit is likely critical to the withdrawal symptom in question. By performing these kinds of experiments, neuronal circuits contributing to the withdrawal syndrome will continue to be defined, and viable targets to enable the development of more specific therapeutics will be identified. Thus far, several brain structures have been already implicated in the withdrawal syndrome.

5.1 Dopaminergic System

The mesocorticolimbic dopamine (DA) system has been identified as critical to the development of addictive behaviors, and DA signaling is involved in reward-based reinforcement of drug-derived behaviors. While the DAergic VTA has been widely implicated in the rewarding aspects of addictive drugs, it has also been shown to participate in the signaling of aversion and lack of expected reward (Schultz et al. 1998; Ungless et al. 2004; Tobler et al. 2007; De Biasi and Dani 2011). Dopaminergic deficits in the mesolimbic pathway, particularly in the nucleus accumbens, are among the neurochemical mechanisms underlying the symptoms of nicotine withdrawal (Hildebrand et al. 1998; Carboni et al. 2000; Rada et al. 2001). Such deficiencies in accumbal dopaminergic transmission are believed to contribute to the aversive anhedonic or dysphoric state experienced during nicotine abstinence (Koob and Le Moal 2008; Zhang et al. 2012). A reduction in basal DA levels was observed to linger for at least 5 days in mice following 12 weeks of chronic nicotine treatment (Zhang et al. 2012). This change in DA activity during withdrawal was correlated with a reduced modulatory influence by β2* nAChRs over DA release in the NAcc. What causes the hypodopaminergic state associated with withdrawal is not yet clear. However, increased inhibitory input to the VTA is a potential mechanism, as GABA input is sufficient to suppress burst firing even with excitatory inputs intact (Lobb et al. 2010; Jalabert et al. 2011). GABAergic inputs arrive at the VTA from the substantia nigra pars reticulata, nucleus accumbens, ventral pallidum/globus pallidus, laterodorsal tegmentum, pedunculopontine nuclei, diagonal band of Broca, bed nucleus of the stria terminalis, and the caudal tip of the VTA, termed the rostromedial tegmental nucleus (RMTg) (Geisler and Zahm 2005; Jhou et al. 2009; Kaufling et al. 2009). Activity in the RMTg increases upon exposure to aversive stimuli and decreases upon exposure to reward stimuli, representing inversely correlated signaling patterns (Hong et al. 2011).

5.2 Habenular Complex and Interpeduncular Nucleus

The habenular complex, comprising the lateral (LHb) and medial (MHb) nuclei, is a diencephalic, epithalamic structure located ventrally along the dorsal third ventricle (Dani and De Biasi 2013). The habenula has been demonstrated to participate in the signaling underlying fear, anxiety, depression, and stress (Viswanath et al. 2013). The LHb sends excitatory glutamatergic projections to the RMTg and is a major component of the circuit underlying negative reward (Dani and De Biasi 2013). Additionally, the cholinoceptive cellular population within the medial habenula (MHb) has been associated with the aversive effects of nicotine (Fowler et al. 2011). The most prominent efferent output from the MHb is to the IPN via the fasciculus retroflexus, forming the MHb IPN axis. Through this projection, the MHb releases acetylcholine (ACh), substance P (SP), and glutamate onto the IPN (Zhao-Shea et al. 2013). The MHb also hosts the expression of norepinephrine (NE), serotonin, ATP, and several neuropeptides (Dao et al. 2014). The IPN is located in the ventral midbrain, with the VTA and median raphe nucleus located dorsally.

Mice chronically treated with nicotine exhibit symptoms of withdrawal upon microinjection of mecamylamine into the MHb and IPN, but fail to manifest withdrawal symptoms when mecamylamine is injected to the cortex, hippocampus, or VTA (Salas et al. 2009). Furthermore, lidocaine infusion into the MHb to inhibit signaling significantly blocks the manifestations of somatic symptoms resulting from both mecamylamine-precipitated and spontaneous induction of nicotine withdrawal (Zhao-Shea et al. 2013). Following precipitated nicotine withdrawal in WT mice, there are elevated markers of neuronal activation in GABAergic IPN cells (Zhao-Shea et al. 2013). The involvement of the IPN in the manifestation of nicotine withdrawal symptoms was demonstrated by expression of channelrhodopsin (ChR2), a light-driven excitatory ion channel, in the GABAergic cells of the IPN (Zhao-Shea et al. 2013). Additionally, infusion of an α3β4* nAChR-selective antagonist into the IPN elicited somatic withdrawal signs in nicotine-naïve mice and an even greater number of total symptoms in mice chronically treated with nicotine (Zhao-Shea et al. 2013). Considered together, these data strongly implicate the MHb-IPN circuit in the nicotine withdrawal syndrome. As previously discussed, the α5, α3, and β4 nAChR subunits participate in withdrawal symptomatology. They are each densely expressed in the MHb and/or IPN (Salas et al. 2003b, 2004) and are potential targets for treatments of the nicotine withdrawal syndrome (Grady et al. 2009).

5.3 Hippocampus

The hippocampus is a crucial structure for the manifestation of cognitive deficits during nicotine withdrawal. Contextual FC is dependent upon hippocampal signaling (Davis et al. 2005), and during spontaneous withdrawal from nicotine, mice exhibit deficient contextual FC relative to saline-treated controls (Davis et al. 2005). Administration of nicotine to mice undergoing withdrawal ameliorates this deficit (Davis et al. 2005). This effect was not attributable simply to a lowered threshold of fear responses inherent to the effects of nicotine because cued FC was equivalent between nicotine-treated and nicotine-naïve groups. Similar deficits of contextual fear learning were observed when a nAChR antagonist (dihydro-β-erythroidine) was infused into the hippocampus of WT mice chronically treated with nicotine (Davis and Gould 2009). The work also implicated β2* nAChRs in the

manifestation of memory-associated deficits during nicotine withdrawal. Furthermore, chronic nicotine treatment upregulated a variety of nAChRs in the hippocampus, and the duration of β2 subunit upregulation relative to other nAChR subunits most closely corresponded to the total duration of memory-associated withdrawal symptoms (Gould et al. 2012).

5.4 Extended Amygdala

The bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), and shell of the nucleus accumbens (NAc-Sh) form the extended amygdala, an anatomically and neurochemically interconnected system located in the basal forebrain (Smith and Aston-Jones 2008). The system has been implicated in stress-related components of drug withdrawal and is a site of interaction between corticotropinreleasing factor (CRF) and NE transmission. Nicotine withdrawal leads to an increase in CRF in the CeA, and blockade of CRF1 receptors diminishes nicotine withdrawalinduced anxiety-like behavior (George et al. 2007). The CRF/CRF1 receptor signaling in the CeA also mediates nicotine withdrawal-induced increases in nociceptive sensitivity in rats that are dependent on nicotine (Baiamonte et al. 2014).

Given the polygenic nature of nicotine addiction, many other mechanisms and many other brain areas are likely to influence the manifestations of withdrawal. Preclinical research is increasingly benefitting from the information coming from genetic studies and pharmacogenetic trials, and the speed of discovery is destined to increase in the coming years.

6 Summary and Research Moving Forward

Individuals undergoing nicotine withdrawal experience both affective and somatic symptoms beginning between 4 and 24 h after ceasing intake. The syndrome is most severe in the first week, but it can persist for longer periods of time. During this time, relapse is incentivized by the ability of nicotine to alleviate or abolish withdrawal symptoms. In addition, there are cognitive deficits that manifest during nicotine withdrawal, including difficulty concentrating, increased reaction times in tasks requiring sustained attention, and impaired episodic and working memory (Myers et al. 2008; Wesnes et al. 2013). Knowledge of the molecular mechanisms that govern the emergence and intensity of withdrawal symptoms, and elucidation of the genetic variants associated with successful smoking cessation, will facilitate the development of personalized treatments.

New techniques in neuroscience are enabling researchers to ask previously unapproachable questions regarding which genes, circuits, and neurochemical systems mediate and modulate different aspects of addiction and withdrawal. Using transgenic mouse lines or viral delivery, designer receptors [DREADDs (Rogan and Roth 2011)] and light-driven ion channels [opsins (Yizhar et al. 2011)] can be expressed in particular anatomical structures or in particular neuronal types. These genetically targeted receptors and ion channels can be used to control the activities of specific circuits and populations of neurons in freely behaving mice. When combined with receptor KO mice, or with the delivery of nAChRs

derived from specific human SNPs, these techniques enable researchers to manipulate neuronal activity while monitoring the dynamics of withdrawal-related behaviors.

Genetic studies of nicotine dependence and smoking cessation have identified several risk factors using GWAS, candidate gene approaches, and pharmacogenetic analyses. These studies provide targets that can be validated with large population samples and across ethnicities. Once putative genes are identified, hypotheses of the functional roles of such candidate genes can be tested in preclinical animal models. The functional consequences of some SNPs can be addressed relatively easily if the SNPs are non-synonymous coding variants. The best example is provided by rs16969968, the SNP that leads to an aspartic acid for an asparagine substitution (D398N) in CHRNA5 (Jackson et al. 2010). The impact of the mutation that defines the risk allele has been validated repeatedly in vitro and in animal models (Kuryatov et al. 2011; Morel et al. 2014). For many of the SNPs, however, there is no change in protein sequence, and therefore, it is harder to formulate clear functional hypothesis. Candidate SNPs can have a multitude of biochemical functions, such as altering DNA methylation, histone modification, or accessibility of DNA to transcription factor binding that can impact when, where, and how much a gene, and its protein, is expressed. Fortunately, analytical tools, such as the Encyclopedia of DNA Elements (ENCODE), can help the design of experiments that explore disease-related variants located within non-coding regions (Siggens and Ekwall 2014). Increasing attention is being paid to the functional analysis of rare variants, as common variants can explain only a small percentage of the variance in smoking-related phenotypes. More work is necessary at the bench and in the clinic, as a collection of genes likely operates collectively to predispose or protect individuals to or from nicotine addiction and withdrawal. Ultimately, this increased capability to comprehensively characterize the etiology of withdrawal symptoms will inform more competent and efficient drug design. The future undoubtedly holds greater development of pharmacological nicotine cessation therapeutics.

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