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The Role of the Habenula in Nicotine Addiction

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Abstract

To thrive in any given environment, mobile creatures must be able to learn from the outcomes of both successful and disappointing events. To learn from success, the brain relies on signals originating in the ventral tegmental area and substantia nigra that result in increased release of dopamine in the striatum. Recently, it was shown that to learn from disappointment the brain relies on signals originating in the lateral habenula, which indirectly inhibit dopaminergic activity. The habenula is a small brain region that has been shown in mice to be critical for the appearance of nicotine withdrawal symptoms. The nicotinic acetylcholine receptor subunits expressed in the medial habenula are necessary to observe withdrawal symptoms in mice, and blocking nicotinic activity in the medial habenula only is sufficient to precipitate withdrawal in dependent mice. In addition, recent genome wide association studies have shown that in humans, genetic variants in the same nicotinic receptor subunits are at least partially responsible for the genetic predisposition to become a smoker. The habenula is linked not only to nicotine, but also to the effects of several other drugs. We postulate that the continuous use of drugs of abuse results in habenular hyperactivity as a compensatory mechanism for artificially elevated dopamine release. Drug withdrawal would then result in non-compensated habenular hyperactivity, and could be thought of as a state of continuous disappointment (or a negative emotional state), driving repeated drug use. We believe that drugs that alter habenular activity may be effective therapies against tobacco smoke and drug addiction in general.

Keywords

Habenula; Tobacco; Nicotine; Nicotinic receptors; Withdrawal

The Habenula

The habenula is a small brain structure that has elicited great interest lately. The name habenula (latin for “little rein”) comes from the shape that resembles a rein, located by the third ventricle. The habenula is divided into two structures termed medial and lateral habenula, that are anatomically and transcriptionally very different [1].

Ramon y Cajal recognized the possible importance of the habenula, based mainly on the prevalence of its connectivity [2]. Among the major axon bundles of the rodent brain are the

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stria medullaris (habenular input) and the fasciculus retroflexus (habenular output). The stria medullaris brings input to the habenula from several areas including the septum and hippocampus, the ventral pallidum, the lateral hypothalamus, the globus pallidus, and other basal ganglia structures [1]. The fasciculus retroflexus is a major axon bundle that brings habenular input to midbrain structures. It is divided into a core region that originates in the medial habenula and ends at the interpeduncular nucleus, and an outer region that originates in the lateral habenula and ends at the rostromedial tegmental nucleus (RMTg). The RMTg (which was sometimes called the "tail" of the ventral tegmental area (VTA)) is a small nucleus that contains mainly inhibitory gabaergic cells. These cells are activated by glutamate released by the lateral habenula via the fasciculus retroflexus and in turn release GABA onto dopaminergic cells in the VTA and substantia nigra pars compacta (SNc). Therefore, lateral habenular activity indirectly inhibits dopaminergic cells in the VTA/SNc. This results in a decrease in dopamine being released in striatal areas (Figure 1) [3,4]. Less is known about the medial than the lateral habenula. The medial habenula receives input mainly from the septum (the stria medullaris brings inputs to both the lateral and medial habenule), and projects to the interpeduncular nucleus [5,6]. The function of the medial habenula is not as clear as for the lateral. Since the interpeduncular nucleus projects to dopaminergic areas, it is possible that the medial habenula is also involved in prediction error as the lateral habenula has been shown to be [7]. Throughout this review, we mention "lateral" or "medial" habenula when the data allows for that distinction and simply "habenula" when the original data leaves that question unresolved. An example of "habenula" happens in functional magnetic resonance experiments, where spatial resolution does not permit the distinction between medial and lateral habenula. In contrast, in electrophysiology the position of the electrodes can be carefully studied and the results can be ascribed to the medial or lateral habenula.

The study of the habenula had a first "golden age" several years ago, when a series of elegant and thoughtful reports where released [5,6,8–13]. Probably because of its small size, the complexity and subtlety of its role, and the lack of suitable pharmacological agents, the study of the habenula has been much slower than that of many other brain regions. There are several reasons why the habenula is currently the object of intense study. The main reasons related to drug abuse are discussed below.

Mouse work points to the medial habenula as a critical region for nicotine withdrawal

Work on several lines of mutant mice pointed to the medial habenula as a critical mediator of nicotine withdrawal signs and several other effects of nicotine. Several groups have created and analyzed nicotinic acetylcholine receptor (nAChR) subunit mutant mice, including both null mice [14–24] and gain-of-function mice [25–27]. From this body of work, a very surprising result emerged: the nAChR subunits that are responsible for many of the effects of nicotine including sedation, nicotine-induced seizures and most importantly, somatic withdrawal symptoms, are the α2, α3, α5 and β4 subunits. This was unexpected since other subunits particularly the α4, α7 and β2 are more ubiquitously expressed and had been hypothesized to be relevant for addiction for many years. The β2 subunit, which together with α4 subunits forms the majority of nicotinic receptors in the brain, had been shown to be necessary for nicotine self-administration in mice [18]. Therefore, it was a surprise when we showed that β2 null mice had normal nicotine withdrawal somatic symptoms [20]. The α2, α3, α5 and β4 subunits which modulate withdrawal are expressed in a highly restricted pattern in the brain: despite some species-specific differences, these subunits are expressed at high levels in the medial habenula and its main output, the interpeduncular nucleus (Ipn) [21,22,28]. Other areas also express these subunits: the hippocampal CA1 area expresses α5, the pineal gland and mitral cells in the olfactory bulb

express α3 and β4, certain areas of the thalamus express α3, etc [19,28,29]. Taken together, the four relevant subunits clearly pointed to the habenula/Ipn axis as a critical mediator of nicotine withdrawal in mice. The role of the habenula in nicotine withdrawal was pinpointed by injecting mecamylamine (a nons-pecific nAChR blocker) in the medial habenula of mice chronically treated with nicotine. This precipitated withdrawal symptoms [22], just as systemic mecamylamine does [30]. Withdrawal was also precipitated when mecamylamine was injected into the Ipn, but not when injected into the cortex, hippocampus or the VTA [22]. In a related line of work, the group lead by Dr. Stanley Glick showed that 18MC, an ibogaine derivative that is an antagonist at β4-containing nAChRs blocks effects not only of nicotine, but also of other drugs of abuse. Using microinjections into rodent brains, they showed that the effects of 18MC are mediated by the medial habenular region [31–34]. Therefore, it is possible that β2 subunit-containing receptors, which are highly expressed in the VTA/SNc and are important for reward-related dopamine release in the striatum control nicotine self-administration in mice, while β4 subunit-containing receptors are associated to nicotine withdrawal. Which of these two effects is more relevant to tobacco addiction has been debated for a long time. One view is that smokers continue to smoke not for the rewarding effects of nicotine, but to mitigate the effects of withdrawal that appear shortly after finishing a cigarette [35–38]. A new cue to support that view recently came from genetic studies in human smokers.

Genome wide association studies in humans

Genome wide association studies (GWAS) have boomed in the last few years. In GWAS, two populations of humans (affected vs. controls, usually several hundreds of subjects per group) are compared by extracting DNA and genotyping hundreds of thousands of single nucleotide polymorphisms (SNPs). After comprehensive statistical analysis including a rigorous multiple comparison problem correction, some polymorphisms are usually found to be differentially represented in both populations. It is then suggested that those polymorphisms may be associated to genetic variants that cause the phenotype, and the genes where those variants are found are further analyzed. Interestingly, tobacco addiction is one of the conditions where GWAS has provided the most reliable and clean data so far. Studies from several groups have shown that a genetic cluster in the chromosome 15q25 region that includes the α3, α5 and β4 subunits of the nAChRs contains a series of polymorphisms that increase or decrease the relative risk of becoming a smoker [39–42]. These polymorphisms are in strong linkage disequilibrium and are located throughout the gene cluster containing the 3 subunits. In addition, the same genetic variants appeared in different types of screens, such as smoking, peripheral arterial disease and lung cancer. Since these subunits are the same (excluding α2) that were previously highlighted by mutant mouse work as important for nicotine withdrawal, it is likely that the habenula, the main site of expression of the gene cluster, is involved in the human phenotypes studied. Together with preclinical work discussed earlier, we hypothesize that nAChRs containing these subunits, located in the habenula are mediators of withdrawal symptoms in humans, and that most people smoke to block the withdrawal symptoms that appear a few hours after the last cigarette. Thus, the habenula and the nAChRs expressed therein are necessary for nicotine withdrawal symptoms. Therefore, to understand nicotine withdrawal we must first know the role that the habenula plays within the brain. A major break-through came from primate electrophysiological work, as described below.

The lateral habenula and the negative prediction error

All drugs of abuse have in common that they increase striatal dopamine release, which is believed to convey a rewarding feeling. Nicotine is no exception, and nicotine infusion has repeatedly been shown to increase dopamine release in the striatum, which is believed to be

the reason why nicotine is addictive. In addition, during withdrawal the dopamine concentration in the striatum is decreased [43]. Therefore, the activity of dopaminergic cells in the VTA/SNc is of importance to addictive behaviors. Although the nicotinic receptors that seem most important for nicotine addiction are expressed in the medial habenula, the lateral habenula may also be involved in the regulation of nicotine's effects: the levels of activity in the lateral habenula are a major modulator of the activity of the VTA/SNc. The relationship between activity in the medial and in the lateral habenula is not at all clear and it should be a research priority for the coming years.

The importance of the dopaminergic system in reward learning was highlighted by primate work in which monkeys were given juice squirts after a visual cue, while the activity of dopaminergic cells was measured with electrodes. At first, juice delivery elicited strong activity in dopaminergic areas, suggesting that reward activated dopamine release in the striatum. After training, monkeys learned that the cue predicts juice, and dopaminergic activity was elicited by the cue itself, but not by juice delivery if it was expected. Therefore, the conclusion was that dopamine does not signal reward itself, but unexpected reward [44,45]. In that sense, dopamine becomes a learning signal: if reward is as much as expected, no learning needs to occur, and therefore no increase in dopamine release is needed. If an unexpected reward (or an unexpected reward prediction signal, as in the appearance of the cue) is delivered, dopamine release is increased to signal a positive prediction error. It was also observed that if expected reward was omitted or delayed, dopaminergic activity decreased at the time of expected reward [45]. The origin of this decrease in activity was not found until many years later, when Dr. Okihide Hikosaka performed similar experiments, implanting electrodes not only in dopaminergic areas, but also in the lateral habenula [7]. In this work, monkeys were trained to perform an eye saccade to the left or to the right, and one side predicted juice while the other side predicted nothing. As expected, saccades to the rewarded side were faster than to the non-reward side, but monkeys did both because the saccade would allow for a new trial to start. As shown previously, dopaminergic area activity was increased at unexpected reward and decreased at expected reward non-delivery. Interestingly, lateral habenular neuronal activity showed the exact opposite: increase at disappointment (negative prediction error), and decrease at unexpected reward (positive prediction error). Therefore, the activity of the lateral habenula and of the VTA/SNc (major dopaminergic site of the brain) are anticorrelated [7]. It needs to be noted that this work was done solely on the lateral habenula, which does not express the α3, α5 or β4 nAChR subunits. In addition, the negative prediction error (or disappointment) is not the only event that triggers habenula activation. Other negative emotional states, such as when receiving an air puff to the face, also activates the habenula in a similar fashion [46]. In humans, the habenula has been severely understudied mainly because of its small size. In non-invasive techniques such as functional magnetic resonance imaging (fMRI) the habenula accounts for just a handful of voxels. The signal, therefore, is typically overwhelmed by noise. A few reports using fMRI have shown habenular activity. In a seminal 2003 paper, Ullsperger and Von Cramon showed that the habenula is activated by negative feedback [47]. Subjects had to guess which one of two balls traveling at different speeds would hit a target first. The difficulty of the task was manipulated such that every subject would make about 30% mistakes, and feedback was given as a happy or frowny face. When negative feedback was received, the habenula activated. To verify that humans signal negative prediction errors similarly to monkeys, we repeated the juice experiments done in primates in humans: subjects received a small fruit-flavored juice squirt after seeing a cue in a computer screen, while their brains were scanned using a high resolution fMRI protocol. The juice was delivered 6 seconds after the cue repeatedly, and once learning on this paradigm was complete the time was delayed in some trials to 10 seconds. We showed that the four seconds of "expected reward non-delivery" activated the habenula in humans, similarly to what was shown in the lateral habenula of monkeys using electrophysiology. In

addition, we showed that habenular and striatal activities are indeed anticorrelated in humans as had been shown in monkeys [48]. Our hypothesis is that the relationship between the negative prediction error and addiction in general is that drugs of abuse, by increasing dopamine release in the striatum, "trick" the brain into feeling that things are better than expected, even when no reward is actually received. We hypothesize that the reward circuitry adapts to repeated drug exposure by dampening dopamine release via increasing lateral habenular activity. This increased lateral habenular activity is what brings negative feelings during withdrawal: when the drug is removed from the system, the dopamine-increasing effect of the drug is gone, but the dopamine-decreasing effect derived from long term changes in the habenula is still present. According to this hypothesis, a person in withdrawal would feel that "things are worse than expected" even if no actual lack of reward is present.

Other habenular functions

Besides the connection to the dopaminergic system, the lateral habenula is also connected to two other major groups of neurons in the brain: the raphe nucleus and the locus coeruleus. Therefore, activity in the lateral habenula has the potential to modulate three major neurotransmitter systems, not only dopamine but also serotonin (raphe nucleus) and norepinephrine (locus coeruleus). Therefore, it should not be surprising that habenular activity has been related to several seemingly unrelated behaviors in a wide variety of animal models. Possibly through its connection to the locus coereleus, the habenula plays a role on stress-induced behavior. In an effort to study the link between the habenula and a stress-induced behavior that may be connected to schizophrenia, Heldt and Ressler used mice with habenular or sham lesions, and measured pre-pulse inhibition. Pre-pulse inhibition is performed by exposing mice to two tones, one loud that makes the mouse startle, and one much lower (just before the loud tone) that inhibits startle. In humans, pre-pulse inhibition is a hallmark of schizophrenia, although it is not diagnostic per se [49]. Pre-pulse inhibition in mice with habenular lesions was normal in basal conditions, but impaired after fear stress training, which was a dopamine-dependent effect. The authors concluded that the habenula may be involved in stress-dependent modulation of monoamine systems [50]. It should be stressed that, as in most habenular lesion studies, these experiments may not allow for medial vs. lateral habenula discrimination.

In addition, the medial and lateral habenule have been associated to maternal, feeding, and sexual behavior, and other behaviors and processes [1]. Even more importantly, the lateral habenula may play a major role on clinical depression. Several lines of evidence support that hypothesis: first, Morris et al showed that when former depressed patients that are in remission are put on a tryptophan-free diet, the habenula becomes hyperactive, and that the severity of depression symptoms upon tryptophan depletion is correlated to the level of coupling between the habenula and the raphe nucleus [51]. In addition, it has been shown that depressed patients have a small but significant difference in habenular size in women [52]. Finally, a clinically depressed, treatment resistant patient was treated for depression by using a deep brain stimulation electrode directed to the lateral habenula and showed striking improvement on depression symptomatology. Although this is just one case, it is interesting to note that the patient became sick again after a bicycle accident. The doctors realized that the deep brain stimulation electrode had been disconnected by the accident, and after re-connection to the battery the depression symptoms disappeared again [53]. The mechanism of these relationships was studied in an excellent report by Li et al. These authors used learned helplessness in rodents as a model for depression and showed that this protocol potentiates synaptic inputs from the lateral habenula. There was a correlation between the level of potentiation and the level of behavioral helplessness, suggesting that at least in this model of depression, increased lateral habenula activity may be implicated in the etiology of

depression [54]. The current hypothesis is that lateral habenular hyperactivity (for example, an excessive response to negative prediction error, or disappointing events) in the long run disrupts not only the dopaminergic but also the serotonergic system, with major effects on mood-controlling circuits in the brain. The connection between tobacco addiction and depression has been well documented. In 1990, two reports using large number of subjects were published in JAMA, describing a strong association between depression symptoms and tobacco smoking [55,56]. Since then, many reports have shown the importance of this association, including during adolescence. The mechanism of the relationship, however, is not clear: it has been suggested that depression causes smoking, smoking causes depression, that there is a bidirectional relationship, or that both conditions are simply related to similar confounders [57]. In terms of lateral vs. medial habenular involvement in depression, the picture is not very clear yet: The effects mentioned above are all in the lateral habenula but bupropion, a drug that affects the receptors expressed in the medial habenula (and other non-nicotinic systems), is a popular antidepressant [58]. In addition, rats with a congenital predisposition to helplessness and depressive behavior showed abnormal metabolism in a series of areas, including both the medial and the lateral habenule [59]. Much work is needed to disentangle this field, and we believe that one of the brain regions to be studied must be the habenula, the nAChRs expressed therein, and the relationship between medial and lateral habenule.

Nicotinic receptor subunits and their roles on nicotine's effects

nAChRs are membrane proteins composed of five subunits that form a central pore permeable to cations, mainly Na+ and in less proportion Ca++. There are two main types of nAChRs: heteropentamers, composed of both alpha and beta subunits, and homo-alpha pentamers, composed of α7 subunits, and in some cases α9 and α10 subunits. Since there are 9 alpha (α2 to α10) and 3 beta (β2 to β4) subunits, many different combinations are theoretically possible. However, only certain combinations are found in nature and the number of different types of receptors expressed in the nervous system is much smaller than the theoretical limit. Among the most commonly expressed combinations are α4β2, α3β4, α6β3, and α7. The nAChR that has received the most attention by the investigators in the field is the α4β2, followed by the α7. The reasons for this preference are several, including most ubiquitous expression, better pharmacological tools, and the fact that α4β2 receptors up-regulate their expression level upon treatment with chronic nicotine [60]. This effect has been hypothesized for years to be critical for addiction, including withdrawal symptoms, but clear evidence of the importance of nAChR up-regulation in addiction is still lacking. The expression patterns of the different nAChR subunits are quite complex and overlapping, giving rise to different subunit combinations in different brain areas. nAChRs are cation channels that are endogenously opened by the neurotransmitter acetylcholine, giving rise to neuronal depolarization and subsequent activation. Nicotine also opens the channel but after nicotine binding, the receptors stay in a desensitized state, in which new binding of acetylcholine or nicotine will not elicit opening [60]. Despite several years of careful electrophysiological experiments, it is not clear whether some of the effects of nicotine are derived from the activation or desensitization of the channels, or both. Some subunit combinations desensitize more readily than others, which also complicates matters.

The role of the α5 subunit in nicotine's effects

The first clear evidence that the α5 subunit of the nAChR plays a major role on nicotine's effects came from mutant mouse work. Mice null for the α5 subunit were shown to have grossly normal behavior but showed a strong resistance to nicotine induced hypolocomotion in the open field and to nicotine induced seizures [16,19]. Although the effects of acute nicotine in rodents are obviously not the best model for nicotine addiction in humans, it

should be noted that the effects of the first cigarette ever is a good predictor of later tobacco addiction in life [61]. Therefore, studying the effects of acute nicotine in rodents may in fact provide clues about mechanisms of tobacco addiction. In addition, the work on the acute effects of nicotine in mutant mice paved the way for subsequent reports where the role of the α5 subunit (and other subunits) was examined in more detail. In mice, the lack of the α5 subunit resulted in an anxiolytic-like phenotype only in females. Interestingly, anxiety-like behavior in mice follows the estrus cycle, but this effect was absent in α5 null mice. In addition, progesterone injections in castrated female mice increased the levels of α5 mRNA, suggesting an estrus cycle-dependent transcriptional mechanism for these effects [62]. Additional work is necessary to study the possible link between α5 expression and function to behavioral changes during the estrus cycle in humans. This could be of importance given the sex-dependent link between addiction and depression.

Therefore, the α5 subunit plays a major role on acute nicotine's effects in mice. A much more relevant question is whether this subunit affects nicotine withdrawal. In nicotine-treated α5 null mice, no mecamylamine-precipitated nicotine withdrawal was observed when nicotine was given either orally or using continuous infusion with minipums. The fact that the medial habenula is the locus of the effects of the α5 subunit was partially answered by injecting mecamylamine in the medial habenula, which precipitated withdrawal in nicotine treated mice [22]. The confirmation that the α5 subunit's roles on the effects of nicotine are mediated by the medial habenula came later in a very elegant report by Fowler et al. In this paper, it was demonstrated that α5 null mice show markedly increased nicotine intake, and that such effect could be rescued by re-expressing the α5 subunit in the medial habenula. Furthermore, α5 subunit knock down in the medial habenula of wild type animals did not alter the rewarding effects of low doses of nicotine but abolished the inhibitory effects of higher doses [63]. This argues that nAChRs containing the α5 subunit in the medial habenula are necessary for the appearance of negative but not positive effects of nicotine, which is in agreement with the previous reports that the α5 subunit is necessary to observe nicotine withdrawal.

In humans, the role of each particular subunit is much more difficult to assess. The SNPs that were shown to affect tobacco addiction are located in a genetic cluster that contains the α3, α5 and β4 subunit. These SNPs are in strong linkage disequilibrium and it is hard to assign their effects to a particular subunit. Most of these SNPs are in non-coding areas, which led to the hypothesis that a particular SNP (rs16969968), which results in an aminoacid change in the α5 subunit (a change from aspartic acid to asparagine in aminoacid 398, or D398N) may be the one responsible for these effects. The combined statistics from several studies for the involvement of this SNP in tobacco addiction are very convincing ($p=5.57 \times 10^{-72}$) [64]. However, although this SNP clearly plays a role in addiction, much work must still be done to assess the effects of different genetic variants on the susceptibility to tobacco addiction. In the α3/α5/β4 gene cluster there are other SNPs that are not genetically linked to rs16969968 and that are also known to play a role on tobacco addiction [64]. Less statistically significant but still relevant are SNPs on other nAChR subunits such as β3 (a subunit expressed in both the medial habenula and the VTA) [65] and α4/β2 (highly expressed in the VTA and many other brain regions including the medial and lateral habenule) and in unrelated genes including proteins involved in nicotine metabolism such as CYP2A6 [66]. It is possible that SNPs in the α4, β2 and β3 subunits affect more the reward from nicotine while SNPs in the α3, α5 and β4 subunits affect withdrawal symptoms. If this is true, the overwhelming genetic evidence would argue that it is withdrawal, and not so much reward, that drive tobacco continuous use, as suggested before [35–38].

The role of the β4 subunit in nicotine's effects

A major role for the β4 subunit of nAChRs in nicotine's effects was first suggested by the lack of hypolocomotion and nicotine induced seizures in β4 null mice [16,29]. These effects were even stronger in β4 that in α5 null mice. As in the case of the α5 subunit, the effects of this mutation on anxiety and nicotine withdrawal were also studied. Interestingly, an anxiolytic phenotype similar to the α5 was found, but no sex differences were reported. More importantly, the somatic signs of nicotine withdrawal were absent on these mice, as was the enhanced nociception exhibited by mice during nicotine withdrawal [21]. A recent report showed using very well designed experiments that the effects of the β4 subunit are indeed mediated by the expression of this subunit in the medial habenula. Frahm et al. used mice that overexpress the β4 subunit using a bacterial artificial chromosome, which directs overexpression of the β4 subunit and not the rest of the cluster mainly to the endogenous expression sites. Those mice showed high aversion to nicotine that could be mitigated by viral-mediated expression of the α5 D398N variant in the medial habenula. This effect was mediated by an increase in current at the channel when β4 is overexpressed. Furthermore, a residue in the β4 subunit that is in close proximity to D398 is necessary for that increase in current to happen, and that effect was mitigated when the α5 D398N was introduced [67]. The authors conclude that that the levels of the β4 subunit in the medial habenula are rate limiting for the amount of current elicited by activation of nAChRs in these cells. In addition, the effects of the D398N genetic variant on nicotine addiction are likely mediated by a decrease of habenular activity of nAChRs containing the α5 and the β4 subunits in the medial habenula. It has been shown that the α5 subunit does not form an active channel with beta subunits unless another alpha subunit is present, and the most likely partner for α5 and β4 subunits in the medial habenula is the α3 subunit (although α4 subunits are highly expressed and known to form active channels in the medial habenula, the sub-habenular expression pattern of the α4 and α3 and β4 subunits suggests that the relevant channels are α3/α5/β4 and not α4/α5/β4-containing [68]). Therefore, this data suggests that the electrophysiological activity of α3/α5/β4-containing nAChRs in the medial habenula is the main cause for the enhanced genetic risk to become a smoker seen in certain genetic populations.

Human genetic work agrees with that conclusion, as SNPs located on those three subunits confer increased risk to become a smoker. As stated before, since most of these genes are in high linkage disequilibrium it is hard to assess the relative contributions of each variant. In addition, rare variants may play a more important role than suggested by GWAS so far: in GWAS there is a major multiple comparison problem: close to a million SNPs are studied in hundreds and even thousands of subjects. Therefore, genetic variants that are rare in the studied population will inevitably fall under detection when multiple comparisons are accounted for during statistical analysis. This kind of variant must be carefully discovered and analyzed using targeted statistical techniques. Showing the possibility that rare variants also play a role, the β4 subunit was shown to have variants that are present in a small percentage of the population but were shown to affect the electrophysiological properties of the channels when expressed in Xenopus oocytes [69]. It is likely that rare variants that affect the nAChR channel activity in the medial habenula also have an impact on habenular activity during tobacco use and withdrawal.

The role of the α3 subunit in nicotine's effects

Mouse work on the α3 subunit has been lagging behind work on the other subunits due to a lethal phenotype: α3 null mice die soon after birth with several peripheral phenotypes. The α3 subunit is highly expressed on peripheral ganglia and α3 null mice show severe defects including extreme bladder enlargement, dribbling urination, and dilated pupils [23].

Interestingly, neither β2 nor β4 null mice have any of these problems despite expression of these subunits in peripheral ganglia. However, mice that lack both β2 and β4 subunits die soon after birth with a phenotype very similar to that of the α3 null mice [24]. Therefore, the β2 and β4 subunits are able to compensate for each other in the periphery, but apparently not so much in the medial habenula, where the lack of β4 subunits results in a major phenotype. Since α3 null mice die before reaching weaning age, only behavior on α3 heterozygous mice was conducted. These mice that carry only one wild type α3 gene showed reduced sensitivity to nicotine-induced seizures that was statistically significant but much lower than in α5 or β4 null mice [29]. In another report using a different technique, an antisense oligonucleotide directed against the α3 subunit was intracerebroventricularly infused in rats. After 7 days of infusion, rats showed decreased α3 expression in brain areas including the medial habenula and thalamus. These rats showed decreased epibatidine-induced seizures. Since epibatidine is a non-specific nAChR agonist, we conclude that those rats with reduced α3 levels in the medial habenula have a similar phenotype to the one that α3 heterozygous mice showed [70]. Similarly to the α5 and β4 subunits, several SNPs in the α3 subunit were discovered during different GWAS screenings [39–42]. As usual, whether the effects of those SNPs are direct or mediated by other SNPs in linkage disequilibrium is a question that needs to be further studied with carefully designed experiments.

The role of the α2 subunit in nicotine's effects

The α2 subunit has not been extensively studied, probably because its pattern of expression is highly restricted. In particular, the α2 subunit is highly expressed in the Ipn, a brain area that has received little scientific attention. Another strong locus of expression is the olfactory bulb [28,71]. Fortunately, a null line of α2 nAChR subunit mice has become available recently. Using these and β2 and β4 null mice, it was shown that the α2 subunit co-precipitates mainly with β2 in the Ipn and with β4 in the olfactory bulb [71]. The α2 null mice were also used on a nicotine withdrawal experiment, and proved resistant similar to β4 and α5 null mice [22]. This effect was hypothesized to be dependent on α2 subunit's expression in the Ipn. Mice null for the β2 subunit (also expressed in the Ipn) showed normal withdrawal signs [20]. We hypothesize that in the Ipn the lack of α2 subunits is enough to significantly alter nicotinic activity, while the lack of β2 is probably masked by availability of β4 subunit, as was demonstrated in peripheral ganglia [24].

Despite being as important as the β4 or the α5 subunits in terms of somatic withdrawal signs in mice, no human genetic data has shown α2 as an important subunit in terms of smoking behaviors. It is possible that once more specific experiments are conducted variants in the α2 subunit are found that regulate addicted behavior, but the importance in humans is probably much less than that of the α3, α5, β4 cluster.

The role of the α4 and β2 subunits in nicotine's effects

The α4β2 subunit-containing receptor was hypothesized for a long time to be the major nicotinic receptor player in the effects of nicotine. In fact, β2 null mice were shown to display deficient nicotine self-administration [18], an effect that was rescued by viral re-expression of β2 subunits in the VTA [17]. In addition, using an α4 gain-of-function mutant mouse, Tapper et al. concluded that activation of α4 subunit-containing nAChRs is sufficient for nicotine reward, tolerance and sensitization [27]. Genetic screens aimed directly at studying the β2 or the α4 subunits in human smokers found associations between genetic variants in these subunits and smoking [72,73]. However, when the more unbiased GWAS experiments came into play, it was clear that the α3/α5/β4 subunits are the major players in the risk to become a smoker, followed by α6/β3 subunits, while variants in α4 subunits appeared only in some populations [74]. Thus, despite being critical mediators of

the rewarding effects of nicotine, the α4 and β2 subunits don't seem to play a major role in smoking behavior. This argues that in smokers the rewarding effects of nicotine are less important than the withdrawal effects mediated by α3, α5 and β4 subunits.

Successful anti-tobacco therapies target β4-containing nicotinic receptors

Currently there are 3 types of pharmacotherapies approved in the USA to help quit smoking: nicotine replacement therapy in its different formulations, varenicline, and bupropion [75]. Interestingly, the only molecular target that these three therapies have in common is the β4-containing nAChR.

Bupropion, initially introduced and still used as an antidepressant, has been used as a pharmacotherapy for smoking cessation for several years. Despite a small incidence of seizures, it is well tolerated and more effective than nicotine replacement therapy to aid in smoking cessation [76]. Bupropion acts mainly at three molecules: it blocks the reuptake of dopamine and norepinephrine, which can account for its antidepressant effects [77], and is also a β4-containing nAChR antagonist [58]. In withdrawal experiments similar to those described on mutant mice, bupropion has been shown to mitigate nicotine withdrawal in rats [78]. Since nicotine withdrawal in rodents has already been shown to depend on β4-containing nAChRs in the medial habenula, it is possible that bupropion's effects on tobacco cessation are at least partially mediated by the same circuit. In addition, bupropion decreased nicotine self-administration in rats [79].

Varenicline is the newest and probably most effective pharmacotherapy against tobacco addiction [75]. It was synthesized using the nicotinic receptor agonist, cytosine as starting point to find nicotinic receptor partial agonists specific for the α4β2 type receptor. The rational was that a partial agonist would first block the effects of nicotine and then block the effects of withdrawal [80]. Accordingly, varenicline is taken first for a week while the patient is still smoking, and then a few more weeks after quitting. According to our hypothesis, varenicline should not be very effective because we believe that β4-containing and not β2-containing receptors are the relevant targets. However, despite hundreds of reports claiming that varenicline is a "specific" α4β2 nAChR partial agonist, varenicline was soon after its synthesis shown to be a good α3β4 partial agonist, and an α7 full agonist [81]. Therefore, we postulate that varenicline's effects on tobacco cessation are due its affinity for α3β4 receptors in the medial habenula and not because it binds to α4β2 receptors elsewhere. It must be noted that varenicline does not decrease nicotine self-administration in β2 null mice as it does in wild type mice [82].

Evidence for the habenula as a general locus for addiction

The medial habenula is most strongly linked to nicotine addiction, and we know now that relevant effects of nicotine are mediated by the medial habenular expression of a specific group of nicotinic receptor subunits. However, there are several lines of evidence for both the medial and the lateral habenule playing a more general role in addiction.

The fasciculus retroflexus degenerates upon treatment with drugs of abuse

Work performed several years ago by Dr. G. Ellison showed that the fasciculus retroflexus, the axon bundle that connects the habenula with its main targets shows the first signs of axonal degeneration in the brain when rodents are treated with stimulants [83,84]. The core region of the fasciculus retroflexus connects the medial habenula to the Ipn, while the external region connects the lateral habenula to the RMTg, which in turn connects to the VTA /SNc. Neurodegeneration was observed in the fasciculus retroflexus upon chronic treatment with several distinct drugs of abuse, including D-amphetamine,

methamphetamine, MDMA, cocaine, and nicotine. With some drugs such as cocaine, the degeneration in the fasciculus retroflexus was virtually the only neurodegeneration observed. This caused the authors to suggest that the fasciculus retroflexus is a “weak link” in the brain that implies loss of forebrain control circuitry in drug abuse [84]. Interestingly, nicotine treatment induced cell death and axonal degeneration in the central core of the fasciculus, while the other stimulants induced neurodegeneration in the external region. In brief, drugs of abuse in general may have a negative effect on the output of the habenula which could be important for the effects of drugs of abuse.

18MC, a β4-containing nAChR antagonist, decreases the effects of several abused drugs

Ibogaine is a hallucinatory alkaloid that is found in the African shrub Tabermanthe iboga. This substance has been used in African cultures for many years in different spiritual practices. In the 60’s, ibogaine was used to treat addictions in general. Ibogaine was later shown to be effective to block cocaine [85], alcohol [86] and morphine [87] self-administration in rodents. Ibogaine was shown to affect several neurotransmitter systems including the cholinergic and glutamatergic. In the USA and many other countries, ibogaine is banned because of severe side effects. Given the documented possible use of ibogaine against drug abuse, a series of ibogaine derivatives was synthesized and their activities studied. The most promising of that group of substances is 18MC, an ibogaine derivative that has been extensively studied in animal models [32]. 18MC decreases the effects of nicotine, cocaine, alcohol and amphetamine in several types of experimental designs in rodents, with no obvious side effects. Interestingly, 18MC has no effect on glutamate receptors, but keeps strong antagonistic activity on β4-containing nAChR. The brain region where 18MC affects drug effects was studied and it was found that the medial habenula and the Ipn are the sites of 18MC’s activity [32]. Thus, a β4-containing nAChR specific antagonist can affect not only nicotine addiction, but also cocaine, methamphetamine and alcohol addiction. It should be noted that although 18MC targets the nicotinic receptors expressed in the medial habenula, its effects are also seen in dopaminergic activity, which is linked to lateral habenula activity. This argues that the medial and the lateral habenula may be functionally connected, as suggested by anatomical data showing connections from the medial to the lateral habenula (but not in the opposite direction) [88].

Varenicline affects dependence to several drugs of abuse

Varenicline’s effects on cocaine self-administration and reinstatement were studied on rats. Low doses of varenicline diminished cocaine reinstatement, while high doses increased it, but decreased self-administration [89]. Varenicline has also been shown to work against alcohol dependence in mice: in doses similar to those used to reduce nicotine reward, varenicline reduced ethanol but not sucrose seeking on a self-administration drinking paradigm. It also decreased voluntary ethanol but not water consumption in mice [90]. These results have lead investigators to postulate that varenicline may be effective against addiction to several drugs, not only nicotine [91]. Although the mechanism proposed in those reports mention α4β2-containing nAChR as the target molecule, we postulate that it is the effect of varenicline at the α3β4-containing receptor that is mainly responsible for the effects observed. In fact, part of the same group that originally developed varenicline and ascribed its properties to effects on α4β2 receptors recently published a report in which they claim that the effect of varenicline on ethanol consumption is likely due to its activity on α3β4 receptors [92].

Electrical stimulation in the lateral habenula reduces cocaine seeking behavior

Deep brain stimulation has been successfully used for a number of conditions. Given the possible roles of the habenula in addiction, the effect of electrical stimulation of the lateral habenula on cocaine self-administration, extinction and reinstatement behavior was studied in rats. Deep brain stimulation reduced cocaine seeking in both the self-administration and the extinction training, and also attenuated the effects of cocaine reinstatement. In contrast, a lateral habenula lesion increased cocaine-seeking behavior. The authors suggest that the effects of lateral habenula stimulation on cocaine seeking behavior may be mediated by a diminished effect of a cocaine-induced increase in glutamatergic input to the VTA [93].

Genetic studies on addictions other than nicotine also point to α3/α5/β4 nAChRs

Two reports have shown that as expected from the prominent role of nAChRs in the habenula and the general role of the habenula in reward prediction and possibly in drug addiction, variants in the α3/α5/β4 gene cluster are involved in addictions other than to nicotine. Wang and colleagues showed that SNPs in the α5 nAChR subunit are associated with alcohol dependence. Since these SNPs are not in high linkage disequilibrium with the SNPs important for tobacco addiction, these are two independent observations [94]. Grucza et al showed that the α5 variant D398N that increases risk of tobacco abuse has the opposite effect on cocaine abuse [95]. This result point to the α5 nAChR subunit as an important player in addiction in general. However, this data is counter-intuitive and should be independently replicated.

Current hypotheses

Given the data from the rodent models, the human genetics and what we know about the role of the habenula in physiology, we postulate that:

- **a.** Once dependence has settled, most people smoke mainly to prevent withdrawal, not for the rewarding effects.
- **b.** Nicotine withdrawal causes the habenula to be in a state of hyperactivity. This habenular hyperactivity produces negative feelings by decreasing dopaminergic activity. Therefore, nicotine withdrawal would be a state of continuous feeling of disappointment or negative emotional state. In fact, negative emotional states have been linked to smoking relapse [96]. Withdrawal to other drugs is likely to be very similar in terms of the circuitry involved.
- **c.** Drugs that block habenular activity should help decrease withdrawal symptoms. These include varenicline and bupropion, arguably the best pharmacotherapies to quit smoking that are currently available.
- **d.** To design new anti-tobacco pharmacotherapies (and possibly anti-drug abuse in general) we should focus on compounds that may decrease habenular activity, such as β4-containing nAChR ligands.

Future experiments

Many experiments need to be done to better understand the role of the habenula in tobacco addiction. Several of these are currently being tackled at our and other labs.

- **a.** It is necessary to measure habenular activity in humans with fMRI during normal smoking and abstinence. We hypothesize that during *ad libitum* smoking,

habenular activity will be comparable to that in non-smoker controls, but during tobacco abstinence, habenular activity (and therefore the effect of negative prediction error or disappointing events) will be higher.

b. The same experiment as in a) needs to be performed on groups of patients that abuse drugs other than nicotine.

c. The effects of nicotinic SNPs, including the α5 D389N, must be studied on habenular activity using fMRI in control and addicted humans during normal drug use and abstinence. We hypothesize that people carrying genetic variants that modulate α3/α5/β4 nAChRs in the habenula will show not only differential risk to nicotine abuse, but also differential levels of basal reward and disappointment (or positive and negative emotional states). It is possible that these genetic variants are important at setting the basal levels of reward and disappointment in human populations, thereby modulating not only tobacco addiction but other addictions and reward-related behavior in general.

d. A major point of study is the relationship between medial and lateral habenula. The relevant nicotinic receptors are all expressed in the medial habenula and its major target, the Ipn. However, neuronal activation upon negative events and subsequent decrease in DA signal happens in the lateral habenula and its major target, the VTA/SNc. Therefore, the major question in terms of circuitry is the relationship between the medial and the lateral habenula. There are connections from the medial to the lateral habenula that could explain the relative contributions of the two structures to addiction [88], but much work must be done to clarify this point.

e. The resting state connectivity of the habenula needs to be studied. When the brain is not engaged into a specific task, groups of neurons that are functionally connected tend to have correlated spontaneous activity: the “noise” in neuronal activity in any given area can be correlated with activity in the rest of the brain, and areas with high correlation are functionally connected [97]. There is a growing body of literature on this effect, including differences in resting state connectivity between patients and controls. A particularly interesting report showed that subjects carrying the α5 D389N variant show decreased connectivity in the cingulated-striatum circuit [98]. The resting state connectivity of the habenula has not been studied yet, mainly because the size of the habenula poses great challenges for this technique. It will be important to study the connectivity of the habenula in control subjects and in drug abusers in both sated and abstinent conditions.

f. Finally, if our hypotheses are correct, a major unanswered question is the mechanism by which the habenula and its connecting structures change activity chronically after repeated nicotine use.

Conclusions

In summary, data from rodent work and data from human genetics point to the medial habenula and the nicotinic receptors expressed therein as critical neuronal mediators of drug abuse, including tobacco. To design improved pharmacotherapies to treat drug addiction, much work must be done to understand the circuitry and molecular mechanisms of habenular activity.

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Abbreviations

Ipn	Interpeduncular Nucleus
VTA	Ventral Tegmental Area
RMTg	Rostromedial Tegmental Nucleus
nAChR	Nicotinic Acetylcholine Receptor
GWAS	Genome-Wide Association Study
SNP	Single Nucleotide Polymorphism
fMRI	Functional Magnetic Resonance Imaging
SNc	Substantia Nigra Compacta

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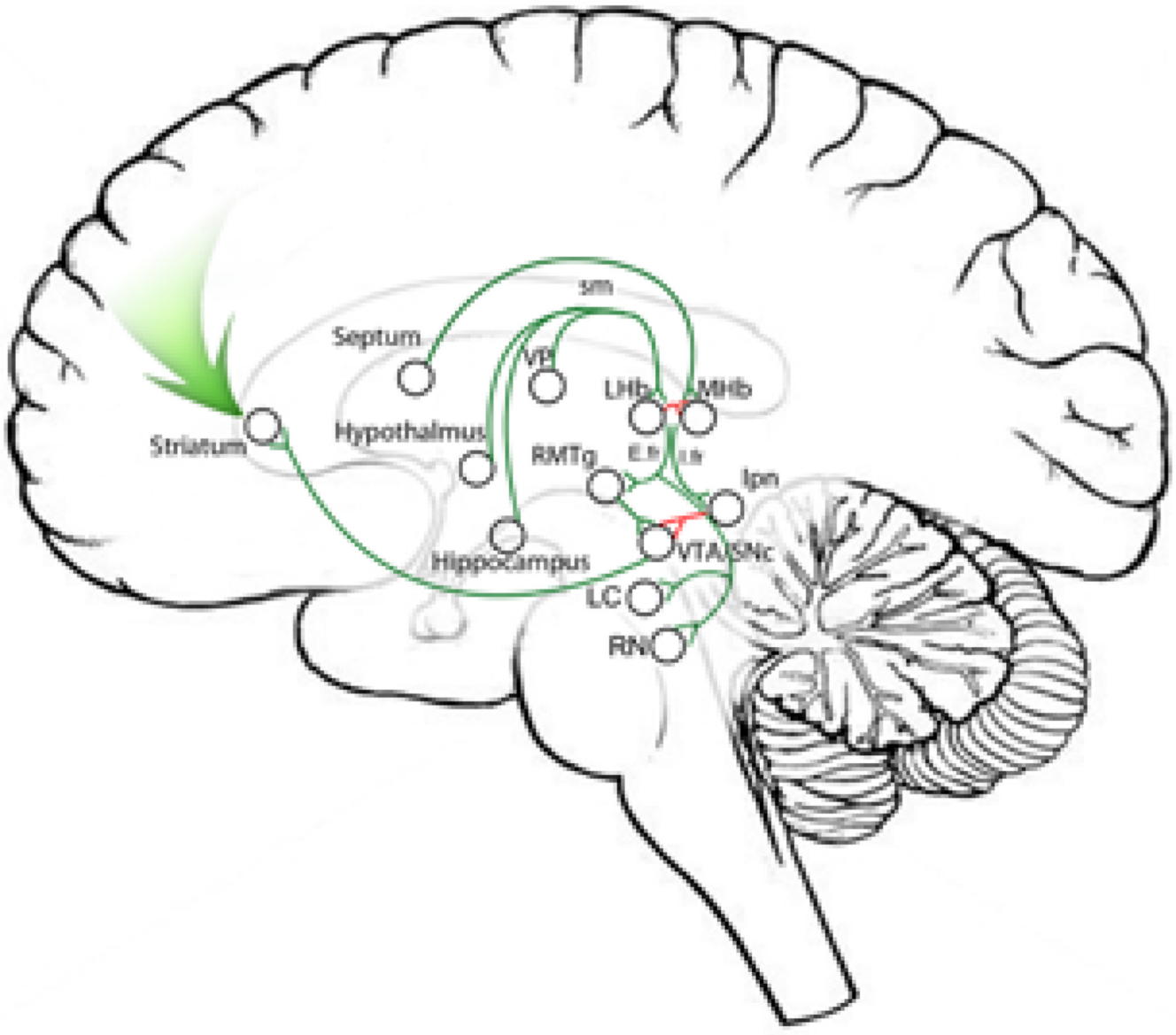

Figure 1.

The habenula and connecting structures. Several areas confer input to the habenule through the stria medullaris (sm). The external part of the fasciculus retroflexus (E.fr) connects the lateral habenula (LHb) to the locus coeruleus (LC), to the raphe nucleus (RN), and via the rostromedial tegmental nucleus (RMTg) to the ventral tegmental area (VTA). The internal part of the fasciculus retroflexus (I.fr) connects the medial habenula (MHb) to the interpeduncular nucleus (Ipn). Medial to lateral habenula connections and Ipn to VTA connections are depicted in red: these connections are hypothesized to be important but their significance is not clear.

Table 1

Nicotine-related phenotypes of mice with mutations in the nAChR subunits.

nAChR subunit	Brain expression pattern	Phenotype	References
α2	Ipn, olfactory bulb	Resistant to mecamylamine-induced nicotine withdrawal somatic signs	[22,28,71]
α3	Medial habenula, Ipn, VTA, thalamus, pineal	Peripheral symptoms (lethal). Decreased nicotine-induced seizures in heterozygous mice	[23,29]
α4	Quasi-ubiquitous	Impaired nicotine reward, tolerance sensitization, and self-administration	[27,99]
α5	Hippocampus CA1, medial habenula, Ipn, VTA, cortex	Resistant to mecamylamine-induced nicotine withdrawal somatic signs, and nicotine-induced seizures and locomotion. α5 knock down in habenula increases nicotine self-administration	[16,19,63]
α6	VTA	Impaired nicotine self-administration	[99]
α7	Quasi-ubiquitous	Increased nicotine induces seizures in gain-of-function mice. Normal nicotine self administration	[25,99]
β2	Quasi-ubiquitous	Impaired nicotine self-administration, rescued by VTA re-expression	[17,18]
β3	Medial habenula, VTA, Locus coeruleus	Modulates dopamine release	[14]
β4	Olfactory bulb, medial habenula, pineal	Resistant to mecamylamine-induced nicotine withdrawal somatic signs, and nicotine-induced seizures and locomotion. Habenular overexpression induces nicotine avoidance	[16,20,29,67]