The nicotinic acetylcholine receptor alpha 4 subunit contains a functionally relevant SNP Haplotype

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Abstract

Background: Non-coding single nucleotide polymorphisms within the nicotinic acetylcholine receptor alpha 4 subunit gene (CHRNA4) are robustly associated with various neurological and behavioral phenotypes including schizophrenia, cognition and smoking. The most commonly associated polymorphisms are located in exon 5 and segregate as part of a haplotype. So far it is unknown if this haplotype is indeed functional, or if the observed associations are an indirect effect caused by linkage disequilibrium with not yet identified adjacent functional variants. We therefore analyzed the functional relevance of the exon 5 haplotype alleles.

Results: Using voltage clamp experiments we were able to show that the CHRNA4 haplotype alleles differ with respect to their functional effects on receptor sensitivity including reversal of receptor sensitivity between low and high acetylcholine concentrations. The results indicate that underlying mechanisms might include differences in codon usage bias and changes in mRNA stability.

Conclusions: Our data demonstrate that the complementary alleles of the CHRNA4 exon 5 haplotype are functionally relevant, and might therefore be causative for the above mentioned associations.

Keywords: CHRNA4, Acetylcholine receptor, ACh sensitivity, Haplotype, mRNA stability

Background

Cholinergic effects on cortical information processing and related cognitive performance are partly mediated through stimulation of high-affinity heteromeric α4β2 nicotinic acetylcholine receptors (nAChRs) [1-4]. α4β2 receptors are abundantly expressed in human cortex and hippocampus and possess high affinity to (partial) agonists including nicotine and varenicline [5-7]. Receptor upregulation occurs with chronic exposure to agonists and is thought to be regulated on the translational/post-translational rather than transcriptional level [8-10].

In earlier work, we reported a causative relationship between mutations in exon 5 of CHRNA4 (the nAChR α4-subunit coding gene) and the autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) - a rare seizure disorder that is frequently associated with neurocognitive deficits or psychiatric affections [11-14]. We then explored in a more recent study, whether association also might exist between human information processing and common CHRNA4 exon 5 single nucleotide polymorphisms (SNPs). Using functional magnetic resonance imaging (fMRI), association, especially for SNP rs1044396, was observed with prefrontal/parietal information processing during a selective attention-requiring task [15]. Complementary behavioral, electrophysiological and neuroimaging studies from other groups have later provided converging evidence supporting the validity of this association. [16-25] Furthermore, we and others repeatedly observed that the common exon 5 SNPs are also associated with endophenotypes of nicotine dependence [26-29].

The CHRNA4 SNPs that repeatedly showed association with neurological and behavioral traits all have in common that they are silent mutations, i.e. are not changing codons and therefore have no apparent effect on the protein sequence. However, during the last decade it has become obvious that not all silent SNPs are functionally neutral. In the present study, we therefore addressed the question if silent CHRNA4 exon 5 SNPs are able to...
modulate mRNA or receptor properties. For this purpose we conducted experiments on receptor sensitivity and mRNA stability, and performed in silico analysis regarding possible codon usage differences introduced by the haplotype alleles.

Results

Receptor sensitivity analysis

Exon 5 of the CHRNA4 gene contains a linkage group (haplotype) of synonymous variants (haplotype 5'-rs1044393, rs1044394, rs2229959, rs2229960, rs1044396, rs1044397-3') of hitherto unknown functional relevance. This haplotype rather than single SNPs was chosen for the here reported functional studies because exon 5 SNPs are in linkage disequilibrium with each other. It is therefore not possible to decide if, for example, associations found for a specific SNP are indeed caused by this SNP or by another one located on the same haplotype. Analysis of the haplotype therefore allowed us to simultaneously include all major exon 5 SNPs into our search for functional effects.

Haplotype allele hap1 (T-T-G-T-C-G) corresponds to the NCBI reference sequence (NM000744.5) and has a frequency of 9% in the general population, while the complementary hap2 allele (C-C-T-C-T-A) accounts for 52% of all alleles (according to our reference student population). Hap 1 and Hap 2 differ in each single SNP position. Besides hap1 and hap2 at least six other alleles of this haplotype are present in the normal population. These haplotypes share one or more SNP allele with the major haplotypes Hap1 and Hap2 and can therefore be expected to produce intermediate results in functional analysis. They were therefore not included in the present study.

Heterologous expression experiments showed that α4β2-receptors with both haplotype yield functional receptors with current amplitudes that increased in a dose-dependent manner with the ACh concentrations (Figure 1). For low ACh doses the currents from hap2 receptors (incl. rs1044396 T-allele) were up to 130% larger than those from hap1 receptors (incl. rs1044396 C-allele), resulting in a shift of the hα4(hap2)β2 curve towards lower concentrations with respect to the curve for hα4(hap1)β2. For higher doses...
of ACh the opposite effect was found with currents for 
ha4(hap2)β2 that were about 13% lower than those ob-
tained for ha4(hap1)β2. The EC50L for ha4(hap2)β2
(0.33 μM ± 0.017) differed significantly (P ≤ 0.001, n = 65)
from ha4(hap1)β2 (0.72 ± 0.04), indicating a higher sensi-
tivity to ACh for ha4(hap2)β2 at low ACh concentrations.
Interestingly, the EC50H data demonstrate a switch in
this behavior, pointing to a lower sensitivity to ACh for
ha4(hap2)β2 (42.5 μM ± 3) compared to ha4(hap1)β2
(33.6 μM ± 2.3, P ≤ 0.05, n = 65) in case of high ACh
concentrations (Figure 1).

mRNA stability analysis
When comparing the mRNA decay time difference of
hap 1 and 2, none of the time differences for the four up-
stream fragments were significant. However, regarding the
time difference 0 to 24 hrs for the most downstream frag-
ment, our results revealed that the 3’ end of hap 1 mRNA
was significantly more slowly degraded than that of hap 2
(P = 0.03) (Table 1).

mRNA secondary structure prediction and codon usage
analysis
Analysis of the predicted mRNA secondary structure
showed marked differences between the two haplotype
alleles (see Additional file 1: Figure S1). Codon usage ana-
lysis showed that most SNPs introduced changes from fre-
cently to more rarely used codons or vice versa. These
effects were most pronounced for rs2229959, rs1044396
and rs1044397 (see Table 2).

Discussion
Taken together, our experimental data show that the
CHRNA4 haplotype alleles exert different functional
effects on mRNA stability as well as on receptor sensi-
tivity including reversal of receptor sensitivity between
low and high ACh concentrations. Furthermore, in silico
analysis predict that the haplotype alleles also differ with respect to codon usage and mRNA secondary
structure. The experiments were conducted using clones that contained identical fragments from the
CHRNA4 coding region, the only differences between
the clones being the respective alleles of the five SNPs
composing the haplotype. Thus the variation observed
in both ACh sensitivity and mRNA stability should be
attributable to the SNPs within the haplotype. Our re-
sults therefore strongly suggest that one or more of
the synonymous SNPs that constitute the haplotype
are functionally relevant. Such a conclusion would not
be too surprising, given that several examples exist in
which silent SNPs have been found to modulate gene
function, for example by altering mRNA stability, transla-
tion efficiency or protein conformation [30-32].

Various mechanisms could explain the distinctive dose-
response curves of the two haplotypes. It is a possibility
that the observed changes in mRNA stability are one
of the mechanisms that contribute to the haplotype-
dependent differences in ACh sensitivity. One explan-
ation could be that the altered mRNA stability may
lead to an increased translation rate of CHRNA4 mRNA
carrying the more stable haplotype 1. Such an increased
translation rate would expand the amount of α4 subunit
protein in hap 1 carriers while the amount of β2 subunit
protein would remain constant. This in turn could alter
the nAChRs stoichiometry so that more (α4)3(β2)3 than
(α4)2(β2)3 receptor subtypes are assembled. Such changes
in stoichiometry are a factor known to influence several
functional receptor characteristics and to increase recep-
tor affinity [33,34]. It is also possible that, apart from
mRNA stability, additional mechanisms are responsible
for the observed differences in agonist sensitivity. For ex-
ample, changes in the mRNA sequence are known to
affect its folding which in turn can influence the efficiency
and speed of protein synthesis [35]. This mechanism
would also be able to affect the ratio of α4 versus β2
subunits within the mature nAChR. Furthermore, codon
bias is discussed as mechanism for a gene expression
regulation because it has been observed that genes with
lower expression levels prefer codons which are recog-
nized by tRNAs with lower gene copy numbers [36].

Another factor that might play a role would be codon
bias at the ribosome. It is assumed that the speed at
which a given mRNA is decoded at the ribosomes largely
depends on the availability of individual tRNA molecules.
However, most amino acids can be encoded by more than
one base pair triplet, and there are significant differences
with respect to the frequency with which individual co-
dons occur in genes. In fact, synonymous codons are used
at nonrandom frequencies, a phenomenon termed codon
usage bias. Such differences in codon usage are not only
found between species, but in some examples also have
been described for different tissues from the same indi-
vidual [37]. Both codon usage and tRNA gene numbers
evolved together, and, consequently, tRNAs that recognize
frequently used codons are usually more abundant at the
ribosome and are therefore more readily available for
translation [37,38]. Thus a silent SNP changing a fre-
cently used codon into a rarer one can slow down the
speed of mRNA translation. In fact, synonymous codon
usage is recognized as the primary cause of non-uniform
translation rates, a mechanism known to cause for differ-
tential maturation and folding of nascent polypeptides
[39]. These differences in polypeptide processing are
possible because the time newly synthesized polypep-
tides spend at the ribosome is used to introduce various
modifications that are important for protein folding, sta-
bility, and interaction with binding partners [40]. The

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| mRNA decay time difference | qPCR fragment | 1 | 2 | 3 | 4 | 5 |
|----------------------------|---------------|---|---|---|---|---|
|                            | Δ Cq mean     | p-value | Δ Cq mean | p-value | Δ Cq mean | p-value | Δ Cq mean | p-value | Δ Cq mean | p-value |
|                            | hap 1 hap 2 hap1/2 hap 1 hap 2 hap1/2 hap 1 hap 2 hap1/2 hap 1 hap 2 hap1/2 hap 1 hap 2 hap1/2 |
| 0 to 3 hrs                 | 1.16          | 0.29 | 0.242 | 0.24    | 1.91 | 0.09 | 0.0291 | 1.61 | 0.26 | 0.066 | 1.07 | -0.07 | 0.08 | 1.17 | 0.37 | 0.436 |
|                            | (-0.72 - 3.04) | (-1.57 -2.15) | (-0.31 -4.12) | (-1.77 -1.94) | (-0.73 -3.95) | (-1.61 -2.13) | (-0.70 -2.83) | (-1.66 -1.51) | (-1.48 -3.81) | (-1.73 -2.47) |
| 0 to 6 hrs                 | 2.54          | 1.95 | 0.403 | 3.34 | 2.41 | 0.313 | 3.04 | 2.48 | 0.546 | 2.23 | 1.7  | 0.503 | 3.69 | 3.34 | 0.821 |
|                            | (0.49-4.58) | (0.63-3.27) | (0.79-5.89) | (0.77-4.04) | (0.43-5.65) | (0.75-4.21) | (0.43-5.65) | (0.75-4.21) | (0.26-4.19) | (0.29-3.11) | (0.72-6.66) | (0.71-5.97) |
| 0 to 24 hrs                | 5.74          | 5.64 | 0.918 | 6.18 | 6.24 | 0.952 | 5.06 | 4.77 | 0.778 | 5.21 | 4.77 | 0.679 | 8.16 | 10.73 | 0.0362 |
|                            | (3.86-7.62) | (3.22-8.06) | (4.02-8.33) | (3.84-8.63) | (2.79-7.34) | (2.55-7.00) | (3.55-6.87) | (2.23-7.32) | (5.42-10.90) | (6.03-13.44) |

Δ Cq mean, delta Cq mean: difference of the mean quantification cycle; confidence intervals are given in brackets.

1 confidence interval for delta Cq-mean values of hap1 and hap2 are not significant.

2 confidence interval for delta Cq-mean values of hap1 and hap2 are significant.
silent SNPs that constitute the CHRNA4 haplotype introduce several changes from frequent to rarer used codons (or vice versa, see Table 2) and could therefore alter, by the above discussed mechanisms, functional characteristics of the nAChR such as stoichiometry, surface expression or function. Pathomechanisms like this have already been reported for other silent SNPs associated with human disorders [31,35,41].

It appears remarkable that, within our experimental setting, mRNA stability was only altered in the downstream (3’) region of the cloned CHRNA4 coding region fragment. These observations suggest that it is the SNPs in the 3’ part of the haplotype that are able to alter mRNA stability. Interestingly, the 3’ end of the CHRNA4 haplotype harbors the two silent SNPs that have most consistently shown association with clinical phenotypes. For example, recent work from several groups including our own reported association between rs1044396/rs1044397 and endophenotypes of schizophrenia as well as nicotine addiction. Both SNPs are significantly associated with cognitive endophenotypes such as brain activation (N100-amplitude – in particular in prefrontal cortex) during selective-attention-requiring tasks [15-19,21,23-26]. With a minor allele frequency above 0.45 both SNPs would be common enough to contribute considerably to the inter-individual variability in the processing of cognitive tasks, addictive behavior and psychiatric disorders within the general population. Additional studies are needed to shed light on the complex interactions between these silent nAChR variants, differences in nAChR function, and the inter-individual variability of neurological and behavioral traits in humans.

Conclusions
Our experimental and in silico data demonstrate that the complementary alleles of the CHRNA4 exon 5 haplotype differ with respect to mRNA stability, codon usage, and agonist sensitivity. These results render it possible that one or more of the haplotype-constituting SNPs are causative for the previously reported associations with neurological and behavioral phenotypes.

Materials and Methods
Receptor sensitivity analysis
The cDNAs with either one of the two complementary CHRNA4 haplotypes and with the CHRN82 wild type sequence were injected in Xenopus oocytes in equal amounts and the electrophysiological properties of the α4β2 nAChR channel were determined using a two-electrode voltage clamp technique (HiClamp, Multichannel System®, Reutlingen Germany) and applying different concentrations of acetylcholine (ACh). Concentration-activation curves were fitted using a Hill equation in the form $Y = \frac{1}{1 + \left( \frac{EC50}{x} \right)^{nH}}$ where: $y =$ the fraction of evoked current, $EC50 =$ concentration for 50% activation of the high affinity, $nH =$ the apparent cooperativity for the high affinity, $x =$ agonist concentration. Concentration-inhibition curves are fit with a comparable equation $Y = \frac{1}{1 + \left( \frac{x}{IC50} \right)^{nH}}$ where: $y =$ the fraction of remaining current, $IC50 =$ concentration for 50% inhibition, $nH =$ the apparent cooperativity, $x =$ antagonist concentration.

mRNA stability testing
The Tet-Off® advanced inducible gene expression system was purchased from Clontech (Saint-Germain-en-Laye, France). The coding sequence of CHRNA4 hap 1, respectively hap 2 was obtained by PCR amplification of human

| dbSNP ID | amino acid positions | Alleles (hap 1/hap 2) | Codon usage frequencies (hap 1/hap 2) |
|----------|----------------------|-----------------------|--------------------------------------|
| rs1044393 | D213 | GAT/GAC | 21.8/25.1 |
| rs1044394 | C226 | TGT/TGC | 10.6/12.6 |
| rs2229959 | P403 | CGG/CCT | 6.9/17.5 |
| rs2229960 | C409 | TGT/TGC | 10.6/12.6 |
| rs1044396 | S543 | AGC/AGT | 19.5/12.1 |
| rs1044397 | A553 | G/A | 74/158 |

Data source for codon usage: http://www.kazusa.or.jp/codon/.

Figure 2 Schematic representation of the CHRNA4 gene. The positions of SNPs that constitute the CHRNA4 haplotype (see main text) are indicated above, the fragments used for mRNA stability testing below the transcript.
DNA. After KpnI and EcorV digestion of the pTRE-Tight-BI-AcGFP1 vector, the coding sequence of CHRNA4 hap 1, respectively hap 2 (1884 nt) was ligated into the multiple cloning site of pTRE-Tight-BI-AcGFP1 downstream of the doxycycline-dependent promoter. The resulting construct had the following structure (origin of fragments given in brackets): (pTRE-Tight) ...GCTGGCTTCTATCG (pTRACER) CGAGCTCGGATCCA (CHRNA4 uncoding) CTAGTAGTGGCC (CHRNA4 coding) ATG...TAG (CHRNA4 uncoding) GAATA (pTRACER) GAATTC TGCAGAT (pTRE-Tight) ATCTC... After cloning the inserts were confirmed by sequencing. Culturing of Tet-Off human embryonic kidney cells (HEK) 293 (Clontech, Saint-Germain-en-Laye, France) was performed using standard protocols. HEK 293 cells were transfected with 10 ng plasmid pTRE-Tight-BI-AcGFP1 containing the coding sequence of CHRNA4 haplotype 1 and 2, respectively, using 3 μl of TransIT®-LT1 Transfection Reagent (MoBiTec GmbH, Göttingen, Germany) with 24 h transfection prior to medium change and addition of 1 μg doxycycline. RNA was extracted after 0; 3; 6 and 24 hours of doxycycline incubation by using QiAshredder and RNaseasy kit, including DNase treatment of 10 min in solution, according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Real-time PCR was performed targeting five fragments of the CHRNA4 coding sequence (primer sequences: 1 F GCTCATATTGAGCTGGATGAGA, 1R CCGTTCAGCAATTGTTGAGA, 2 F GCTGGAATCTGGGAGAGTGAGT2R AGGGGAAGTAGGAGTTGATG, 3 F TGCTCATACGAGGGAATCATC, 3R ATGACGATGACGAGGGGAGTTT, 5R GCTGGACTTGCAAGGATGTTT, 6R CATGATGTATCGCCCTCGAAC, AcGFP_F ATGATGTATCGCCCTCGAAC, AcGFP_R CA CATGATGTATCGCCCTCGAAC, AcGFP_R CA CATGATGTATCGCCCTCGAAC) (Figure 2). Amplification efficiency and test linearity (correlation coefficient R2) were assessed for each primer pair. The reactions were carried out in the Mini Opticon CFD-3120 cycler (Bio-Rad, Munich, Germany). All experiments were repeated independently three times with triplicate biological and triplicate technical samples (nine experiments each in total). Statistical analysis was performed with program R to compare the haplotype 1 and 2 RNA degradation rate for each target fragment. A p value of p < 0.05 was considered statistically significant.

### Prediction of mRNA secondary structure and codon usage

Changes in the minimum free energy (MFE) secondary structure caused by the haplotype alleles were predicted by the use of the RNA fold web server, Vienna RNA package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). A prediction software was employed for the codon usage analysis (http://www.kazusa.or.jp/codon).
9. Wever A, Burghaus L, Moser N, Witter B, Steinlein OK, Schulte U, et al. Expression of nicotinic acetylcholine receptors in Alzheimer’s disease: postmortem investigations and experimental approaches. Behav Brain Res. 2000;113(1):207–15.

10. Moberger A, Winterer G. The molecular and cellular neurobiology of nicotine abuse in schizophrenia. Pharmacopsychiatry. 2008:41:551–9.

11. Steinlein OK, Mulley JC, Propping P, Wallace RH, Phillips HA, Sutherland GR, et al. A Misseense mutation in the neuronal nicotinic Acetylcholine-receptor alpha-4 subunit is associated with Autosomal-dominant nocturnal frontal lobe Epilepsy. Nat Genet. 1995;11:201–3.

12. Magnusson A, Stordal E, Brodtkorb E, Steinlein OK. Schizophrenia, psychotic illness and other psychiatric symptoms in families with autosomal dominant nocturnal frontal lobe epilepsy caused by different mutations. Psychiat Research. 2003;132(2):91–5.

13. Bertrand D, Emslie F, Hughes E, Tronche J, Sander T, Bertrand S, et al. The CHRNA2 mutation (I312M) is associated with epilepsy and distinct memory deficits. Neurobiol Dis. 2005;20(3):799–804.

14. Bertrand S, Weiland S, Berkovic SF, Steinlein OK, Bertrand D. Properties of neuronal nicotinic acetylcholine receptor mutants from humans suffering from autosomal dominant nocturnal frontal lobe epilepsy. Brit J Pharmacol. 1998;125(4):751–60.

15. Winterer G, Musso F, Konrad A, Vucurevic G, Stoetter P, Sander T, et al. Association of attentional network function with exons 5 variations of the CHRNA4 gene. Hum Mol Genet. 2007;16(18):2165–74.

16. Parasaruram R, Greenwood PM, Kumar R, Fossella J. Beyond heritability - Neurotransmitter genes differentially modulate visuospatial attention and working memory. Psychol Med. 2015;45:291–300.

17. Epseseth T, Endestad T, Rootwelt H, Reinvang I. Nicotine receptor gene CHRNA4 modulates early event-related potentials in auditory and visual oddball target detection tasks. Neuroscience. 2007;147(4):974–85.

18. Greenwood PM, Fossella JA, Parasaruram R. Specificity of the effect of a nicotinic receptor polymorphism on individual differences in visuospatial attention. J Cognitive Neurosci. 2002;14(10):1611–20.

19. Greenwood PM, Lin MK, Sundaranjan R, Finkxh KJ, Parasaruram R. Synergistic effects of genetic variation in nicotinic and muscarinic receptors on visual attention but not working memory. Proc Natl Acad Sci U S A. 2009;106(9):3633–8.

20. Greenwood PM, Sundaranjan R, Lin MK, Kumar R, Finkxh KJ, Parasaruram R. Both a Nicotinic single Nucleotide Polymorphism (SNP) and a noradrenergic SNP modulate working memory performance when attention is manipulated. J Cognitive Neurosci. 2009;21(11):1219–33.

21. Reinvang I, Lundervold AJ, Rootwelt H, Wehling E, Epseseth T. Individual variation in a cholinergic receptor gene modulates attention. Neurosci Lett. 2009;453(3):130–4.

22. Markett S, Montag C, Reuter M. The association between dopamine DRD2 polymorphisms and working memory capacity is modulated by a functional Polymorphism on the nicotinic receptor gene CHRNA4. J Cognitive Neurosci. 2012;22(9):1944–54.

23. Markett S, Montag C, Walter NT, Reuter M. Evidence for the modality independence of the genetic epistasis between the dopaminergic and cholinergic system on working memory capacity. Eur Neuropsychopharm. 2011;21(2):161–20.

24. Markett S, Reuter M, Montag C, Weber B. The dopamine D2 receptor gene DRD2 and the nicotinic acetylcholine receptor gene CHRNA4 interact on striatal gray matter volume evidence from a genomic imaging study. Neuroimage. 2013;64:167–72.

25. Gessing C, Neber T, Thiel CM. Genetic variation in nicotinic receptors affects brain networks involved in reorienting attention. Neuroimage. 2012;59(1):831–9.

26. Feng Y, Niu TH, Xing HX, Xu X, Chen CZ, Peng SJ, et al. A common haplotype of the nicotine acetylcholine receptor alpha 4 subtype gene is associated with vulnerability to nicotine addiction in men. Am J Hum Genet. 2004;75(1):1112–21.

27. Li MD, Beuten J, Ma JZ, Payne TJ, Lou XY, Garcia V, et al. Ethnic- and gender-specific association of the nicotinic acetylcholine receptor alpha 4 subtype gene (CHRNA4) with nicotine dependence. Hum Mol Genet. 2005;14(9):1211–9.

28. Streltitz LP, Dahmen N, Mittelstrasz K, Rujescu D, Gallinat J, Fehr C, et al. Association of nicotinic acetylcholine receptor subtype alpha 4 polymorphisms with nicotine dependence in 5500 Germans. Pharmacogenomics. 2009;9(4):219–24.

29. Kamens HM, Corley RP, McQueen MB, Stallings MC, Hopper CJ, Crowley TJ, et al. Nominal association with CHRNA4 variants and nicotine dependence. Genes Brain Behav. 2013;12(3):297–304.

30. Nackley AG, Shabalina SA, Tchivileva IE, Satterfield K, Korchnyskyi O, Makarov SS, et al. Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science. 2006;314(5807):1930–3.

31. Kimchi-Sarfaty C. A ‘silent’ polymorphism in the MDRI gene changes substrate specificity (vol 315, pg 525, 2007). Science. 2007;318(5855):1382–3.

32. Tsai CJ, Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, Gottesman MM, Nussinov R. Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct Minima. J Mol Biol. 2008;383(2):281–91.

33. Krashia P, Moroni M, Broadbent S, Hofmann G, Krakun S, Beato M, et al. Human alpha 3 beta 4 neuronal nicotinic receptors show different stoichiometry if they are expressed in xenopus oocytes or Mammalian HEK293 cells. Plos One. 2010;5(10):e13611.

34. d’Incamps BL, Ascher P. High affinity and low affinity heteromeric nicotinic acetylcholine receptors at central synapses. J Physiol Lond. 2014;592(19):4131–6.

35. Bartoszewski RA, Jablonsky M, Bartoszewska S, Stevenson L, Dai Q, Kappes J, et al. a synonymous single nucleotide polymorphism in delta F508 cfr alters the secondary structure of the mRNA and the expression of the mutant protein. J Biol Chem. 2012;287(37):30741–8.

36. Lavner Y, Kotlar D. Codon bias as a factor in regulating expression via translation rate in the human genome. Gene. 2005;345(1):127–38.

37. Dittmar KA, Goedenbour JM, Pan T. Tissue-specific differences in human transfer RNA expression. Plos Genet. 2006(2):e12107–15.

38. Novoa EM, de Pouplana LR. Speeding with control: codon usage, tRNAs, and ribosomes. Trends Genet. 2012;28(11):574–81.

39. Lavner Y, Kotlar D. Codon bias as a factor in regulating expression via translation rate in the human genome. Gene. 2005;345(1):127–38.

40. Giglione C, Fieulaine S, Meinnel T. Crotanslational processing mechanisms: towards a dynamic 3D model. Trends Biochem Sci. 2005;30(8):417–26.

41. Jacobo SMP, DeAngelis MM, Kim K, Kazlauskaus A. Age-related macular degeneration-associated silent polymorphisms in IntrA1 impair its ability to antagonize insulin-like growth factor 1. Mol Cell Biol. 2013;33(10):1976–90.

42. Gruber AR, Lorenz B, Bennhart SH, Neubock R, Hofacker II. The Venna RNA website. Nucleic Acids Res. 2008;36:W70–4.