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Nicotine aversion: Neurobiological mechanisms and relevance to tobacco dependence vulnerability

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Nicotine stimulates brain reward circuitries, most prominently the mesocorticolimbic dopamine system, and this action is considered critical in establishing and maintaining the tobacco smoking habit. Compounds that attenuate the rewarding effects of nicotine are considered promising therapeutic candidates for tobacco dependence, although many of these drugs have off-target effects that limit their translation to the human condition. Consequently, the neurobiological mechanisms of nicotine reward are the subject of intense investigation. Nicotine is also highly noxious, particularly at higher doses, and aversive reactions to nicotine after initial exposure can decrease the likelihood of developing a tobacco habit in many first time smokers. Nevertheless, relatively little is known about the mechanisms of nicotine aversion. The purpose of this review is to present recent new insights into the neurobiological mechanisms that regulate avoidance of nicotine. First, the role of the mesocorticolimbic system, so often associated with nicotine reward, in regulating nicotine aversion is highlighted. Second, genetic variation that modifies noxious responses to nicotine and thereby influences vulnerability to tobacco dependence, in particular variation in the *CHRNA5-CHRNA3-CHRNB4* nicotinic acetylcholine receptor (nAChR) subunit gene cluster, will be discussed. Third, the role of the habenular complex in nicotine aversion, primarily medial habenular projections to the interpeduncular nucleus (IPN) but also lateral habenular projections to rostromedial tegmental nucleus (RMTg) and ventral tegmental area (VTA) are reviewed. Forth, brain circuits that are enriched in nAChRs, but whose role in nicotine avoidance has not yet been assessed, will be proposed. Finally, the feasibility of developing novel therapeutic agents for tobacco dependence that act not by blocking nicotine reward but by enhancing nicotine avoidance will be considered.

Introduction

Nicotine is considered the major reinforcing component of tobacco responsible for addiction in human smokers (Stolerman and Jarvis, 1995), and it has been shown that humans, non-human primates and rodents will volitionally self-administer the drug (Corrigall and Coen, 1989; Goldberg et al., 1981; Harvey et al., 2004; Watkins et al., 1999). Volitionally consumed nicotine is known to stimulate activity in brain reward circuitries (Kenny and Markou, 2006), with this action considered central to the establishment and maintenance of the tobacco habit in human smokers. It is important to note, however, that instead of hedonic reactions, most smokers report their initial smoking experiences as unpleasant. This reflects the fact that in addition to its rewarding effects, nicotine is also highly noxious. Highlighting this dichotomous nature of nicotine, doses of the drug that support maximal rates of responding in squirrel monkeys also induce marked symptoms of aversion, such as vomiting, when the drug-taking habit is being acquired. Moreover, monkeys work to avoid non-contingent delivery of intravenous nicotine infusions even though they will work equally hard to obtain those same nicotine infusions when they are available for contingent delivery (Goldberg and Spealman, 1982, 1983; Goldberg et al., 1981; Goldberg et al., 1983; Spealman and Goldberg, 1982). These aversive reactions to nicotine are important in the context of tobacco dependence, as stronger aversive reactions to nicotine after initial exposure are negatively correlated with the development of habitual tobacco use in first time smokers (Sartor et al., 2010).

Aversive responses to nicotine also appear to play key roles in determining the overall amounts of tobacco smoke consumed and patterns of intake. Indeed, when levels of nicotine contained in tobacco are varied, smokers are far more

efficient at titrating their intake downwards when consuming high-nicotine-content tobacco to avoid noxious effects of the drug (Henningfield and Goldberg, 1983a; Henningfield et al., 1986; Russell et al., 1975), than they are at adjusting their intake upward to compensate for reduced nicotine in low-content tobacco (Sutton et al., 1978). Hence, self-regulation of consumption to avoid noxious effects of nicotine is far better regulated that compensation upwards to avoid a reduction in nicotine intake. Also consistent with a key role for noxious nicotine effects in controlling tobacco consumption, a treatment strategy previously employed to facilitate smoking cessation, but no longer typically used (Hajek and Stead, 2004), is to encourage smokers to inhale tobacco smoke more rapidly and deeply than usual. This results in aversive reactions to nicotine, with this increased nicotine exposure from more rapid consumption resulting in persistent suppression of intake (Norton and Barske, 1977). It is likely, therefore, that tolerance to the unpleasant effects of nicotine, and learning to efficiently control tobacco smoking to avoid these effects, must develop in order for habitual tobacco use to be established (Russell, 1979). As such, it is probable that discrete circuitries in the brain respond to the noxious properties of nicotine and that learning to titrate patterns of tobacco consumption in order to avoid activation of these circuitries plays a key role in the acquisition of smoking behavior. Indeed, the nicotinic acetylcholine receptor antagonist mecamylamine has been shown to block both the rewarding and aversive effects of nicotine, delivered by intravenous infusions to human volunteers (Lundahl et al., 2000), consistent with their being at least two discrete populations of nAChRs with each regulating either rewarding or aversive effects of the drug. Diminished sensitivity of nicotine-related aversion systems in the brain is therefore likely to increase vulnerability to develop habitual smoking. As such, it may be

possible to target such circuitries in brain to enhance the noxious properties of nicotine with small molecule drugs, offering a novel treatment strategy to facilitate lower levels of tobacco consumption, and perhaps increased ability to cease tobacco smoking altogether. Nevertheless, until recently relatively little was known about which circuits in the brain regulate nicotine aversion, in sharp contrast to our burgeoning knowledge on mechanisms of nicotine reward. Here, we summarize much of the current knowledge on the mechanisms of nicotine aversion.

The mesocorticolimbic system and nicotine aversion

As noted above, the reward-enhancing actions of nicotine are hypothesized to play a key role in the establishment and maintenance of the tobacco habit in human smokers (Kenny and Markou, 2006). The reward-related actions of nicotine are thought to be related to the stimulatory effects of the drug on neuronal nicotinic acetylcholine receptors (nAChRs) containing α4 and β2 subunits (denoted α4β2* nAChRs), particularly those located in the ventral tegmental area (VTA) (Corrigall et al., 1992; Ikemoto et al., 2006a; Maskos et al., 2005; Picciotto et al., 1998; Tapper et al., 2004). Indeed, nicotine-induced activation of α4β2* nAChRs in the VTA increases forebrain dopamine transmission, most prominently in the shell region of the nucleus accumbens (NAc), and this has been shown to contribute to the reinforcing properties of the drug in laboratory animals (Iyaniwura et al., 2001; Nisell et al., 1997; Pontieri et al., 1996). Because of their central role in nicotine reinforcement, α4β2* nAChRs are considered important targets for the development of smoking cessation agents. Varenicline (Chantix) is the only FDA-approved smoking cessation agent that was rationally designed through traditional drug

discovery and was developed as an α4β2* nAChR partial agonist (Coe et al., 2005; Dwoskin et al., 2009; Lerman et al., 2007; Reus et al., 2007).

In addition to α4β2* nAChRs in VTA, accumulating evidence suggests that α7 nAChRs in this site may also play a role in nicotine reinforcement. Nicotine-induced increases in glutamatergic transmission in the VTA is thought to occur through actions at both α4β2* and α7 nAChRs, leading to increases in mesoaccumbens dopamine transmission and regulation of nicotine self-administration behavior (Besson et al., 2012; Gao et al., 2010; Mansvelder and McGehee, 2000; Mao et al., 2011; Melis et al., 2013; Pons et al., 2008; Schilstrom et al., 2000). Supporting a key role for nicotine-induced increases in glutamatergic transmission in the VTA with nicotine reinforcement, blockade of local NMDA receptors profoundly decreases nicotine intake in rats (Kenny et al., 2009b). Considering the well-established role for the VTA and mesocorticolimbic dopamine transmission in regulating the reinforcing properties of nicotine and other drugs of abuse, it is interesting to note that accumulating evidence implicates this same system in nicotine aversion. Lesions of cholinergic innervation of the VTA arising from the pedunculopontine tegmental nucleus (PPTg) blocked the expression of reward-related behaviors in response to nicotine and rendered 'rewarding' doses of nicotine aversive, reflected in increased avoidance of the drug (Laviolette et al., 2002). Similarly, blockade of α7 nAChRs or NMDA receptors in the VTA switched the effects of nicotine from rewarding to aversive (Kenny et al., 2009b; Laviolette and van der Kooy, 2003b). Projections from the VTA into the striatum and NAc also appear to be influenced by nicotinic signaling. Presynaptic nAChRs directly regulate the release of dopamine from dopaminergic terminals (Rice and Cragg, 2004; Zhou et al., 2001), in which

α4α5β2 nAChR subtype regulates DA release in the dorsal caudate/putamen and the α4α6β2β3 nAChR is involved in the NAc core (Exley et al., 2012). In these regions, several subtypes of nAChRs have been identified, including the α4, α5, α6, α7, β2 and β3 (Exley et al., 2012; Marks et al., 1992; Seguela et al., 1993; Wada et al., 1989), and as such, nicotine has the potential to directly and/or indirectly modulate various types of neurotransmission. An intricate network of cholinergic interneurons is present throughout the NAc and striatum. In addition to releasing acetylcholine, these interneurons may also corelease glutamate (Higley et al., 2011). Presynaptic α4β2* nAChRs have been shown to enhance GABA release from inhibitory interneurons, which may subsequently inhibit the cholinergic interneurons (Grilli et al., 2009; Koos and Tepper, 2002; Sullivan et al., 2008). Increased accumbal acetylcholine release is found during expression of a conditioned taste aversion (Mark et al., 1995), and pharmacologically enhancing cholinergic signaling in the NAc is sufficient to produce a conditioned taste aversion (Taylor et al., 2011). It has been proposed that dopamine and acetylcholine act in an opposing manner within the NAc, in which dopamine mediates reward-related processing, whereas acetylcholine signals aversion-related events (Hoebel et al., 2007). Support for this hypothesis derives from the findings that drugs of abuse, such as nicotine, stimulate mesolimbic dopamine release (Mifsud et al., 1989), and in contrast, increased acetylcholine in the NAc attenuates drug self-administration and promotes escape behavior (Glowa et al., 1997; Rada and Hoebel, 2001; Williams and Adinoff, 2008). Furthermore, nicotine withdrawal elicits a decrease in dopaminergic signaling that is paralleled by an increase in acetylcholine (Rada et al., 2001). Although evidence supports this concept of dopamine/acetylcholine opponent processing, more recent findings suggest that acetylcholine can directly enhance dopamine release in this brain region

as well (Cachope et al., 2012; Threlfell et al., 2012). Moreover, rather than diminishing its rewarding effects, blockade of dopamine receptors can in some instances enhance the rewarding effects of nicotine, as measured using place conditioning procedures (Grieder et al., 2012; Laviolette and van der Kooy, 2003a). Laviolette and colleagues have shown that blockade of dopamine D2 receptors in the shell region of the nucleus accumbens (NAc), or D1 receptors in the core region of the accumbens, can switch the effects of intra-VTA infusions of higher doses of nicotine from aversive to rewarding during place conditioning (Laviolette et al., 2008). Clarke and co-workers have similarly shown that lesioning dopamine inputs to the NAc shell, achieved using local infusions of the toxin 6-hydroxydopamine, abolished the rewarding effects of intravenously delivered nicotine infusions (Sellings et al., 2008). Conversely, lesions of dopamine inputs into the NAc core region increased the rewarding effects and abolished the aversion-related responses to nicotine, as measured by conditioned taste aversion (Sellings et al., 2008). These findings suggest that dopamine signaling, at least somewhat compartmentalized between the NAc shell and core regions, regulate the rewarding and aversive effects of nicotine, respectively.

One may question how mesoaccumbens dopamine transmission can be critical for both the reinforcing properties of nicotine that support consumption of the drug and also the aversive effects of the drug that support avoidance? Two recent findings appear to shed important light on this issue. First, Malenka and colleagues have shown that distinct populations of VTA dopamine neurons may separately encode reward-related and aversion-related information, and thus guide approach or avoidance behaviors accordingly. Specifically, they found that the cholinergic inputs to the VTA from the laterodorsal tegmental

nucleus (LDTg), and inputs to the VTA from the lateral habenula (LHb), regulate reward and aversion behaviors in mice, respectively (Lammel et al., 2012). Moreover, LDTg neurons preferentially synapse onto dopamine neurons that project to the NAc shell, whereas LHb neurons synapse onto dopamine neurons that project to the medial prefrontal cortex or the rostromedial tegmental nucleus (RMTg), both areas of the brain involved in response suppression (Lammel et al., 2012). Hence, these findings are consistent with the concept that discrete populations of dopamine neurons receive information from brain regions that regulate appetitive or aversive responses, which in turn project to discrete areas of the accumbens (and other brain sites) to regulate approach versus avoidance behaviors. Consistent with this possibility, Suto and Wise have shown recently that enhancing dopamine transmission by co-infusion of a D1 and a D2 receptor agonist in the NAc core, but not shell, increased satiety-like responses to intravenously self-administered cocaine infusions (Suto and Wise, 2011); see also (Suto et al., 2009; Suto et al., 2010).

In addition to dopamine and acetylcholine, opioid signaling also appears to be an important mediator of aversive processing within the NAc, most notably through the kappa opioid receptor (KOR) (McCutcheon et al., 2012; Resendez et al., 2012), and interactions between nicotinic and KOR signaling have been established. Administration of KOR agonists can attenuate nicotine-induced hyperlocomotion in rats (Hahn et al., 2000), and chronic nicotine pretreatment can enhance the effectiveness of KOR agonists to induce a place aversion or anxiolytic-like response in adult rats (Tejeda et al., 2012). Following chronic nicotine exposure, the somatic signs of nicotine withdrawal may also be potentiated by KOR activation, an effect that can be reversed by co-

administration of the KOR antagonist, nor-BNI (Tejeda et al., 2012). At the intracellular level, it is an interesting possibility that nicotine signaling in accumbens may exert its aversive effects by stimulating cAMP signaling cascades, resulting in activation of cAMP response element-binding protein (CREB) and consequently the increased transcription of the endogenous KOR ligand, dynorphin (McCarthy et al., 2012). Taken together, the above findings suggest that neurotransmission in the NAc shell regulates the reinforcing effects of nicotine and other drugs of abuse that drive their consumption. Conversely, the NAc core may regulate satiety responses and avoidance behaviors that limit consumption of nicotine and other addictive drugs.

Genetics of tobacco dependence and nicotine aversion

In mice, it has been shown that genetic factors play a key role in regulating sensitivity to the aversive effects of nicotine (Risinger and Brown, 1996). Emerging data from genome-wide association studies (GWAS) are identifying polymorphisms that increase vulnerability to tobacco dependence in humans, and also support the notion that sensitivity to nicotine aversion may be influenced by genetics. A prominent gene in which allelic variation has been associated with risk of developing tobacco dependence is *CYP2A6*. This gene encodes the cytochrome P450 enzyme responsible for ~80% of nicotine metabolism in the human liver (Raunio and Rahnasto-Rilla, 2012; Ray et al., 2009; Thorgeirsson et al., 2010). CYP2A6 polymorphisms are more commonly found in individuals of Asian descent and less frequently in Caucasians (Johansson and Ingelman-Sundberg, 2011). The *CYP2A6* gene is highly polymorphic, with over 60 allelic variants known. Of these, 17 alleles have been shown to exhibit reduced function, whereas two of the variants result in increased nicotine metabolism (Mwenifumbo et al., 2008; Raunio and

Rahnasto-Rilla, 2012). Allelic variations that result in a slower metabolism would be expected to allow for a more prolonged action of nicotine. As such, nicotine clearance is expected to be decreased and doses required to experience the rewarding and aversive effects of the drug would be lower (e.g., leftward shift of the dose-response curve). Consistent with this prediction, individuals with a slow metabolizer *CYP2A6* genotype are less vulnerable to develop tobacco dependence than those with normal metabolism (Audrain-McGovern et al., 2007; Bloom et al., 2011; Thorgeirsson et al., 2010), potentially due to a greater aversive response when similar amounts of nicotine are consumed. In further support of this notion, the slow metabolizers intake less nicotine per day and are more successful when attempting to quit smoking than individuals with a normal metabolism (Patterson et al., 2008; Rodriguez et al., 2011; Strasser et al., 2007). Thus, a slower metabolism permits smaller quantities of the drug to be consumed that lead to prolonged activity at nAChRs. As such, it may be more difficult for the individual behaviorally titrate their intake to avoid synaptic accumulation and subsequent activation of lower affinity nAChRs involved in aversive signaling (see nAChR subtype discussion below). In contrast, faster rates of nicotine metabolism would allow for quicker recovery from the actions of nicotine, resulting in diminished sensitivity to the aversive effects of the drug. Indeed, individuals that rapidly metabolize nicotine exhibit lower cessation rates and more severe withdrawal symptoms (Bloom et al., 2011; Patterson et al., 2008). Given that both withdrawal duration and severity predict likelihood to relapse (Piasecki et al., 2000), the aversive state elicited during nicotine withdrawal likely motivates the individual to seek and continue to consume the drug, thus maintaining the tobacco smoking habit. Interestingly, metabolism regulated by *CYP2A6* may be directly modulated by tobacco exposure and contribute to

the development of dependence. Smokers exhibit a slower clearance of nicotine compared to non-smokers (Hukkanen et al., 2005), and nicotine appears to reduce *CYP2A6* transcript and protein levels in monkeys (Ferguson et al., 2012; Schoedel et al., 2003). Finally, given that the *CYP2A6* gene is also expressed in respiratory tissues, it is perhaps not surprising that *CYP2A6* allelic variation is associated with smoking-related diseases, such as COPD and cancer (Ariyoshi et al., 2002; Hukkanen et al., 2002; Wassenaar et al., 2011).

Insert Fig. 1 about here

Since the main site of action of nicotine in the brain is the nAChRs, it may be expected that genetic variation in the genes encoding nAChR receptor subunits would be implicated in tobacco dependence vulnerability. However, what may be surprising is the fact that the subunit genes most implicated in dependence vulnerability are not those that encode the high-affinity nAChRs responsible for regulating the rewarding effects of nicotine ($\alpha4\beta2$* nAChRs), but instead lesser studies $\alpha5$, $\alpha3$ and $\beta4$ nAChRs nAChR subunit genes best known for their role in regulating ganglionic nAChR transmission (Fig. 1). Indeed, recent GWAS studies have shown that variation in the *CHRNA3-CHRNA5-CHRNAB4* gene cluster, which encodes the $\alpha3$, $\alpha5$ and $\beta4$ nAChR receptor subunits, respectively, is prominently associated with predisposition to develop tobacco dependence. Variation in this cluster is also associated with vulnerability to many smoking-associated diseases such as chronic obstructive pulmonary disease (COPD) and lung cancer (Bierut et al., 2008; Hung et al., 2008; Saccone et al., 2010; Saccone et al., 2007; Thorgeirsson et al., 2008). Strikingly, the risk of developing tobacco dependence appears to be more than doubled in individuals carrying two copies of the *CHRNA5* risk

allele, rs16969968, a polymorphism that results in an aspartic acid to asparagine substitution at amino acid residue 398 (D398N). The D398N risk variant is also associated with early onset of smoking behavior (Weiss et al., 2008), a self-reported “pleasurable buzz” during smoking (Sherva et al., 2008), and heavy smoking (Berrettini et al., 2008; Bierut et al., 2008; Grucza et al., 2008; Stevens et al., 2008). As described in detail below, genetic variation in the *CHRNA5-CHRNA3-CHRNB4* subunit cluster, and resultant alterations in the function of mature nAChRs incorporating these altered subunits, appears to regulate tobacco dependence vulnerability not by altering the stimulatory effects of nicotine on brain reward systems, but instead by diminishing the aversive effects of nicotine.

The *CHRNA5-CHRNA3-CHRNB3* gene cluster and nicotine aversion

Genetic variation in enzymes responsible for nicotine metabolism can influence tobacco dependence vulnerability as noted above, perhaps by regulating sensitivity to the aversive effects of nicotine. Considering that genetic variation in the *CHRNA5-CHRNA3-CHRNB4* gene cluster, particularly genetic variation that diminishes □5* nAChR activity, also influences tobacco dependence vulnerability, our laboratory sought to determine if this effect was because of enhanced rewarding effects of nicotine or instead diminished sensitivity the aversive properties of the drug.

Using a mouse intravenous nicotine self-administration procedure, we assessed nicotine intake in wild-type (WT) and α5 subunit knockout (KO) mice on a C57BL6 background; for full details, see (Fowler et al., 2011). We found that both genotypes responded for intravenous nicotine infusions according to an inverted U-shaped dose-response (D-R) curve (Fig. 1). Levels of intake were

similar between WT and KO mice when lower doses of nicotine were available. The ascending portion of the D-R curve is thought to reflect increasing reinforcing properties of nicotine as the unit dose increases (Henningfield and Goldberg, 1983b; Lynch and Carroll, 2001). Therefore, our data suggest that the rewarding effects of nicotine are unaltered by α5 subunit deletion. In contrast, strikingly different patterns of intake were revealed when higher unit doses of nicotine were available, with the KO mice responding far more vigorously than WT mice (Fig. 1). In control experiments, it was shown that this increased nicotine intake in KO mice was not secondary to alterations in operant performance or alterations in sensitivity to drug or non-drug paired conditioned stimuli.

When we examined total amounts of nicotine consumed at each dose available in the WT and α5 KO mice, we found that WT mice consumed ~1.5 mg kg^{-1} per session independent of the available dose (Fig. 1). In contrast, KO mice consumed progressively greater amounts of the drug as the dose increased. This suggests that WT mice efficiently titrate their intake whereas this behavior is largely absent in α5 subunit KO mice. Similar to human smokers, diminished responding of rodents for nicotine as the unit dose increases is thought to reflect greater restraint over intake to avoid the increasingly aversive effects of the drug (Henningfield and Goldberg, 1983b; Lynch and Carroll, 2001). As such, these findings suggest that diminished aversive effects of nicotine, measured by increased responding on the descending portion of the D-R curve for self-administered nicotine, likely explains the increased nicotine intake in α5 KO mice see (Fowler et al., 2011) and perhaps in humans carrying CHRNA5 risk alleles (Bierut et al., 2008). Consistent with these findings, it was also reported that high nicotine doses

continued to support place-conditioning behavior in the knockout mice even though these doses were no longer rewarding in WT mice (Jackson et al., 2010). While it is plausible that rodents vary in their aversive responses to nicotine as compared to humans, the similarities between the findings in both species supports the notion that similar aversive states underlie nicotine's effects. The plasma concentrations achieved by self-administering WT mice are comparable to those in humans after 5 hours of smoking their preferred cigarette (Fowler and Kenny, 2011; Fowler et al., 2011; Matta et al., 2007; Russell et al., 1975). Moreover, self-administration of intravenous nicotine in both rodents and humans decreases when higher doses of the drug are provided (Fowler and Kenny, 2011; Harvey et al., 2004), similar to that found with cigarette smoking in humans (Henningfield and Goldberg, 1983a; Henningfield et al., 1986; Russell et al., 1975). These data, combined with the findings that altered expression of the α5* nAChRs in mice, rats and humans results in a similar behavior profile in relation to nicotine consumption, support the contention that the aversion induced by nicotine is likely conserved across species. Even so it remains unclear what precise psychological state(s) are achieved with these high doses of nicotine, and while this type of assessment may be more readily investigated in humans, it will likely remain unclear in rodents until significant advancements are achieved in assessing rodent psychological states. In addition to α5* nAChRs, 4* nAChRs also play an important role in regulating the aversive properties of nicotine (Frahm et al., 2011). Specifically, it was shown that transgenic overexpression of this subunit in mice increased their sensitivity to the aversive properties of the drug and consequently decreased nicotine drinking behavior (Frahm et al., 2011). No studies to date have reported the effects of genetic ablation or modification of 3 nAChR subunit expression on nicotine intake, likely because deletion of this

subunit results in post-natal lethality. Taken together, the above findings suggest that the *CHRNA5-CHRNA3-CHRNB4* gene cluster plays a key role in nicotine aversion but not nicotine reward, with genetic variation in this cluster increasing vulnerability to tobacco dependence by attenuating the aversive effects of nicotine and thereby diminishing avoidance of the drug.

The medial habenula-interpeduncular systems and nicotine aversion

The above findings show that disruption of α5* nAChR signaling increases, whereas transgenic overexpression of 4 nAChR subunits decreases, nicotine intake. These findings may appear counterintuitive when the role for nAChRs in nicotine addiction has traditionally been to consider their involvement in nicotine reward. Indeed, disruption of nAChR signaling, particularly the high-affinity nAChRs (42*), usually results in diminished reinforcing effects of nicotine and consequently reduced consumption of the drug. Pharmacological blockade of 42* nAChRs, or genetic disruption of 2 nAChR subunits, almost invariably decreases nicotine intake in rats and mice (Corrigall et al., 1994; Corrigall et al., 1999; Corrigall et al., 2000; David et al., 2006; Ikemoto et al., 2006b; Kenny et al., 2009a; Maskos et al., 2005; Molles et al., 2006). Disruption of nAChR function in midbrain dopamine systems, particularly the VTA, is generally thought to be responsible for the decreased reinforcing effects of nicotine reported in these studies. In light of these previous studies, it is somewhat paradoxical that genetic ablation of α5* nAChR signaling increases nicotine intake in rats and mice. Hence, these findings suggest that α5* nAChR receptors, and perhaps also α3* and 4* nAChRs, regulate nicotine intake in a manner distinctly different from nAChRs in midbrain dopamine systems.

The α5 nAChR subunit mRNA is expressed in the VTA and substantia nigra pars compacta (SNc), as well as in deep layers of the cortex. However, the α5 nAChR subunit mRNA is most densely expressed in the medial habenula-interpeduncular nucleus (IPN) system (Marks et al., 1992). The β3 and β4 subunits are also predominately found in the MHb-IPN system. The MHb projects almost exclusively to the IPN via the fasciculus retroflexus (Fr) (Herkenham and Nauta, 1979). Functional α5* nAChRs are expressed on MHb afferents to IPN (Grady et al., 2009; Sheffield et al., 2000). Intriguingly, the MHb-IPN tract is known to limit consumption of noxious substances (Donovick et al., 1970; Thornton et al., 1994) and regulate avoidance of aversive stimuli (Donovick et al., 1970; Hammer and Klingberg, 1990; Meszaros et al., 1985; Thompson, 1960; Thornton et al., 1994; Wirtshafter, 1981). This system is also involved in the expression of somatic aspects of the nicotine withdrawal syndrome (Salas et al., 2009). We therefore hypothesized that α5* nAChRs, and perhaps β3* and β4* nAChRs, in the MHb-IPN regulate aversive effects of nicotine. Consistent with this hypothesis, we found that virus-mediated re-expression of the otherwise absent α5 subunit in the MHb-IPN system of the KO mice completely rescued their behavioral phenotype; these "rescued" α5 KO mice reduced their level of nicotine intake at higher doses consistent with the behavioral profile exhibited by WT mice. Conversely, virus-mediated knockdown of this subunit in the MHb-IPN system of rats resulted in greater intake, particularly when high unit doses of the drug were available for consumption (Fowler et al., 2011). These findings suggest that the *CHRNA5-CHRNA3-CHRNB4* gene cluster in the MHb-IPN system, and nAChRs in these sites that incorporate the subunits encoded by these genes, regulate the aversive effects of higher nicotine doses that serve to suppress intake. To more directly test this hypothesis, we have examined the effects of nicotine on

intracranial self-stimulation (ICSS) thresholds in α5 KO mice and in rats after knockdown of α5 subunits in the MHb-IPN system. Nicotine-mediated lowering of ICSS thresholds are thought to reflect the stimulatory actions of the drug on the brain reward systems responsible for establishing and maintaining the nicotine-taking habit in rodents and perhaps human tobacco smokers (Kenny, 2007). Conversely, elevations of ICSS thresholds induced by higher nicotine doses are thought to reflect an inhibitory action on brain reward systems that drives avoidance of the drug (Fowler et al., 2011). We found that global deletion of α5 nAChR subunits in the knockout mice (Fowler et al., 2013), or restricted knockdown of the subunits in the MHb-IPN system of rats, abolished the ICSS threshold-elevating effects of higher doses of nicotine but did not impact the threshold-lowering effects of lower nicotine doses. The specific role for α5* nAChRs in nicotine aversion but not nicotine reward may explain the shape of the D-R curve for nicotine self-administration in humans, primates and rodents, and why it is altered by α5* nAChR deficiency. Specifically, we propose that nicotine reward and aversion are dissociable effects, with the mesoaccumbens dopamine neurons that project to the accumbens shell region likely playing an important role in the positive reinforcing effects of nicotine, and the habenula-interpeduncular systems regulating nicotine aversion. The rewarding actions of nicotine may occur through the α4α6β2β3* nAChR subtype (Grady et al., 2007), which has the highest sensitivity to nicotine of any native nAChR so far identified (Grady et al., 2007) and would explain the “ascending” portion of the D-R curve. As α6 nAChR subunits are not expressed in the MHb-IPN aversion pathway, the high-affinity α4α6β2β3 nAChR subtype is not expressed locally. Instead, nicotine activates this pathway through lower affinity α5* nAChRs, accounting for the descending portion of the D-R curve at higher nicotine doses. The combinatorial effects of

nicotine at the high-affinity α4α6β2β3* nAChRs in the mesoaccumbens systems, and lower affinity α5* nAChRs in the MHb-IPN therefore likely explains the “window” of nicotine doses that are reinforcing, and the emergence of aversion at higher doses resulting in a U-shaped dose-response curve. Taking all the above data together, it seems that nicotine stimulates the MHb-IPN pathway through α5* nAChRs and thereby enhances glutamatergic transmission in, and consequent activation of, the IPN. Most available smoking cessation agents are thought to attenuate smoking behavior by targeting nAChRs in midbrain dopamine systems, perhaps by targeting the α4α6β2β3* nAChRs. As discussed below, it may be possible to rationally design new smoking cessation agents that act independent of nicotine reward and instead act by targeting α5* nAChRs in habenular aversion systems.

Finally, almost exclusively, regions of the posterior septum are responsible for providing afferent input to the MHb. Specifically, the triangular septal and septofimbrial nuclei and the bed nucleus of the anterior commissure (BAC) densely innervate the MHb (Yamaguchi et al., 2013). Interestingly, lesions to the septum or the interpeduncular nucleus can abolish aversive responses to, and avoidance of, noxious bitter tastants such as quinine (Donovick et al., 1970). This suggests that major functions of the septo-habenulo-interpeduncular pathway include regulation of food consumption by controlling avoidance of noxious substances. Interestingly, individuals with deficits in the ability to detect bitter tastants are much more likely to be regular smokers (Saper et al., 2002). Hence, it is an intriguing possibility that constitutive deficits in septo-habenulo-interpeduncular function, reflected in diminished ability to detect bitter tastes, may also result in diminished sensitivity to the

aversive effects of nicotine and account for the increased vulnerability to developing tobacco dependence in such individuals. More recently, it was shown that septal inputs to MHb may also regulate anxiety and fear-related behaviors in rodents (Yamaguchi et al., 2013). It is therefore interesting to speculate that mood-regulated effects mediated by the septo-habenulo-interpeduncular system may also influence vulnerability to tobacco dependence.

The lateral habenula-rostromedial tegmental area pathway and nicotine aversion

In addition to the MHb projection to the IPN, recent evidence suggests that the lateral habenula (LHb) may also play a role in nicotine aversion. Unlike the MHb, which projects almost exclusively to IPN, the LHb projects only sparely to IPN and instead sends prominent projections to the rostromedial tegmental nucleus (RMTg) (Jhou et al., 2009), and less prominent projects to the VTA. Through these projections, the LHb inhibits the firing of midbrain dopamine neurons directly (via VTA projections) or indirectly (via RMTg projections) (Bromberg-Martin and Hikosaka, 2011; Hikosaka, 2010; Jhou et al., 2009; Lecourtier and Kelly, 2007; Matsumoto and Hikosaka, 2009). LHb neurons are activated by aversive stimuli or omission of anticipated rewards (Bromberg-Martin and Hikosaka, 2011; Hikosaka, 2010; Lecourtier and Kelly, 2007; Matsumoto and Hikosaka, 2009). This suggests that LHb transmission, and its inputs to RMTg, could encode aspects of nicotine aversion and influence responses to the drug. Consistent with this possibility, Pistis and colleagues have recently shown that nicotine potently and robustly excites neurons in the RMTg (Lecca et al., 2012). This effect was likely related to a stimulatory action of nicotine on α7 nAChRs located presynaptically on excitatory glutamatergic

inputs from the LHb (Lecca et al., 2012). The functional consequent of this stimulatory effect of nicotine on RMTg transmission in regulating nicotine aversion and nicotine consumption has not been directly investigated.

Other brain circuitries that may play a role in nicotine aversion

As described above, the MHb-IPN system densely expresses nAChRs containing α5, α3 and/or β4 subunits. Indeed, it was based on the dense expression of these subunits in the MHb-IPN system that the role of α5* nAChRs in these sites in nicotine aversion was first investigated in mice (Fowler et al., 2011). Interestingly, the nucleus tractus solitarius (NTS) is a hindbrain site that also displays very dense expression of these subunits. The NTS contains at least three types of neurons: catecholaminergic neurons that produce the neurotransmitter norepinephrine (and to a lesser extent epinephrine); glucagon-producing neurons that synthesize the neuropeptide glucagon-like peptide-1 (GLP-1); and neurons that synthesize the feeding-related neuropeptide proopiomelanocortin (POMC). The NTS is perhaps best known for its role in regulating taste reactivity, as it receives dense innervation from the buccal cavity (Appleyard et al., 2005; Grill and Hayes, 2009). NTS neurons also receive vagal inputs from the viscera and NTS activation in response to vagal stimulation can induce cessation of food intake. Specifically, catecholaminergic neurons relay signals to higher feeding centers in the brain from the gastrointestinal (GI) tract related to meal ingestion or gastric distension, and respond also to circulating satiety signals including cholecystokinin (CCK) (Appleyard et al., 2007; Monnikes et al., 1997; Rinaman et al., 1998; Willing and Berthoud, 1997). Catecholaminergic neurons in the NTS have been implicated in the expression of aversive aspects of drug withdrawal (Delfs et al., 2000; Taylor et al., 1998). It has been shown that

nicotine can activate NTS catecholaminergic neurons, likely though a mechanism involving increased local glutamatergic transmission (Feng et al., 2012; Hong et al., 2012; Kalappa et al., 2011; Shiraki et al., 1997; Zhao et al., 2007). Moreover, the and adrenergic receptor antagonist carvedilol can reduce self-reported aversive responses to nicotine, delivered as a lozenge to human volunteers (Sofuoglu et al., 2006). These findings suggest that nicotine may activate NTS catecholaminergic neurons, increasing adrenergic transmission in forebrain regions, with this effect contributing to aversive aspects of the drug, a hypothesis that has yet to be tested. NTS catecholaminergic neurons have instead been implicated in drug reward rather than aversion. Specifically, it was shown that the rewarding effects of morphine are greatly diminished in dopamine β-hydroxylase knockout (DBH-KO) mice that are unable to synthesize norepinephrine (Olson et al., 2006), and virus-mediated re-expression of DBH in the NTS of the KO mice restores their sensitivity to morphine reward (Olson et al., 2006). As noted above, neurons that produce the neuropeptide GLP-1 are also a major population of neurons in the NTS. Activation of GLP-1 occurs in response to gastric distention, nausea, stress and illness and results in suppression of food intake (Barrera et al., 2011; Hayes et al., 2009; Turton et al., 1996). Hence, the NTS, and in particular GLP-1 neurons, seem well placed to regulate aversion-related actions of nicotine. Nevertheless, the role of the NTS in nicotine avoidance behavior has not yet been investigated.

Efferents of the IPN are also of interest as potential mediators of aversive processing. Indeed, it is currently unknown how the MHb-IPN circuit integrates aversion-related information with the reward-related processing of the mesocorticolimbic pathway. The IPN has broad ascending and descending

projections to various brain regions. The most prominent of these projections are the medial septum/diagonal band of Broca, hippocampus, dorsal tegmental nucleus, raphe and periaqueductal gray (Groenewegen et al., 1986; Klemm, 2004; Montone et al., 1988; Shibata and Suzuki, 1984). Many of these regions, including the diagonal band of Broca, dorsal tegmental nucleus, and raphe, send projections to the VTA (Groenewegen et al., 1986; Oades and Halliday, 1987; Phillipson, 1979; Wirtshafter, 1981). Thus, MHb-IPN signaling may be integrated with mesocorticolimbic processing via an intermediate brain region that then projects to the VTA, similar to that found with the LHb-RMTg-VTA circuit. Thus, identification of the predominant neural circuit that mediates a motivational response to consume, or cease consuming, nicotine will be important to delineate in future investigations.

Novel smoking cessation agents that modulate nicotine aversion

Data described above demonstrate that deficient α5* nAChR signaling, particularly in the MHb-IPN system, increases nicotine intake in rats and mice. Hence, an intriguing approach to facilitate smoking cessation may be the development of small molecule compounds that amplify α5* nAChR signaling. Before such selective compounds can be developed, it is critical to know which subtype of α5* nAChRs regulates nicotine aversion. In heterologous expression systems, α5 subunits can co-assemble into α4β2, α3β2, and α3β4 nAChR subtypes (Fucile et al., 1997; Gerzanich et al., 1998; Tapia et al., 2007). However, in the mammalian brain it appears that α5 subunits predominantly assembles into α4β2* nAChR subtypes (Gotti et al., 2007; Kuryatov et al., 2008; Mao et al., 2008; Perry et al., 2007). Indeed, using immunoprecipitation, it was reported that α5 subunits are almost exclusively in complex with α4β2 subunits in the hippocampus, striatum, cortex and thalamus, with almost

undetectable levels of α3* nAChRs containing the α5 subunit in these regions (Mao et al., 2008). In the MHb-IPN pathway ~11% of β2* nAChRs express α5 subunits (Grady et al., 2009), whereas only ~5% of β4* nAChRs express the subunit (Grady et al., 2009). Importantly, in the MHb-IPN pathway α3β4* nAChRs are thought to exclusively regulate acetylcholine release (Grady et al., 2009), whereas α4β2α5* nAChRs regulate glutamate release (Girod et al., 2000). We previously found that disruption of glutamatergic transmission in the IPN increases nicotine intake in rats in a manner similar to genetic disruption of α5* nAChR signaling in rats or mice (Fowler et al., 2011). Hence, α4β2α5* nAChRs are likely to be a functional subtype in the MHb-IPN that negatively regulates nicotine intake. Boosting the activity of this particular nAChR subtype in response to nicotine consumption may therefore be a novel strategy to decrease nicotine intake and facilitate smoking cessation efforts.

Nicotinic receptors are pentameric complexes in which acetylcholine (and nicotine) binds at the interface between α and β subunits (orthosteric sites). It is hypothesized that agonist binding at orthosteric sites stabilizes the receptor channel complex in the “open” conformation, thereby increasing receptor activity. Partial agonists less efficiently stabilize the receptor “open” state, and conversely, competitive antagonists stabilize the receptor channel in the “closed” state. Agonists may also stabilize a receptor transition from “active” to an inactive “desensitized” state. There are multiple allosteric sites elsewhere on the multimeric nAChR complex, which by themselves do not stimulate opening or closing of the receptor channel, but instead modify the activity of the receptor once activated by orthosteric ligands (Changeux, 1990; Changeux et al., 1984; Changeux et al., 1992; Chemouilli et al., 1985; Lena and Changeux, 1993). Positive allosteric modulators (PAMs) are ligands that

bind to allosteric sites to facilitate agonist-induced stabilization of the “open” conformation or to reduce agonist-facilitated receptor desensitization, with PAMs unable to influence receptor function in the absence of orthosteric agonists. Hence, PAMs can potentiate the stimulatory effects of low agonist concentrations on nAChR function in much the same manner that benzodiazepines potentiate the actions of GABA at the $GABA_A$ receptor. A number of features of PAM acting at $\alpha4\beta2\alpha5$* suggest that they may be particularly attractive candidates as novel smoking cessation agents. First, because the orthosteric binding site is so well conserved between various nAChR subtypes, it is difficult to engineer agonists with receptor selectivity (Albrecht et al., 2008; Armishaw et al., 2009). Moreover, α5 nAChR subunits co-expressed with β2 or β4 subunits do not co-assemble into functional heteropentameric nAChRs without the presence of another α subunit (Boulter et al., 1987; Couturier et al., 1990). Instead, α5 subunits act as accessory subunits that modulate receptor activation/desensitization kinetics (Ramirez-Latorre et al., 1996). Moreover, α5 subunits play a key role in generating novel allosteric modulatory sites on α5* nAChRs (Taly et al., 2009). Thus, it is likely to be far easier to develop PAMs that are highly selective for α4β2α5* nAChRs compared to the development of orthosteric agonists. Second, it is expected that α4β2α5* PAMs have low intrinsic activity at these nAChRs in the MHb-IPN tract or other brain areas (depending on cholinergic tone). Instead PAMs should potentiate α4β2α5* nAChRs most efficiently only when activated by nicotine in tobacco smoke. This is an important point when considering that MHb-IPN activation typically occurs in response to aversive stimuli (Donovick et al., 1970; Hammer and Klingberg, 1990; Meszaros et al., 1985; Thompson, 1960; Thornton et al., 1994; Wirtshafter, 1981), suggesting that full α4β2α5* nAChR agonists may possess intrinsic aversive properties that would limit their

clinical utility. Furthermore, unlike orthosteric agonists, PAMs are unlikely to desensitize and thereby inhibit α4β2α5* nAChRs. Desensitization of nAChRs by full agonists could paradoxically decrease MHb-IPN sensitivity to nicotine, resulting in an increase in the motivational properties of the drug and an undesired increase in tobacco consumption. Finally, by potentiating the deficient function of α4β2α5* nAChRs in individuals carrying *CHRNA5* risk alleles, PAMs may be able to attenuate genetic vulnerability to tobacco dependence.

In the context of developing α4β2α5* nAChR PAMs for smoking cessation, it is interesting to note that that the acetylcholinesterase (AChE) inhibitors galantamine and physostigmine are PAMs of α4β2* nAChRs (Maelicke et al., 2001; Pereira et al., 1994; Pereira et al., 1993; Samochocki et al., 2003; Samochocki et al., 2000; Storch et al., 1995), and codeine may also be an α4β2* nAChR PAM (Storch et al., 1995). This action is not thought to be related to AChE inhibitor activity, as other AChE inhibitors including tacrine, metrifonate, rivastigmine and donepezil do not share this action (Samochocki et al., 2000). Importantly, the FK1 monoclonal antibody, which binds selectively to α nAChR subunits (Schroder et al., 1994), completely abolishes the PAM effects of galantamine, physostigmine and codeine on α4β2* nAChR function (Pereira et al., 1994; Storch et al., 1995), thereby verifying a direct allosteric action on the α subunit, and not an orthosteric action at the interface between and subunits. Intriguingly, a recent study reported that galantamine is a PAM only at α4β2α5* nAChRs, and is practically inactive α4β2* nAChRs that do not contain α5 subunits (Kuryatov et al., 2008). Galantamine has been shown to reduce the number of cigarettes smoked in a recent clinical trial (Diehl et al., 2006) and also reduced intravenous nicotine

self-administration behavior in rats (Hopkins et al., 2012; Liu and Stewart, 2009). These actions of galantamine may be related in part to its PAM action at α4β2α5* nAChRs. Nevertheless, galantamine is likely to have very limited clinical utility for smoking cessation. Its PAM action occurs only at low concentrations, and at higher concentrations it inhibits α4β2α5* nAChRs. Also, AChE inhibition by galantamine and other known PAMs is likely to be a major confound. Therefore, it will be important to develop and test novel α5* nAChR PAMs for smoking cessation that are efficacious across a broad dose-range and are devoid of "off-target" effects.

While the concept of enhancing nicotine aversion as a therapeutic strategy may be novel, this type of approach has been used with other drugs of abuse, such as alcohol. Disulfiram (Antabuse) is an aversive therapy to attain abstinence from alcohol, which acts by irreversibly inhibiting an enzyme involved in alcohol metabolism, acetaldehyde dehydrogenase (Center for Substance Abuse Treatment, 2009). During alcohol consumption, disulfiram promotes the accumulation of acetaldehyde, resulting in moderate to severe physical reactions that include nausea, vomiting, hypotension and facial flushing. When alcohol is not consumed, side effects are minimal and may include headache and fatigue (Fuller and Gordis, 2004). The clinical effectiveness of disulfiram has been variable (Brewer et al., 2000; Fuller and Gordis, 2004), most notably due to issues with patient compliance (Suh et al., 2006). While abstinence may be improved by promoting patient involvement in support groups and extensive physician supervision (Brewer et al., 2000), treatment effectiveness can also be enhanced by concomitant use of other therapeutics, such as acamprosate, a glutamate antagonist, or naltrexone, an opioid receptor antagonist (Mason et al., 2006; Suh et al., 2006). Given these

considerations, a therapeutic that enhances the aversive effects of nicotine may not be as straightforward as one would hope in the clinical setting due to the potential confound of patient adherence to the dosing schedule. However, compared to other drugs of abuse, nicotine's aversive effects are more readily induced, as evidenced by the narrow D-R range for intake. In contrast, aversive reactions to alcohol are usually minimal in the absence of disulfiram, as evidenced by it being more readily consumed in excess. Thus, by choosing to not take disulfiram, a patient with alcoholism may largely avoid any physiological aversion associated with alcohol consumption, whereas this will be less likely with nicotine consumption. Nevertheless, treatments that include physician supervision, support of family and friends, and education regarding the severe and detrimental health consequences of continued tobacco use will likely be more effective than pharmacotherapies alone. Finally, a novel 'aversion-inducing' therapeutic for smoking cessation may be most efficacious when used in conjunction with drugs that limit the rewarding properties of the drug, such as Chantix. By concurrently enhancing the aversive while reducing the rewarding properties of the drug, it is possible that long-term abstinence may be more readily attainable for the patient.

Summary

The findings reviewed above demonstrate that, in addition to the rewarding effects of nicotine, noxious effects of the drug also likely influence the development and persistence of the tobacco smoking habit in humans. Specifically, avoidance of the aversive properties of nicotine play a key role in determining the amounts of nicotine consumed, patterns of consumption, and hence the magnitude by which nicotine induces neuroplasticity in addiction-relevant brain reinforcement circuits. Habenula-interpedunular glutamatergic

transmission and aspects of mesoaccumbens dopamine, acetylcholine and opioid transmission appear to regulate the aversive effects of nicotine, and thereby control avoidance of the drug. Moreover, allelic variation in genes highly expressed in the aversion-related circuitries, in particular the *CHRNA5* gene that encodes the 5 nAChR subunit, can influence vulnerability to tobacco dependence in humans, highlighting the importance of nicotine aversion in controlling vulnerability to addiction. Much work still remains to precisely understand how the aversive effects of nicotine are encoded in the brain, and how aversion-related circuits may interact with reward circuits to control nicotine intake. Nevertheless, the available data support the interesting possibility that amplifying the noxious properties of nicotine via small molecule drugs may serve as a novel strategy to develop efficacious smoking cessation agents.

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Figure legends:

Allelic variation in the *CHRNA5-CHRNA3-CHRNB4* gene cluster contributes to vulnerability to tobacco dependence. (**a**) Graphical representation of the genomic organization of the *CHRNA5-CHRNA3-CHRNB4* nAChR subunit gene cluster on chromosome 15 (Chr15 q25.1). (**b**) Mice with null mutation in the α5 subunit gene self-administer more nicotine than wildtype mice. Mice were responding under a fixed-ratio 5 time-out 20 sec schedule of reinforcement. (**c**) Data from panel B were transformed such that the total amount of nicotine self-administered by wildtype and knockout mice could be examine. Data are modified with permission from (Fowler et al., 2011).