Association of Impulsivity and Polymorphic MicroRNA-641 Target Sites in the SNAP-25 Gene

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Abstract

Impulsivity is a personality trait of high impact and is connected with several types of maladaptive behavior and psychiatric diseases, such as attention deficit hyperactivity disorder, alcohol and drug abuse, as well as pathological gambling and mood disorders. Polymorphic variants of the SNAP-25 gene emerged as putative genetic components of impulsivity, as SNAP-25 protein plays an important role in the central nervous system, and its SNPs are associated with several psychiatric disorders. In this study we aimed to investigate if polymorphisms in the regulatory regions of the SNAP-25 gene are in association with normal variability of impulsivity. Genotypes and haplotypes of two polymorphisms in the promoter (rs6077690 and rs6039769) and two SNPs in the 3’ UTR (rs3746544 and rs1051312) of the SNAP-25 gene were determined in a healthy Hungarian population (N = 901) using PCR–RFLP or real-time PCR in combination with sequence specific probes. Significant association was found between the T–T 3’ UTR haplotype and impulsivity, whereas no association could be detected with genotypes or haplotypes of the promoter loci. According to sequence alignment, the polymorphisms in the 3’ UTR of the gene alter the binding site of microRNA-641, which was analyzed by luciferase reporter system. It was observed that haplotypes altering one or two nucleotides in the binding site of the seed region of microRNA-641 significantly increased the amount of generated protein in vitro. These findings support the role of polymorphic SNAP-25 variants both at psychogenetic and molecular biological levels.

Introduction

Impulsivity is a multidimensional personality trait characterized by action without planning and lack of consciousness to guide acts and behavior [1]. The phenotype is based on a complex neurochemical background, and genetic factors play a crucial role in its development. Twin studies suggested that inheritance plays approximately 45% role in its determination [2]. There is a relatively broad spectrum of individual differences in healthy subjects regarding their impulsivity, therefore to summarize this trait in one specific definition has been a challenge for many decades. There is an agreement however that major deflection of impulsivity, thus, candidate genes shown to associate with ADHD might emerge as putative genetic components of impulsivity. A number of association studies as well as meta-analyses suggested the role of

DrD4, DrD5, HTR1B and SLC6A3 genes, reviewed by Faraone [10]. Moreover, several studies pointed out an association between two SNPs (rs3746544 and rs1051312) in the 3’ UTR of the SNAP-25 gene and ADHD [11,12,13], and the role of the former polymorphism was also confirmed in a comprehensive meta-analysis [14]. Genetic variants of SNAP-25 including promoter polymorphisms [10,12,15], have already been investigated as putative risk factors of other psychiatric disorders as well [16,17], but it has not yet been studied in the background of impulsivity in a non-clinical sample.

SNAP-25 (synaptosomal-associated protein, 25 kDa) plays a crucial role in the central nervous system, being one essential component of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex and contributes to exocytosis by targeting and fusion of vesicles to the cell membrane [18]. There are two SNPs (rs3746544 and rs1051312) in the 3’ UTR of the SNAP-25 gene with special interest, we previously demonstrated by in silico sequence analysis that they are localized in the putative target site of miR-641 [19]. As microRNAs are known to play a role as translational regulators of protein synthesis, SNPs located either in the coding
sequences of microRNAs or in their binding sites might cause an imbalance of this regulation. SNPs have been described in genomic regions encoding miRNAs [20], the rs1625579 polymorphism is located in the coding region of miR-137, which has been shown to target the SNPs in the 3' UTR of target genes altering the binding region of a miRNA to its specific target mRNA [20]. Polymorphisms in the microRNA-binding sites have been suggested to contribute to the genetic background of various diseases, such as coronary heart disease [22], different types of cancer [23, 24] or ADHD and other co-morbid psychiatric illnesses [12, 25]. A polymorphic variant (rs13212041) in the HTR1B gene 3' UTR was demonstrated to influence miR-96 binding and to be associated with conduct-disorder phenotype in a sample of 359 students [26].

Here we present data on the functional effect of rs3746544 and rs1051312 SNPs and their haplotypes on miR-641 regulated reporter gene expression in cell culture. We also aimed to carry out an association study to analyze whether either these loci or the polymorphic variants of the SNAP-25 promoter (rs6077690 AT and rs6039769 AC), studied so far in psychiatric disorders, contribute to the genetic background of impulsivity in a non-clinical sample.

Methods

Ethics Statement, Participants

901 healthy Hungarian young adults participated in the study. Selection criteria included no past or present psychiatric history (based on self-report). Before providing buccal samples for genetic analysis, participants signed written informed consent. The study protocol was approved by the Scientific and Research Ethics Committee of the Medical Research Council (ETT TÜKEB). The mean age of the investigated population was 21.3 (±3.3) years, 45.1% were males and 54.9% were females.

Phenotype analysis

Hungarian version of the 11th revised Barratt Impulsiveness Scale [27] was used to measure impulsivity. The questionnaire was originally published by Barratt. Translation into Hungarian was carried out by a “forward-backward” procedure [6], Cronbach alpha value for the total score of the scale was 0.808.

The Barratt Scale is a self-reporting measure widely used both by clinicians and in research settings. It consists of 30 items regarding acting and thinking in different situations. These statements are asked to be rated by the participant on a four-point-scale, reaching from “occasionally” to “always”. The highest theoretically possible total score is 120 and impulsivity is considered “normal” in the range from 52 to 71.

DNA isolation

DNA purification was initiated by incubating the buccal samples at 56°C overnight in 0.2 mg/ml Proteinase K cell lysis buffer. It was followed by protein denaturation using saturated NaCl solution. Finally, DNA was precipitated using isopropanol and ethanol by standard procedures and DNA pellet was resuspended in 100 μl 0.5× TE (1× TE: 10 mM Tris pH = 8, 1 mM EDTA) buffer. Concentration of each DNA-sample was measured by Varioscan Flash spectral scanning multimode reader.

Genotyping of SNAP-25 SNPs

The promoter SNP rs6039769 was genotyped by the C_29497340_10 (Life Technologies) pre-designed primer- and TaqMan probe-set. A 7300 Real-Time PCR System (Life Technologies) was employed to detect the FAM and VIC signals corresponding to the C and A alleles, respectively.

Genotypes of rs6077690 promoter SNP were determined by PCR-RFLP. Flanking region of the polymorphic site was amplified using the 5' ATG TCA GTG TGG GGC ATC 3' sense and 5' AGG CAT GTT GCT GAA ATT TGT T 3' antisense primers. The Qiagen HotStarTaq DNA-polymerase system was applied for PCR amplification, the reactions were carried out in a total volume of 10 μL containing 1 μM sense and antisense primers, 0.2 mM of each deoxyribonucleotide-triphosphate, 0.25 U Hot-StarTaq DNA-polymerase together with 1× buffer and 1× Q- solution and approximately 4 ng genomic DNA sample. Thermocycle was initiated by 15 min at 95°C initial denaturation and polymerase activation. It was followed by 40 cycles of 1 min denaturation at 94°C, 30 sec annealing at 63°C and 1 min extension at 72°C. The last step of the PCR was a final extension at 72°C for 10 minutes, amplicons were then kept at 8°C for downstream processing. In the next step PCR-products were digested by Tgl 509 restriction endonuclease. Two non-specific recognition sites were incorporated in the amplicons to verify optimal conditions of digestion. Reactions were carried out according to manufacturer’s instructions. A 301- and a 110-bp-long product (together with the two control fragments) were generated in the presence of the A allele, whereas the 411-bp long fragment could be seen in case of the T allele. Digestion pattern was analyzed by traditional submarine gel electrophoresis. Call rate of genotyping was 97%.

Direct haplotyping of SNAP-25 3' UTR SNPs

As the two SNPs (rs3746544 and rs1051312) in the 3' UTR are separated by only 3 basepairs from each other, it was possible to identify haplotypes directly in each individual sample using the published “double-tube” method [19]. In summary, the method is based on the application of haplotype-specific TaqMan probes in a real-time polymerase chain reaction. Two simultaneous analyses contained the four different probes corresponding to the four haplotypes labeled by FAM and VIC, respectively. Amplification and data-collection were done by a 7300 Real-Time PCR System (Life Technologies). Call rate was 98%. Individual genotypes of rs3746544 and rs1051312 were deduced from haplotype data.

Haplotype analysis of SNAP-25 promoter SNPs

SNPs in the promoter were about 1.5 kb apart from each other which provided rather limited possibilities for simultaneous analysis and thus direct haplotype determination. Therefore individual haplotypes for these SNPs were calculated from genotype data. Haplotype was ambiguous only in case of double heterozygote individuals (rs6077690 AT and rs6039769 AC; i.e. either A–A/T–C or A–C/T–A). Linkage disequilibrium analysis, however, revealed that the frequency of the T–A haplotype was as low as 0.6%. As a total of 103 double heterozygotes were identified in our sample, approximately 1 participant was mathematically expected to possess the rare T–A haplotype (i.e. A–C/T–A), which was neglected. Using this assumption, the haplotypes of double heterozygotes were also able to be identified from the genotypes, as these haplotypes were considered to be A–A/T–C. To confirm this approach, haplotypes were also reconstructed by Phase 2.1, which provided the same result for each sample [28].

Plasmid constructs

The entire 3' UTR region of the human SNAP-25 gene was cloned behind the firefly luciferase gene at the multicloning site of the pMIR-REPORT Luciferase miRNA Expression Reporter
Vector (Life Technologies), using the 5’ TGT AAT GAG CTC CTG GGA AGT GGT TAA GTG T 3’ sense and antisense 5’ CCC GAG AAG CTT AAA CTA GCT ACA AAA TGT CAA TCA 3’ primers. Bold italic letters show the recognition sites of Sac I and Hind III restriction endonucleases in the sense and antisense primers, respectively. Genomic DNA possessing a TT haplotype was applied to amplify the SNAP-25 3’ UTR, constructs with the GC, GT and TC haplotypes were subcloned by QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). All four constructs were verified by direct sequencing. Another pMIR construct was used as internal control, which contained an insert with same length but different sequence and most importantly missing the binding site of the analyzed miR-641.

Cell culture and transient transfections

Human embryonic kidney (HEK) 293 cells (purchased from Sigma-Aldrich Ltd. Budapest, Hungary) were cultured in 24-well tissue culture plates in DMEM supplemented with 10% fetal bovine serum and 1% penicillin solution at 37°C in a humidified atmosphere containing 5% CO2. After optimization experiments were in Hardy–Weinberg-equilibrium for each SNP, minor allele frequencies varied between 0.255 and 0.417 (Table 1). As the two SNPs of the 3’ UTR were in close proximity (see Table 1 for genomic localization), a direct haplotype determination was possible to perform for each individual DNA samples using haplotype-specific TaqMan probes, and genotypes were deduced from haplotype data. On the other hand, the two SNPs of the promoter region were genotyped individually as their greater distance did not allow a direct haplotype analysis, and haplotype frequencies were estimated by calculation. Analysis of the 3’ UTR haplotypes confirmed our previous result [19], that the G-C haplotype did not occur at all in the population. Interestingly a similar situation was observed regarding the promoter haplotypes. Although the T-A haplotype was not absent completely, its frequency (0.006) was much lower than expected (0.161). This resulted in a special type of linkage disequilibrium characterized by a high Lewontin’s $D'$ value, together with a relatively low $R^2$. On the other hand absolutely no linkage disequilibrium could be observed between the promoter and the 3’ UTR region (Figure 1).

Association analysis of SNAP-25 SNPs and impulsivity

As a first step, a single marker analysis was used by comparing the average impulsivity scores of participants in the different genotype categories (Table 2). No association could be detected with the promoter SNPs. On the other hand, one of the 3’ UTR (rs1051312) polymorphism showed a nominal association with Barratt-scores of impulsivity ($p = 0.042$) which disappeared after Bonferroni correction for multiple test ($4 \text{ SNPs: } p < 0.0125$).

As a second step, we applied a haplotype-based allele-wise analysis for the promoter and the 3’ UTR SNPs, separately. No association was found between the estimated promoter haplotypes and total impulsivity scores (data not shown). It is important to note that the 3’ UTR haplotypes were not estimated but determined in each subject individually by molecular methods (see the “Methods” section). It was observed, that individuals possessing the T-T haplotype of the rs3746544 and rs1051312 SNPs achieved a lower total Barratt-score (58.24) compared to participants without this haplotype (59.63), and this effect was significant either calculating for the three haplotype categories ($p = 0.009$), or opposing the T–T haplotype (associating with lower

![Figure 1. Linkage disequilibrium analysis of the two promoter (rs6077690 AT and rs6039769 AC) and two 3’ UTR (rs3746544 GT and rs1051312 CG) SNPs.](doi:10.1371/journal.pone.0084207.g001)
Impulsivity and miRNA Target SNPs in the SNAP-25 gene.

Table 1. Genotype and haplotype frequencies of the assessed SNPs in the SNAP-25 gene.

| SNP         | Position on chromosome 20 | Hardy–Weinberg p | Minor allele and its frequency (MAF) |
|-------------|---------------------------|------------------|-------------------------------------|
| rs6077690 AT| 10,197,461                | 0.8899           | A: 0.417                            |
| rs6039769 AC| 10,198,954                | 0.4417           | A: 0.276                            |
| rs3746544 GT| 10,287,084                | 0.7394           | G: 0.391                            |
| rs1051312 CG| 10,287,088                | 0.1909           | C: 0.255                            |
| rs6077690 AT – rs6039769 AC| rs3746544 GT – rs1051312 CG |                |                                      |

Haplotype  | Frequency | Haplotype  | Frequency |
-----------|-----------|-----------|-----------|
           |           | TC        | 0.579     |
           |           | AA        | 0.269     |
           |           | AC        | 0.146     |
           |           | TA        | 0.006     |

Table 2. Association analysis between the SNAP-25 SNPs and impulsivity.

| SNP         | Genotype | N  | Mean   | STD  | P        |
|-------------|----------|----|--------|------|----------|
| rs3746544 GT| 393      | 59.30| 10.0   | 0.335|          |
| rs1051312 CT| 326      | 59.02| 9.6    | 0.042|          |
| rs6039769 CA| 80       | 62.89| 10.0   | 0.934|          |
| rs6077690 AT| 95       | 62.26| 10.3   | 0.411|          |

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The rs3746544 and rs1051312 SNPs in the 3’ UTR region of the SNAP-25 gene had a more pronounced effect on the measured phenotype than the contributing SNPs, separately.

Functional analysis of SNAP-25 3’ UTR haplotypes

Based on the results of the association study, the 3’ UTR haplotypes showing an association with impulsivity were subjected to in vitro functional analysis. According to sequence alignment analysis both SNPs (rs3746544 and rs1051312) in the 3’ UTR are supposed to alter duplex formation of miR-641 and SNAP-25 mRNA, as the two polymorphisms are localized in the target site of miR-641, but the position of the SNP within the seed region does not play a crucial role in this system. The G–C haplotype resulted in two mismatches in the target region of miR-641 and caused a 4.6-fold higher luciferase activity compared to the T–T form (Figure 2B), demonstrating that two mismatches in the target sequence have a more pronounced effect on miRNA function than one mismatch. The control construct had the same length, but an independent sequence without any binding site of miR-641. As expected, the corresponding luciferase activity was significantly higher than that of any of the constructs containing the 3’ UTR of the SNAP-25 gene.

Discussion

Recent studies have demonstrated that regulation of protein synthesis is much more complex than was earlier predicted. One major component of this network is the system of micro-RNAs (miRNAs) demonstrated by the fact that as high as 5% of the genes are predicted to encode miRNAs. It is also noteworthy that at least 30% of protein coding genes are suggested to be regulated by miRNAs [29], while this number could reach even 60% according to other estimations [30]. Alterations in miRNA profiles have been shown in the background of several illnesses, such as cancers, autoimmune diseases or cardiovascular disorders [31]. MiRNA profiling can be a useful tool in tumor diagnostics [32], and several miRNA-based therapeutic protocols have been elaborated. For example a miR-122 inhibitor was shown to decrease the amount of hepatitis C virus RNA, whereas a modulator of miR-208 level seemed to be effective against cardiac hypertrophy [33].

As a single miRNA might regulate a set of target genes, or the 3’ UTR of a specific miRNA might bind a number of miRNAs, the translational regulation by microRNAs is a complex system. Interestingly, as few as 20 SNPs have been identified in genomic regions coding for miRNA seed sequences. On the other hand, more than a hundred thousand SNPs are predicted to change miRNA target sites, but less than 1% of these functional variants have been validated experimentally [30].

The rs3746544 and rs1051312 SNPs in the 3’ UTR region of the SNAP-25 gene have been thoroughly investigated as possible...
risk factors of ADHD [34,35,36] or bipolar disorder [12], however the molecular function of these variants has not yet been studied. Thus the question has been raised if these SNPs are genetic markers or causal polymorphisms [14], and according to our knowledge, our study is the first to analyze the effect of these SNPs on miRNA binding efficiency. Both studied SNPs are localized in the binding region of miR-641 in a close proximity of each other. Here we demonstrated that a single mismatch in the 3' UTR of the SNAP-25 gene caused by any of the two SNPs resulted in about 2-fold reporter activity, while the rs3746544 G–rs1051312 C haplotype possessing two mismatches in the miR-641 target site led to a more than 4-fold elevation in the luciferase level. These results confirm the molecular functional role of rs3746544 and rs1051312 haplotypes in the SNAP-25 gene and are in agreement with the results of previous studies demonstrating the importance of optimal SNAP-25 level. The coloboma mouse, which is the animal model of ADHD has SNAP-25 deficiency and changes in SNAP-25 expression were shown to play a role in altered neuronal function [37]. It was also demonstrated, that atomoxetine which is an orally administered medicine used for the treatment of ADHD resulted in the significant up-regulation of SNAP-25 both on mRNA and protein level [38].

On the other hand the T allele of both SNPs was also shown to be the risk factor of ADHD. It is also notable, however, that the association had a modest statistical significance [10] and odds ratio was 1.15 and 1.06 for the rs3746544 and rs1051312 SNPs, respectively [14]. This shows that SNAP-25 is only one of the several genetic factors of impulsivity and related psychiatric disorders. Interestingly the haplotype with double mismatch (G–C) in the miRNA target site of SNAP-25 gene was completely undetectable in our healthy volunteer population (Hungarian) according to our previous [19] and current studies. It is important to note that haplotypes were not estimated but individually measured by a direct molecular haplotype analysis method developed earlier [19,41]. A theoretically possible explanation of the absence of the G–C haplotype in our healthy volunteer population could be the more severe effect of this haplotype on SNAP-25 gene expression leading to pathophysiological consequences. Alternatively, the missing haplotype might be explained by the evolutionally young age of one of the SNPs, therefore recombinant haplotypes of these 3' UTR SNPs, could not spread out in the population yet.

Although the putative biological effect of SNPs in regulatory regions both in 5' or 3' region is the modulation of protein level by...
different mechanisms, interestingly, much less data are available about the role of 5’ polymorphisms of the SNAP-25 gene. The rs6039769 SNP was shown to be in association with early-onset bipolar disorder [15], however no association could be found between ADHD and this locus [13]. On the other hand, the other polymorphism located 2 kb 3’ from the transcriptional start site (rs6077690) was demonstrated to be in association with ADHD [13]. Analysis of the two promoter SNPs and their haplotypes did not reveal any association between the polymorphisms and impulsivity. In conclusion, our results confirmed the findings of previous studies investigating SNAP-25 and ADHD.

Our study focused on a healthy population, but even in this setting an association could be observed between the haplotypes of the 3’ UTR SNPs (rs3746544 and rs1051312) and normal individual variability of impulsivity. In vitro functional analyses suggested that these loci are miRNA-SNPs altering the binding efficiency of miR614 in a luciferase reporter system.

Author Contributions
Conceived and designed the experiments: MS-S. Performed the experiments: NN RK-N. Analyzed the data: AS. Wrote the paper: ZR.

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