The nicotinic receptor alpha5 coding polymorphism rs16969968 as a major target in disease: Functional dissection and remaining challenges

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Abstract

Nicotinic acetylcholine receptors (nAChRs) are major signalling molecules in the central and peripheral nervous system. Over the last decade, they have been linked to a number of major human psychiatric and neurological conditions, like smoking, schizophrenia, Alzheimer's disease, and many others. Human Genome-Wide Association Studies (GWAS) have robustly identified genetic alterations at a locus of chromosome 15q to several of these diseases. In this review, we discuss a major coding polymorphism in the alpha5 subunit, referred to as α5SNP, and its functional dissection *in vitro* and *in vivo*. Its presence at high frequency in many human populations lends itself to pharmaceutical intervention in the context of "positive allosteric modulators" (PAMs). We will present the prospects of this novel treatment, and the remaining challenges to identify suitable molecules.

Introduction

Acetylcholine is a major signalling molecule in most biological systems, and has been so for the last three billion years (Yamada *et al.* 2005). This includes also a pivotal role in the central and peripheral nervous system of animals (Le Novère *et al.* 2002). It exerts its function through two types of receptors, the ionotropic nicotinic acetylcholine receptors (nAChRs), and the metabotropic muscarinic acetylcholine receptors.

We will focus here on the ionotropic receptors. nAChRs in the mammalian genome are encoded by a total of 16 genes, comprising alpha and beta subunits (Corringer *et al.* 2000). These come together in a myriad of combinations comprising obligatory alpha subunits providing the essential binding sites for ACh and the exogenous ligand nicotine. And accessory beta subunits contributing to alpha/beta subunit containing hetero-pentameric receptors. They have been considered important drug targets for a number of indications, from smoking cessation to Alzheimer's disease (AD) (Taly *et al.* 2009; Lombardo and Maskos 2015).

The human genetics of alpha5 nAChRs

Further support for this translational interest stems from findings in humans themselves. Over the last ten years, human genetic studies, mostly through genome-wide association studies (GWAS) have highlighted genetic alterations in nAChRs linked to a number of major diseases. These include nicotine addiction (Tobacco_and_Genetics_Consortium. 2010), schizophrenia (Ripke *et al.* 2014), cannabis use disorder (Demontis *et al.* 2019), lung cancer (Hung *et al.* 2008; Thorgeirsson *et al.* 2008; Amos *et al.* 2008), Chronic Obstructive Pulmonary Disease (COPD) (Pillai *et al.* 2009; Wilk *et al.* 2012; Busch *et al.* 2017) and lung function (Shrine *et al.* 2019). They have also established links to Body-Mass Index (BMI) (Taylor *et al.* 2014), and longevity (Joshi *et al.* 2016). The link with lung cancer was to some extent the most surprising, as the hit to the nAChRs was the only locus identified in these original studies. However, as pointed out immediately (Chanock and Hunter 2008), smoking is known to be the most important risk factor for lung cancer, and the initial assumption was that these human polymorphisms would have a role in nicotine addiction, a link discussed further in the following sections.

Furthermore, a large number of candidate-gene studies has identified additional potential roles for nicotinic subunits in other addictions, to alcohol, cocaine (Grucza *et al.* 2008), and opioids (Curtis *et al.* 2017), or even all three of them (Sherva *et al.* 2010).

Most of these studies have identified Single Nucleotide Polymorphisms (SNPs) in one particular part of the human genome, at position 15q, where the hits span three different genes *CHRNA3*, *CHRNA5* and *CHRNB4*, coding for the alpha3, alpha5 and beta4 subunit proteins, respectively, see **Figure 1**. Interestingly, these three genes are co-expressed in a number of tissues, and also specific cell types in the nervous system, like peripheral ganglia, and also the interpeduncular nucleus (IPN) in the central nervous system (CNS).

These human genetic studies and associated polymorphisms have been summarised in a number of reviews, and will not be discussed further here.

We will focus here on a particular non-synonymous (coding) SNP, rs16969968, changing one amino acid in the alpha5 protein, at position 398, from aspartate (D) to asparagine (N), an amino acid perfectly conserved in evolution (Bierut *et al.* 2008). This mutation was first identified in a candidate gene study for smoking in 2007 (Saccone *et al.* 2007). We will pursue the working hypothesis that the important effect on "odds ratios" identified in the 15q cluster are due to a large extent to this amino acid change, found in linkage disequilibrium with other non-coding SNPs. An important point is the relative frequency of the mutation in many human populations, up to 37% in Europeans, and even higher in the Middle East (Bierut *et al.* 2008). Comparing the human protein with the sequence in other species, **Figure 2**, one realises an almost perfect conservation of amino acids around the 398 position, which corresponds to position 397 in mice and rats.

The available evidence will be discussed, and missing pieces in the mechanistic dissection identified. It is important to point out that the alpha5 subunit can only serve as an "accessory" fifth subunit in a nicotinic receptor pentamer (Corringer *et al.* 2000). This is because it is unable, like the beta3 subunit, to contribute to an ACh binding site that is always formed between a beta subunit, beta2 or beta4, and the other alpha subunits, mostly alpha4, and alpha6. However, the presence of the alpha5 has a marked influence on the properties of the pentamer (Ramirez-Latorre *et al.* 1996).

The alpha3 subunit is the main component in the peripheral nervous system, and present in the central nervous system in very specific locations, like the IPN. Some of the possible combinations of pentamers including the alpha5 subunit are indicated in **Figure 3**.

Functional consequences of rs16969968 *in vitro*

A. HEK cells

Bierut and colleagues started a functional analysis of the coding polymorphism, from here on referred to as α5SNP. They used the expression of the cloned human alpha5 subunit, either wild-type (WT), or the mutated α5SNP, together with alpha4 and beta2 subunits, in HEK cells. Calcium imaging was the readout (Bierut *et al.* 2008). In the following, we will always look in detail at the experimental procedures employed, as important questions will remain with respect to the technologies used to identify functional consequences. The co-expression of the three subunits leads to the presence of putative alpha5 containing pentamers, with alpha4 and beta2 at a stoichiometry of two subunits each. The expression levels of the pentamer(s) were measured using epibatidine binding, a compound used to identify fully formed pentamers (Scholze *et al.* 2012), and by Western blotting of the two alpha5 isoforms, WT and SNP. The main finding was that the calcium response, measured as total intra-cellular calcium, was significantly different between the two: the dose-response curve of epibatidine-evoked calcium release was shifted for α5SNP, and the maximal response to stimulation was lower in the α5SNP. However, the EC50 values did not differ between the two. This was thus an indication of a (partial) loss of function elicited by α5SNP.

These initial findings were rapidly followed by more advanced studies using Xenopus oocytes, once again injected with individual, or "single", nicotinic receptor subunits.

Stitzel and collaborators continued the analysis of α5SNP function in HEK cells (Tammimaki *et al.* 2012). They used aequorin-based calcium imaging, and focused on the alpha5 association with the alpha3 and beta4 subunits, the combination found in peripheral ganglia, but also the IPN. The cell lines used stably expressed the different subunits, and surface expression of α5-containing receptors was similar to α3β4-only receptors. Inclusion of alpha5 subunits reduced calcium responses, and the α5SNP elicited a further reduction compared to the WT alpha5

subunit. This reduced response to agonist was most pronounced at high intracellular calcium levels.

A study from the Steinbach laboratory using a similar expression approach came to a different conclusion (Li *et al.* 2011). When carrying out single channel recordings of the same receptor subunits, no differences between the presence of the α5SNP or WT subunits were observed concerning the pharmacology of receptor activation, when using ACh, nicotine, cytisine or DMPP (Dimethylphenylpiperazinium), or the nicotine metabolites cotinine, NNN (*N*-Nitrosonornicotine), or NNK (Nicotine-derived nitrosamine ketone), as agonists. The desensitisation behaviour, or the individual single-channel properties, were also not altered in their hands.

A more recent paper by Fucile and collaborators used a number of different model systems to identify mechanisms of action of α5SNP (Sciaccaluga *et al.* 2015). For the heterologous expression, they used a different cell line for the study of concatemers of the alpha4 beta2 type in GH4C1 cells, a rat pituitary derived line. The co-expression of α5SNP or WT subunits caused only very minor differences in the densensitisation rate. Importantly, this differential response of α5SNP and WT expressing cells was altered when the internal calcium concentration of the cells was modified. This served as an indication that intra-cellular modulation could be an important mechanism of action for the α5SNP.

B. Xenopus oocytes

The studies above were complemented by a number of analyses using Xenopus oocytes. One early contribution came from the laboratory of Jon Lindstrom (Kuryatov *et al.* 2011). For the expression of alpha3 containing receptors, they used "single" subunits as above, but to circumvent the issues potentially associated with the use of individual subunits, like the occurrence of multiple different pentameric combinations, they opted for a "concatemeric" approach: an expression construct linking two alpha4 and two beta2 subunits into a tetramer, with the individual subunits being separated by short amino acid linkers. The subunit order was alpha4-beta2-alpha4-beta2. They then co-expressed the WT or α5SNP subunit and compared the consequences.

There were no differences between WT and α5SNP in the alpha3* context, but a number of phenotypes were revealed in the (alpha4 beta2)2 context. Importantly, the authors found a partial loss of calcium permeability in the α5SNP containing receptor, when calcium was the only cation in the buffer. In addition, an important change in desensitisation of the alpha4beta2 containg receptors was identified when α5SNP was present, compared to WT.

In direct contrast, a study one year later re-examined the role of α5SNP in alpha3beta4 containing receptors, and came to a different conclusion (George *et al.* 2012). Importantly, they were the first to use a fully concatenated pentamer for their functional studies in oocytes. They could show conclusively that the inclusion of α5SNP reduces the acetylcholine-mediated function of the pentamer, compared to WT.

A similar full pentameric concatemer molecule was used by Pierre-Jean Corringer and co-workers. In a forthcoming paper (Prevost *et al.* 2020), they carried out an elegant dissection of pharmacological and functional properties. They describe the design of an α4β2α5 concatemer that allows the expression of a robust and fully controlled population of nAChRs incorporating the α5 subunit to characterise its properties in two-electrode voltage-clamp electrophysiology after expression in Xenopus oocytes. They examined the electrophysiological and pharmacological properties of this concatemer as opposed to alpha4 and beta2-only containing pentameric concatemers, and found that the α5 can form an ACh binding site as a complementary component. In these experiments methanethiosulfonate ethylammonium (MTSEA) was able to specifically and irreversibly block the α5-containing concatemer. The concatemeric strategy applied here to the α4β2α5 nAChRs allowed the re-investigation of their intrinsic properties and provided a new reliable tool to screen specific compounds.
Importantly, Corringer and colleagues have developed here a convenient concatemeric system to "force" the subunit stoichiometry of the pentamer, which display currents large enough for characterisation. They are thereby fully assigning the electrophysiological currents to α5-containing receptors. Additionally, they provide evidence that the concatemers assemble according to two orientations, clockwise and anticlockwise, a feature that must be considered when interpreting pharmacological data. In the literature, the other study describing pentameric

concatemeric α4β2α5 nAChRs (Jin *et al.* 2014a) differs from theirs in the linker used and the subunit order. Jin and collaborators inserted alpha5 between two alpha4s, with the final subunit order of beta2-alpha4-alpha5-alpha4-beta2. The resulting constructs displayed low current amplitudes, with maximal currents around 30nA, precluding their use for systematic functional and pharmacological investigation.

The important finding of Prévost *et al.* is that they found no significant effect of the α5SNP mutation on the concatemeric function. This contrasts with its strong effect in human populations and preclinical models, see below. They thus speculate that α5SNP might rather alter other features of the receptor, such as its biosynthesis and/or trafficking. In this line, it is noteworthy that the α5SNP mutation is located in the intra-cellular loop of the receptor subunit, the domain for which we have little structural information. This intra-cellular loop mutation could thus affect export to the cell surface, the "upregulation" of the receptor under chronic nicotine treatment, diffusion in the plasma membrane, or internalisation processes, rather than intrinsic functional properties of the channel. Unfortunately, these "cell-biological" processes cannot be studied in Xenopus oocytes, that are far from recapitulating the conditions in neuronal cells.

More generally, despite these caveats, the concatemeric designs of Prévost et al., which force the incorporation of the α5 subunit, offer a well characterised platform to screen for allosteric modulators of α5-containing nAChRs, and decipher their mechanisms of action. These concatemers will also allow for a precise dissection of the gating mechanisms, since they enable to introduce mutations one subunit at a time into the entire pentamer to assess the interactions between subunits, as well as the long-range allosteric communication between distant binding sites.

The results contrast with Marotta *et al.* (2014) who showed that, for a 1:1:10 ratio for α4:β2:α5(V9'S) expressed by RNA injection in the oocyte, a nearly homogeneous population containing the α5(V9'S) subunit was present, which specifically shows fast-reversible mecamylamine inhibition. It is difficult to reconcile these two studies. It is possible that the discrepancy could be due to RNA versus cDNA injection. Another factor to take into account is the presence of a mutation in the channel pore in the Marotta *et al.* (2014) study (V9'S), since single mutations in the transmembrane domain have been reported to strongly alter subunit

assemblies in $GABA_A$ receptors that belong to the same family of ligand-gate ion channels (Hannan and Smart 2018).

C. Primary neurons

Surprisingly little work has been carried out in primary neurons, although there the native environment of nAChRs would be mostly preserved, unlike in the above heterologous sytems, human embryonic kidney cells, or even more so, Xenopus oocytes.

The laboratory of Sergio Fucile used a preparation of ventral mesencephalic neurons from E14 mouse embryos of the three genotypes (Sciaccaluga *et al.* 2015). They carried out calcium imaging, using Fura2, of both the processes and somata of these neurons. Nicotine elicited calcium transients were larger in the two cell compartments in WT compared to α5SNP. Generally, the precentage of cells responding to nicotine treatment was significantly lower in α5SNP, and zero for the KO.

D. Human induced pluripotent stem cell (iPSC) derived neurons

When addressing a human polymorphism, it makes perfect sense to use human cells with the background of a human genome in these studies. Our laboratory published a study using iPSC-derived cells from subjects with the three genotypes, wild-type DD, heterozygous, and homozygous NN. The cell type used was dopaminergic neurons in culture, studied with patch clamp electrophysiology, coupled to single-cell qPCR analysis of selected genes for the different nAChR subunits (Deflorio *et al.* 2017). The alpha5 subunit is highly expressed in rodent dopaminergic neurons (Klink *et al.* 2001), and the presence of its RNA was verified from individual patched neurons. The response of nicotinic currents to commonly used antagonists was established in dose-response curves for mecamylamine, bungarotoxin and especially conotoxin MII, a specific antagonist of alpha6 containing nAChRs, and a specific marker for DA neurons. Then, ACh and nicotine were applied to the three genotypes to establish a dose response curve. For both ACh and nicotine, a significant difference was observed for the EC50 between homozygous NN and DD in the human DA neurons (Deflorio *et al.* 2017).

Others used a different means to analyse phenotypes due to the α5SNP (Oni *et al.* 2016). They generated both human iPSC-derived dopaminergic neurons similar to the protocol used above, but also a population of glutamatergic neurons. Rather than analysing the direct response of the expressed nAChRs, they incubated the cultures with low-dose nicotine, 0.1uM, and measured the postsynaptic response due to glutamatergic activation of the neurons. These responses were significantly enhanced in both cell types for the α5SNP homozygous genotype. Adding higher doses of nicotine then led to a differential desensitisation of the α5SNP containing receptors.
Oni et al. (2016) did link this to a comprehensive RNA-seq analysis of the transcriptome of the two genotypes, wild-type and α5SNP. GO analysis suggests a differential upregulation of genes in the α5SNP cells, that taken together could modify the responses to many neurotransmitters. This paper thus exemplifies a potentially enormous transcriptomic change in α5SNP expressing neurons. It will therefore be important to continue this dissection and identify the intracellular signaling pathways responsible for the phenotypes.

Generally, the use of iPSC in the dissection of α5SNP can still be improved. For example, in both papers the different genotypes were analysed in different cell lines from separate donors. Isogenic lines will allow to identify more subtle differences in the consequences of nAChR function.

E. Brain slices

Similarly, maybe because transgenic mice and rats expressing α5SNP have only recently been published, little work has focused on the analysis of phenotypes observable using slice electrophysiology.

Working with the groups of Philippe Faure and Betrand Lambolez, we carried out slice recordings from ventral tegmental area (VTA) of adult mice (Morel *et al.* 2014). Using DMPP (Dimethylphenylpiperazinium, 100uM) as a canonical agonist of nAChRs, reponses from α5KO mice showed a clear reduction in currents, whereas α5SNP and WT expressing mice, through the

expression of the corresponding lentiviral vectors, showed no significant difference. However, only the dose of 100uM was used, and no dose-response curve was established

Using a number of different model systems, Fucile and collaborators also recorded from ventral midbrain slices of the three different genotypes of three week-old males (Sciaccaluga *et al.* 2015). They could show that the alpha5 subunit is essential for the presence of a high amplitude subpopulation of inward currents mediated by heteromeric nAChRs in neurons in mouse VTA slices, with α5SNP being less efficient than the WT subunit. Two major current classes in the dopaminergic neurons recorded were identified, of around -10 pA and -45 pA. In the alpha5 KO mouse, the -45 pA current was no longer detectable. The occurrence of that same current was significantly lower in α5SNP compared to wild-type mice.

The main difference observed in slice electrophysiology between α5SNP and wild-type neurons came from a study using transgenic rats, whose generation is detailed below (Forget *et al.* 2018). Slice electrophysiology was carried out on IPN neurons of the three genotypes, of adult rats. The important finding was that nicotine elicited currents, in response to puff application for 200ms of 30uM, of both α5SNP and α5KO animals were indistinguishable, far inferior to the large currents obtained in wild-types, on the order of 300pA. The IPN is the structure in the rodent brain with the highest expression of alpha5* nAChR receptors, and therefore nicotine-related behaviours linked to this structure are likely very important. This is a primary example for a considerable loss of function by α5SNP, and similar in consequence to the full KO.

Functional consequences of rs16969968 *in vivo*

A. Humans

Surprisingly, the first demonstration of a functional impact of α5SNP *in vivo* came from imaging studies in humans (Hong *et al.* 2010). Hong and collaborators carried out magnetic resonance imaging (MRI) studies comparing the three genotypes, humans homozygous and heterozygous for α5SNP, and homozygous for the major allele. The cohort included smokers and also psychiatric patients. The readout was resting-state functional connectivity, a measure of the

strength of connections between different brain structures. As a function of α5SNP, a significant difference was observed for the three genotypes, for a brain network comprising the dorsal anterior cingulate cortex, and multiple brain regions, including bilateral insula, medial frontal, middle frontal and temporal cortices, middle and posterior cingulate and precuneus, striatum, amygdala, hippocampus, thalamus, and brainstem. Interestingly, the functional activity was also altered in smokers versus non-smokers, and was further impaired in the patients. This functional measure can now serve as a biomarker for potential pharmaceutical intervention for both smoking and schizophrenia.

B. Non-human primates

Spindel and collaborators carried out a preliminary analysis of α5SNP action in non-human primates, a species of cynomolgus macaques, originating from Southeast Asia (Shorey-Kendrick *et al.* 2015). This species expresses the same variant coding polymorphism as humans, unlike rhesus macaques. Interestingly, the frequency of the monkey α5SNP of 37.4% is almost the same as in the "average" human European population, 35%. The authors identified two homozygous α5SNP carriers, and carried out a comparison with a "control" group comprising five subjects that were wild-type or heterozygous. They set up a two-bottle choice paradigm for nicotine in the drinking water. Controls consumed significantly more nicotine at low doses, and were indistinguishable from α5SNP at high doses. However, nocturnal nicotine consumption for α5SNP showed a close to significant trend towards increased intake. Interestingly, whereas nicotine intake was related to the consumption of food in wild-types, this was unrelated in α5SNP. Clearly, the limited *n* in this study has not allowed to draw very firm conclusions, but this species would represent a major advantage to further dissect the role of α5SNP in a primate model, and to test potential medication.

C. Rodents

Use of transgenic mice

A comprehensive analysis of a role for α5SNP *in vivo* was carried out in the habenulo-interpeduncular system of mice (Frahm *et al.* 2011). The role of the medial habenula, and the expression of alpha5, in the aversive effects of nicotine, and in regulating nicotine intake, was

also highlighted by a paper from Paul Kenny and collaborators that same year (Fowler *et al.* 2011). The laboratory of Ines Ibanez-Tallon used bacterial artifical chromosome (BAC) mediated over-expression of the alpha3 and beta4 subunits from the 15q cluster where many non-coding SNPs had been identified. This led to the strong enhancement of binding sites for alpha3beta4* nAChRs in many parts of the brain, including the VTA, but also the IPN and medial habenula. Using a two-bottle choice paradigm of nicotine self-administration from the drinking water, the transgenic mice reduced their nicotine intake compared to controls. This was seen as an increase in the aversion to nicotine mediated by the over-expression of the receptors in areas that had previously been linked to aversion. Using a lentiviral expression of α5SNP specifically in the medial habenula (mHb), the authors were able to reduce the "protective" effect of alpha3beta4* nAChR in this paradigm. This thus identified a role for α5SNP in that brain structure. **Figure 4** gives an example of the modelling carried out on the IPN containing receptor, where the presence of the α5SNP alters markedly the charge distribution in what can be seen as an intra-cellular "pore" potentially altering the influx of cations.

Our laboratory expressed the α5SNP in the reward system of mice, and studied the consequences at the level of electrophysiology, and behaviour (Morel *et al.* 2014). Using a lentiviral expression construct as before for most other nAChR subunits, like beta2 (Maskos *et al.* 2005), alpha4 (Pons *et al.* 2008) and alpha6 (Exley *et al.* 2011), the α5SNP and WT subunits were expressed exclusively in the VTA of mice on an alpha5 KO background. An intra-venous short-term nicotine self-administration procedure was employed, and the doses of nicotine that gave *active* nicotine intake of "active" versus "passive" mice was scored. The presence of α5SNP led to active self-administration at a significantly higher dose in α5SNP versus alpha5 WT mice. *In vivo* electrophysiology using extracellular recordings in the VTA of anesthetised mice identified a difference in the respone to intra-venous injection of nicotine. At a dose of 15ug/kg a dopaminergic neuron response was detectable in WT and alpha5 WT re-expressed mice, but not in α5SNP re-expressed mice. At 30ug/kg, the three groups became indistinguishable.

Another study from our laboratory used a similar lentiviral expression study, and extended the targeted area from VTA to also hippocampal expression of α5SNP versus alpha5 WT on an alpha5

KO genetic background (Besson *et al.* 2016). An initial behavioural analysis identified anxiety-like behaviour in α5KO mice that could be reduced by application of high doses of nicotine. This phenotype was also rescued by the re-expression of α5 WT lentiviral vector in the hippocampus, whereas a similar re-epression of α5SNP failed to do so. This work extends the role of α5SNP to a new modality, anxiety, and a new brain structure, the hippocampus. Further work will be needed to identify the hippocampal cell types, most likely interneurons, expressing α5 nAChRs underlying this behaviour.

Our laboratory then extended the analysis of α5SNP to its function in cortex (Koukouli *et al.* 2017). Cortical nAChRs had been implicated in a number of important executive functions, decision making, social behaviour, and attention (Guillem *et al.* 2011; Avale *et al.* 2011). This study was inspired by a major GWAS study published in 2014 that linked the same human polymorphisms in the *CHRNA3/A5/B4* cluster to schizophrenia (Ripke *et al.* 2014). The laboratory of Jerry Stitzel generated the α5SNP knock-in mouse using standard embryonic stem cell technology. An analysis of behavioural features indicated schizophrenia-linked symptoms, such as altered social behaviour in the context of a congener, and altered pre-pulse inhibition, a characteristic in human patients. Using functional two-photon calcium imaging employing the GCaMP6f indicator, Koukouli et al. identified a major reduction in average cortical activity of upper cortical layers 2 and 3, reminiscent of "hypo-frontality" in humans. This hypoactivity could be linked to the presence of α5SNP in a particular class of cortical interneuron, the vasopressin intestinal peptide (VIP) expressing subtype, that inhibits the two main classes of cortical interneurons, the parvalbumin (PV) and somatostatin (SOM) containing subtypes. A partial loss of VIP function through the presence of α5SNP leads to a disinhibition of mainly SOM interneurons, and a reduction in pyramidal cell activity. This can be restored by the application of chronic nicotine, at concentrations obtained in the serum of smokers, through the desensitisation of these same SOM interneurons, and a consequent loss of inhibition of principal cells.

Stitzel and collaborators made another interesting use of the α5SNP mice they had generated (O'Neill *et al.* 2018). Pregnant dams of both genotypes were exposed to nicotine in the drinking

water. The offspring were then tested in a two-bottle choice paradigm for nicotine consumption, and the α5SNP and homozygous wild-types were compared. α5SNP exposed to nicotine *in utero* consumed significantly more in this test than the other groups, wereas wild-types exposed to nicotine *in utero* consumed significantly less. The behaviour could be correlated with nicotine-elicited dopamine release from striatal synaptosomes. This was significantly enhanced in the low-consumption wild-type group, and reduced in α5SNP. These results highlight a trans-generational influence of nicotine exposure in α5SNP mice, and an example of *environment x gene* interaction.

Use of transgenic rats

Our group followed up the transgenic mouse studies with more advanced behavioural studies only possible in rats. Nicotine dependence can only be satisfactorily modeled in rats, as it is possible to take them through long-term chronic intra-venous nicotine self-administration studies, using fixed-ratio and progressive-ratio paradigms, followed by an extinction of the acquired behaviour, and then relapse elicited by a number of ways to provoke nicotine seeking behaviour. Especially, this last point is a crucial element in the human case.

To generate transgenic rats expressing constitutively the α5SNP, a knock-in strategy was used employing the technique of zinc finger nuclease (ZFN) directed genome engineering (Forget *et al.* 2018). The α5SNP transgenic rats, α5 knock-out rats and wild-types were then first tested in fixed-ratio and progressive ratio nicotine intra-venous self-administration (SA) studies. Importantly, α5 knock-out rats did not acquire any SA at the doses chosen. This lends support to the interpretation that the presence of α5 in the reward system, and mainly dopaminergic neurons, is a key factor for the mechanism of action of the polymorphism. α5SNP and wild-type rats acquired SA similarly, but α5SNP administered more nicotine at higher doses, a behaviour that reflects the human situation.

A key role for α5SNP was identified after extinction of SA behaviour, and relapse to nicotine seeking. Three paradigms were used, cue-only (light) induced reinstatement, nicotine-injection induced reinstatement, and a combination of the two. Whereas the cue-alone elicited response was not different between genotypes, the other two showed a marked increase in nicotine seeking from the α5SNP rats. This important finding was followed up with c-fos staining after

relapse, and a clear difference in the neuronal activity was identified in the IPN. As indicated above, slice electrophysiology of the IPN demonstrated a knock-out like response to nicotine for the α5SNP neurons compared to α5KO. This further highlights the need to dissect more the role of the IPN in nicotine dependence.

We then used the same transgenic rats to analyse the role of α5SNP in alcohol dependence and feeding behaviour (Besson *et al.* 2019). SA protocols were set up for both oral alcohol and food pellets. Relapse after abstinence was then provoked similar to the work described for nicotine. α5SNP rats consumed more alcohol than wild-types, when administered as drops of 12% EtOH in water. They also relapsed to a higher extent when challenged with EtOH plus cue after abstinence. For food, no differences were obtained in the amount of "self-administration" between the two phenotypes. However, relapse after abstinence, provoked by both food-relapse and cue-relapse, were significantly enhanced in the α5SNP.

A further analysis in these transgenic rats was carried out for cocaine dependence (Forget *et al., submitted*). Cocaine addiction is a chronic and relapsing disorder with an important genetic component. Human candidate gene association studies showed that α5SNP was linked to a lower prevalence of cocaine use disorder (CUD). Three additional SNPs in the α5 subunit, previously shown to modify α5 mRNA levels, were also associated with CUD, suggesting an important role of the subunit in this pathology. We therefore submitted rats knockout for the α5 subunit gene (α5KO), or carrying the α5SNP, to cocaine SA and evaluated the association of the α5SNP and of SNPs associated with changes in α5 nAChR subunit expression with CUD in a human cohort. The acquisition of cocaine-SA was impaired in α5SNP while α5KO rats exhibited enhanced cocaine-induced relapse associated with altered neuronal activity in the Nucleus Accumbens (NAcc).

The α5SNP seems to protect against CUD by influencing early stages of cocaine exposure while *CHRNA5* expression levels may represent a biomarker for the risk to relapse to cocaine use. Drugs modulating α5 containing nAChR activity may represent a novel therapeutic strategy against CUD.

Possibility of pharmaceutical intervention

From the above, it has become clear that despite some important inconsistencies in the literature, that should be revisited urgently, the presence of the α5SNP constitutes a partial loss-of-function of the corresponding pentamer. We and others (Kuryatov *et al.* 2011; Jin *et al.* 2014b) have proposed that this partial loss of function could potentially be restored, to therapeutic benefit, by small molecules acting as "positive allosteric modulators" (PAMs) of channel function in the high-affinity alpha4beta2* context, and potentially also for alpha3* containing receptors. PAMs would have the property of not binding to the orthosteric site, thus not interfering with the action of the agonist, acetylcholine.

Given the prevalence of the rs16969968 polymorphism in several populations, especially caucasian, homozygous carriers would potentially benefit from this novel class of medication for the indications desribed above. Primary examples would include smoking cessation as demonstrated with the transgenic rat work linking the α5SNP specifically to relapse (Forget *et al.* 2018), and also schizophrenia. These two indications are based on the strong link of the disease to robust human genetic findings, and a fairly complete understanding of the underlying mechanisms.

Conclusions

It has become clear, over the last decade, that the α5SNP represents an important change in the cholinergic-nicotinic system of many human carriers. Initial human genetic findings, and first human imaging studies, are now being dissected in transgenic mouse and rat models. However, the precise mechanistic basis for the changes elicited by this single amino acid substitution are far from clear, even in the most basic *in vitro* studies. Numerous analyses in heterologous expression systems, notably Xenopus oocytes and HEK cells, contradict each other directly. The key aspect that has to be taken into account is the way the different nAChR subunits are expressed, and assembled, in most of the published work so far. Transfecting "single" subunits into oocytes or fibroblasts leads to the presence and assembly of multiple forms of receptors, as the only requirement for a functional receptor lies in the presence of two alpha/beta interfaces, which is additionally modulated by the potential binding site obtained for example between two adjacent alpha4 subunits, as in (alpha4)3 (beta2)2 receptors.

Therefore, in Xenopus oocytes the most robust findings will stem from fully concatemeric expression constructs, constraining the presence to one identified pentamer in the sample. This is so far not possible, or has not been achieved, in mammalian expression systems. It will make a difference for ongoing approaches to screen for potential modulators of alpha5 containing receptors, PAMs, whether defined pentamers will also be able to be obtained in the standard screening systems employed by pharmaceutical companies. There are potentially a number of ways to achieve this, but it will require extensive genetic engineering of the currently used mammalian cell lines.

--Human subjects --
Involves human subjects:
If yes: Informed consent & ethics approval achieved:
=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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Figure legends

Figure 1. The genomic cluster on chromosome 15q

An outline of the relative positions of the three nAChR genes in the 15q cluster. The direction of transcription is indicated, as are major non-coding polymorphisms identified in large scale GWAS. The position of the α5SNP coding polymorphism, rs16969968, is highlighted.

Figure 2. Conservation of amino acid sequences around position 398 in the alpha5 protein

The amino acid sequences surrounding the α5SNP (D**, in bold**) are indicated, showing their strong conservation across species. A main difference lies in the serine **S** moiety. It is present in humans, non-human primates and amphibians, but not rodents.

Figure 3. Possible subunit combinations including a single alpha5 subunit

The alpha5 subunit is expressed in both the central and peripheral nervous system. The figure outlines some potential subunit combinations resulting in high affinity nicotinic receptors containing the beta2 subunit, to the right. In the dopaminergic system, the alpha6 subunit can potentially enter into the composition. The three genes expressed from the 15q cluster can assemble into low-affinity nicotinic receptors, expressed in the IPN, and also peripheral ganglia. There, a beta2 subunit can replace one beta4 subunit. Additional subunits can also enter into the composition of the receptor, like alpha2 (Grady *et al.* 2009; Balestra *et al.* 2000).

Figure 4. Model of an alpha5 containing nicotinic receptor pentamer

A. Model of the 3D structure of the (alpha3)2 (beta4)2 alpha5 nAChR, from (Frahm *et al.* 2011). Transmembrane and intracellular domains of α3, α5, and β4 subunits are shown in orange, red, and green, respectively. EC, extracellular space; IC, intracellular space. The indicated S435 residue in β4 and the D397 residue in α5 are located at the tip of the intracellular vestibule.

B. Electrostatic potential surface representations showing frontal (upper panel) and back (lower panel) views of the intracellular vestibule formed by the (alpha3)2 (beta4)2 alpha5 nAChR. One α3 subunit and one β4 subunit are removed for visualisation of the cavity. Horizontal gray bars indicate the plasma membrane. The electrostatic surface was contoured between -15kT/e and +15kT/e; negative and positive charges are marked in red and blue, respectively. Residues corresponding to the β4-potentiating residue S435 and D397 in α5 are indicated. See (Frahm *et al.* 2011) for further details on the modeling strategy. Figure used with permission from Elsevier.

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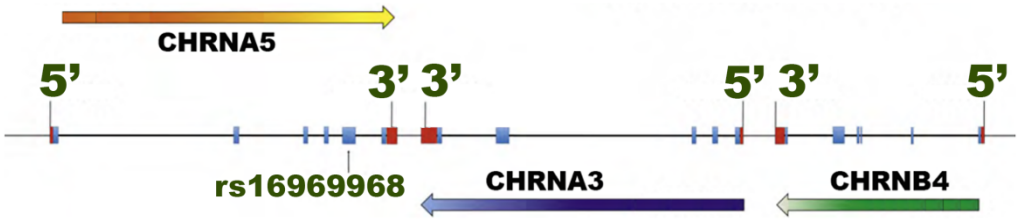

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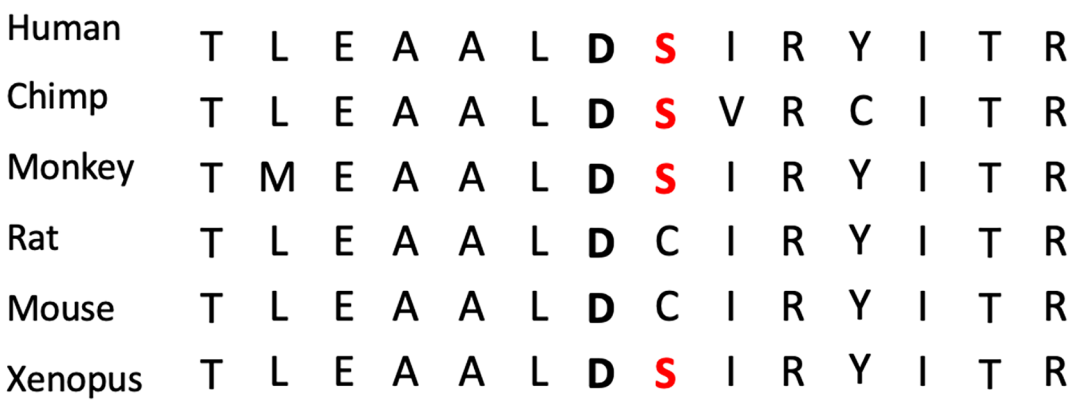

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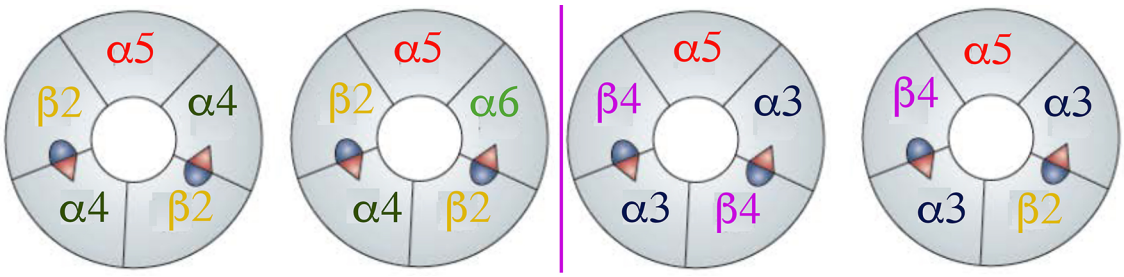

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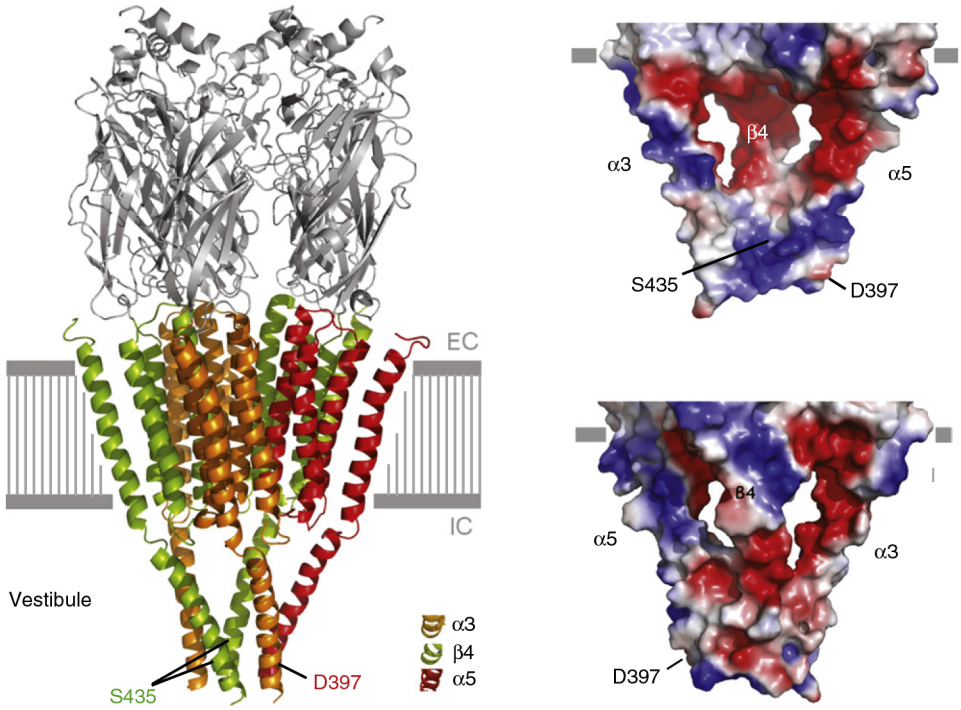

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