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Association analysis of norepinephrine transporter polymorphisms and methylphenidate response in ADHD patients

Nora Angyal[a], Erzsebet Zsofia Horvath[a], Zsanett Tarnok[b], Mara J. Richman[c], Emese Bognar[b], Krisztina Lakatos[d], Maria Sasvari-Szekely[a], Zsofia Nemoda[a,*]

[a] Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary
[b] Vadaskert Child and Adolescent Psychiatric Hospital, Budapest, Hungary
[c] Department of Clinical Psychology and Addiction, Eötvös Loránd University, Budapest, Hungary
[d] Institute of Cognitive Neuroscience and Psychology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary

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ABSTRACT

Aims: Methylphenidate (MPH) is the most frequently prescribed drug in Attention Deficit Hyperactivity Disorder (ADHD). Hitherto mostly the dopamine transporter gene has been studied in MPH-response and only a few studies analyzed the norepinephrine transporter (*NET*, *SLC6A2*) gene, although MPH is a potent inhibitor of both dopamine and norepinephrine transporters. We aimed to analyze this monoamine transporter gene in relation to ADHD per se and MPH-response in particular to gain further knowledge in ADHD pharmacogenetics using a Caucasian sample.

Methods: Six single nucleotide polymorphisms (rs28386840, rs2242446, rs3785143, rs3785157, rs5569, rs7194256 SNP) were studied across the *NET* gene in 163 ADHD children (age: 9.3 ± 2.6; 86.5% male) using ADHD-RS hyperactivity-impulsivity and inattention scales. For case-control analysis 486 control subjects were also genotyped. At the MPH-response analysis responders had minimum 25% decrease of ADHD-RS total score after 2 months of treatment, and chi-square test compared 90 responders and 32 non-responders, whereas ANOVA was used to assess symptom improvement after the first month among the 122 ADHD patients.

Results: The classical case-control analysis did not yield any association with ADHD diagnosis, which was supported by meta-analysis conducted on the available genetic data (combining previously published and the present studies). On the other hand, the intronic rs3785143 showed nominal association with inattention symptoms ($p = 0.01$). The haplotype analysis supported this association, and indicated the importance of the first haploblock encompassing the intronic and 2 promoter SNPs. With MPH-response only the promoter rs28386840 showed nominal association: Those with at least one T-allele were overrepresented in the responder group (42% vs 19%, $p = 0.08$), and they had better improvement on the hyperactivity-impulsivity scale compared to the AA genotype ($p = 0.04$).

Conclusion: Although none of our single SNP findings remained significant after correcting for multiple testing, our results from the MPH-response analysis indicate the potential importance of promoter variants in the *NET* gene.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most prevalent childhood-onset psychiatric disorders, affecting 5% of school-age children worldwide (Polanczyk et al., 2007). The genetic component of ADHD has been demonstrated by family- and twin-studies, but genome-wide association studies have not yielded any main, ADHD-specific genetic locus, only candidate gene studies showed small but significant effect at a number of monoaminergic polymorphisms (Bonvicini et al., 2016; Gizer et al., 2009). Genetic factors determining drug responsiveness have been also intensively studied in the last decade, mostly in connection with methylphenidate (MPH) response in ADHD treatment (Froehlich et al., 2010), because only 65–70% of ADHD patients benefit from MPH treatment (Biederman and Spencer, 2008). The dopamine and norepinephrine transporter genes have been in the center of these studies, because MPH can compete with both catecholamines at their transporter binding, while it does not inhibit the serotonin transporter (Gatley et al., 1996; Han and Gu, 2006;

* Corresponding author at: Institute of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, POB 2, H-1428, Hungary.
E-mail address: nemoda.zsofia@med.semmelweis-univ.hu (Z. Nemoda).

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Markowitz et al., 2006). Furthermore, an in vivo study showed that the usual clinical dose of orally administered MPH produces 70–80% inhibition of norepinephrine transporter (NET, *SLC6A2*) in humans (Hannestad et al., 2010).

Genetic association studies using single nucleotide polymorphisms (SNPs) of the *NET* gene have been controversial both in terms of ADHD diagnosis and MPH-response in ADHD patients. Although association studies pointed repeatedly to intronic variants at the first half of the *NET* gene (rs3785143 from intron 1 and rs11568324 from intron 5) in ADHD (Brookes et al., 2006; Kim et al., 2008), not all subsequent studies could replicate these associations (Hawi et al., 2013; Tzang et al., 2014; Xu et al., 2008). Therefore, further association studies using independent patient populations are still important. Our aim was to carry out case-control and dimensional association analyses of *NET* gene variants (located both on the 5′ and 3′ end) in ADHD patients. Meta-analysis was planned to support our single SNP analysis results, and haplotype analysis was carried out to assess further details of *NET* SNPs in ADHD.

Among the *NET* polymorphisms, the rs11568324 minor allele has a potential protective effect. However, the frequency of this allele is below 2%, therefore, we did not include this SNP in our analysis, and chose instead rs3785157 (from intron 7), which was previously indicated in ADHD by different research groups (Bobb et al., 2005; Hawi et al., 2013; Hohmann et al., 2015; Xu et al., 2005). The other selected *NET* polymorphisms included widely investigated SNPs, namely the intronic rs3785143 and exon 9 rs5569 (1287 A/G SNP). To cover the 5′ and 3′ non-coding gene regions of the *NET* gene we selected possibly functional gene variants. From the promoter region the rs2242446 (− 182C/T SNP) and rs28386840 (− 3081 A/T SNP) were chosen, because of their potential or proven influence on gene transcriptional activity (Kim et al., 2006; Sigurdardottir et al., 2016; Zill et al., 2002). From the 3′ end we selected a SNP (rs7194256) which has been shown to affect gene expression in an in vitro luciferase reporter gene assay (Marques et al., 2017). We hypothesized that either the 5′ or 3′ non-coding region's functional genetic variant(s) would have bigger effect compared to the intronic or synonymous polymorphisms in the NET gene.

Beside the association analyses of the *NET* genetic variants with ADHD diagnosis and symptoms, we aimed to characterize MPH-response in our ADHD patient sample. Hitherto, the *NET* pharmacogenetic findings have been mixed. The first pharmacogenetic study of the *NET* gene indicated better improvement on hyperactivity-impulsivity score (but not on inattentive scores) in rs5569 G-allele carriers among Chinese Han youths (Yang et al., 2004). Among the subsequent studies conducted in Korean populations two supported the better response of the GG genotype (Park et al., 2012a; Park et al., 2012b; Song et al., 2011), but other two studies did not (Lee et al., 2011; Kim et al., 2010). In addition, an American (double-blind, placebo-controlled) study did not find association between the rs5569 and MPH-response (McGough et al., 2009). However, a detailed genetic analysis in an open-label study of a MPH transdermal system indicated two other *NET* SNPs (rs17841329 and rs192303) from intron 1 (Mick et al., 2008). Concerning the promoter variants, two Korean studies showed better MPH-response at subjects with at least one rs28386840 (− 3081) T-allele. One of the studies showed better improvement at the Clinical Global Impression-Improvement score (Kim et al., 2010), and the other showed greater decrease in the mean commission error scores in a continuous performance test (Park et al., 2012b). Our pharmacological study was similar to these Asian ones, it was a prospective study using symptom severity scores assessed each month after starting an MPH treatment.

2. Methods

2.1. Subjects and clinical assessments

The study was designed in compliance with the Helsinki Declaration and was approved by the Local Scientific and Research Ethics Committee of the Medical Research Council. Patients and their parents provided written informed consent for their participation. Children with IQ < 80 (estimated from the Raven progressive matrices test; Raven, 1965), as well as those who had severe medical or neurological conditions, or pervasive psychiatric disorder were excluded from the study. Among the selected 163 children (mean age: 9.3 ± 2.6; 86.5% male) diagnosed with ADHD according to DSM-IV criteria (American Psychiatric Association, 1994) by two independent child psychiatrists, 122 children (mean age: 9.6 ± 2.6; 88.5% male) participated in a prospective MPH-response analysis. Comorbid conditions were assessed with the Hungarian child version of the Mini-International Neuropsychiatric Interview (MINI-Kid; Balazs et al., 2004), for detailed demographic and clinical characteristics see Supplementary Table 1.

The MPH-response criteria has been published previously (Kereszturi et al., 2008), in short, creating the category of non-responders was based on < 10% decrease in the total score of the ADHD Rating Scale (ADHD-RS, DuPaul, 1998) after 2 months of treatment (all of the non-responders discontinued the treatment after 3 months). The responders had at least 25% decrease in the ADHD-RS total score after 2 months of treatment; also their symptoms reduced to minimal after 5 months of treatment as measured by the Severity of Illness subscale of the Clinical Global Impression scale (CGI-S; Guy, 1976, point 1–2 corresponding to no or minimal symptoms) at the follow-up visits after 5 and 6 months of treatment. According to these criteria, 90 children were categorized as responder, whereas 32 children as non-responder. Patients participating in the drug response study were given 10–30 mg methylphenidate (Ritalin 10 mg, immediate-release) according to their body weight, in two doses (morning and noon). The daily dose thereby ranged from 0.22 to 0.95 mg/kg/day, in average 0.55 ± 0.15 mg/kg/day. Patients did not receive any other psychoactive medication.

For case-control analyses two control samples were used: 400 sex-matched healthy young adults were selected from the general population (mostly university students, see Varga et al., 2012) who did not report psychiatric disorder diagnosis in their lifetime on a self-report questionnaire. Additionally, a smaller group of children was tested (n = 86, 58.1% male, aged 6–7 years), which was screened for psychiatric diagnoses and also specifically for ADHD symptoms with the Hungarian version of the Child Behavior Checklist (Achenbach, 1991; Gadoros, 1996) rated by the mother as part of a longitudinal study (Birkas et al., 2006). Both the clinical and control samples were ethnically homogenous and of Caucasian origin (based on both biological parents' Hungarian origin), and consisted of unrelated individuals.

2.2. Isolation of DNA and genotyping

Genomic DNA was isolated from buccal cells by the DNA purification kit obtained from Gentra (Minneapolis, USA). The SNPs were genotyped with pre-designed TaqMan probes (rs28386840: C_60398891_10, rs2242446: C_26354911_10, rs3785143: C_27481932_10, rs3785157: C_27481947_10, rs5569: C__3020068_10, rs7194256: C_29079520_10) on 7300 Real-Time PCR System (Applied BioSystem, Foster City, USA). No significant deviations from Hardy–Weinberg equilibrium (p > 0.05) were detected for any of the polymorphisms either in the case or in the control groups.

2.3. Association analyses

In the categorical analyses chi-square tests were carried out, and p-value threshold for multiple comparisons was calculated by the False Discovery Rate adjustment (Benjamini et al., 2001), setting the significance threshold to $p < 0.008$. In the quantitative analyses MPH-effect was assessed with the ADHD-RS Inattention and Hyperactivity/Impulsivity score differences after the first month by analysis of variance. Haplotype frequencies were checked with the Haploview program (Barrett et al., 2005) and quantitative analyses of estimated

haplotypes were performed with the Thesias program (Tregouet and Garelle, 2007).

2.4. Meta-analyses

Published articles were identified through a computerized literature search using PubMed, MEDLINE (Ovid) and PsycINFO (Ovid) electronic databases. Search terms included "ADHD/inattention/hyperactivity/hyperkinetic" combined with "norepinephrine transporter/noradrenaline transporter/SLC6A2/NET/NAT" and "gene/polymorphism". The search was limited to articles that were published in English until April 2017. Additionally, a manual review was performed utilizing cross-references from the selected articles and review papers. We searched for original research articles reporting genetic associations or pharmacogenetics in patients diagnosed with ADHD as compared to healthy controls or family based studies. The search yielded 206 individual publications on PubMed, 38 on PsycINFO (Ovid) and 46 Medline (Ovid) that met the requirements. Inclusion criteria were: (1) original research article (2) published in English, (3) conducted on human samples with ADHD patients (4) genotyping *NET* polymorphisms. When detailed genotype data were not available, authors were contacted. After further review, 20 published research articles and the present study were included in the analyses. Exclusion criteria were: other (less frequently studied) *NET* polymorphism (n = 4), not case-control design (n = 11), overlapping data (n = 10), or not detailed genotype data (in 4 cases there was no response to request), see PRISMA flow diagram presented on Fig. 1.

The Comprehensive Meta-Analysis Version 3.0 software (Borenstein et al., 2005) was used for the meta-analysis. Separate analyses were conducted for the family-based allele transmission (TDT) studies, and for the allele- or genotype-wise case-control studies (papers reporting only one type of data were included only in one meta-analysis). Scores were standardized by calculating the odd's ratio of the studies by comparing the total events presented in the cases versus that in the controls. Odds ratio, confidence intervals (CI) and Z-values of the effect sizes were used to assess statistical significance. The Cochran Q-statistic was used to assess homogeneity of the effect sizes across studies between clinical diagnoses of ADHD versus case controls.

3. Results

3.1. Association analyses with ADHD: case-control design

First, case-control analyses were carried out using the child psychiatry patient group (n = 163) diagnosed with ADHD (according to DSM-IV. criteria) and either a child control group (n = 86) or an adult sex-matched control group (n = 400). There were no significant differences ($p > 0.1$) in the allele or genotype frequencies of the investigated *NET* SNPs between the ADHD and control groups (Table 1). Neither the two types of control groups differed significantly ($p > 0.1$) in their genotype frequencies.

Neither haplotype frequencies were significantly different between the patient and control groups. Based on the genetic data of 486 control subjects and 163 ADHD patients, the linkage disequilibrium (LD) between the investigated SNPs is shown in Fig. 2. None of the SNPs were in perfect LD ($r^2 < 1$) but rs3785157 in intron 7 and rs5569 in exon 9 had near perfect LD ($D' = 0.98$, $r^2 = 0.94$), creating a haploblock. In addition, the two promoter SNPs (rs28386840 and rs2242446) and rs3785143 from intron 1 had high D′ values, indicating linkage between the 5′ end polymorphisms.

Meta-analysis of the available case-control studies revealed no significant association either (for detailed statistics of the allele-wise analysis see Fig. 3, the genotype-wise analysis is available as Supplementary information).

3.2. Association analyses with ADHD symptom severity: dimensional approach

The possible genetic associations with the two ADHD-RS subscales were checked in analysis of variance among the 163 ADHD patients. In these quantitative analyses the rare homozygote and heterozygote genotypes were grouped together to increase the statistical power. The MANOVA indicated a significant association of intronic rs3785143 with inattention symptoms ($F(1,161) = 6.57$, $p = 0.011$) as the T-allele carriers showed significantly lower scores (CC: 16.28 ± 4.67 vs CT or TT: 13.88 ± 5.15). The neighboring rs2242446 from the promoter region also showed a tendentious association with inattention symptoms ($F(1,161) = 3.73$, $p = 0.055$) with the C-allele carriers having lower scores (CC or CT: 15.10 ± 4.92 vs TT: 16.55 ± 4.68). Similar tendency ($0.05 < p < 0.1$) was observed at the total ADHD-RS score. However, after correcting for multiple comparisons, none of the

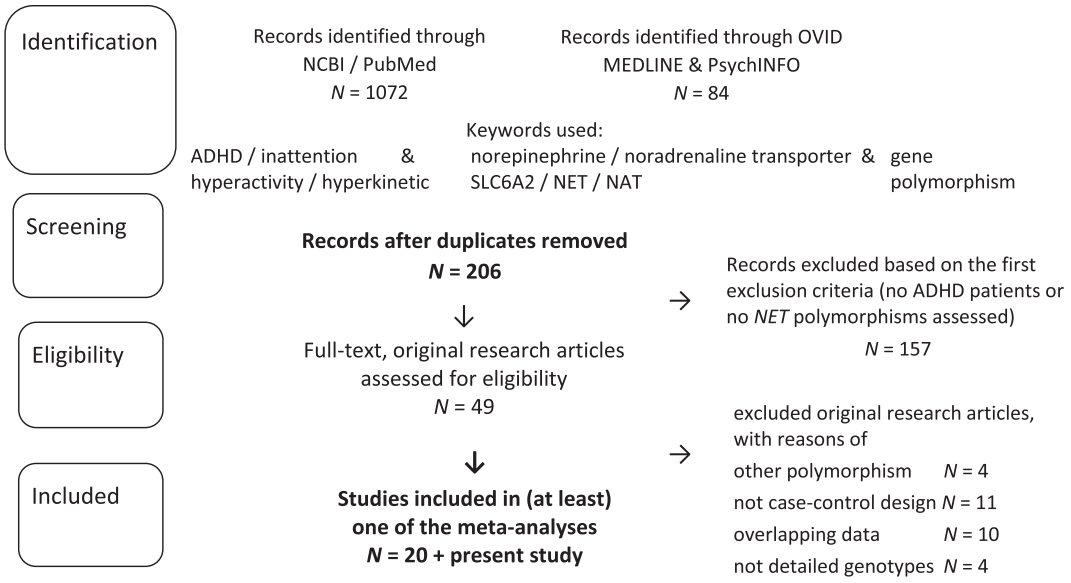

Fig. 1. Flow chart process of identifying studies for meta-analysis.

Table 1
Genotype frequencies of NET polymorphisms in the child ADHD patient, child control, and sex-matched control (from the general population) groups. None of the case-control comparison resulted in significant differences between patient vs. control groups at any of the SNPs in the chi-square analysis.

Polymorphic sites		Promoter rs28386840 (− 3081 A/T)			Promoter rs2242446 (− 182 C/T)			Intron 1 rs3785143 (intron 2 C/T)			Intron 7 rs3785157 (intron 8C/T)			Exon 9 rs5569 (1287 A/G)			3′ UTR rs7194256 (3338 C/T)		
Genotype		AA	AT	TT	CC	CT	TT	CC	CT	TT	CC	CT	TT	AA	AG	GG	CC	CT	TT
Child ADHD patients	N	85	60	18	19	64	80	131	31	1	79	70	14	13	72	78	121	39	3
	%	52.1	36.8	11.0	11.7	39.3	49.1	80.4	19.0	0.6	48.5	42.9	8.6	8.0	44.2	47.9	74.2	23.9	1.8
Child control (age 6–7)	N	40	37	9	9	37	40	66	20	0	41	38	7	7	38	41	57	26	3
	%	46.5	43.0	10.5	10.5	43.0	46.5	76.7	23.3	0.0	47.7	44.2	8.1	8.1	44.2	47.7	66.3	30.2	3.5
Adult control (sex-matched)	N	196	159	45	38	170	192	327	69	4	214	147	39	41	150	209	290	102	8
	%	49.0	39.8	11.3	9.5	42.5	48.0	81.8	17.3	1.0	53.5	36.8	9.8	10.3	37.5	52.3	72.5	25.5	2.0

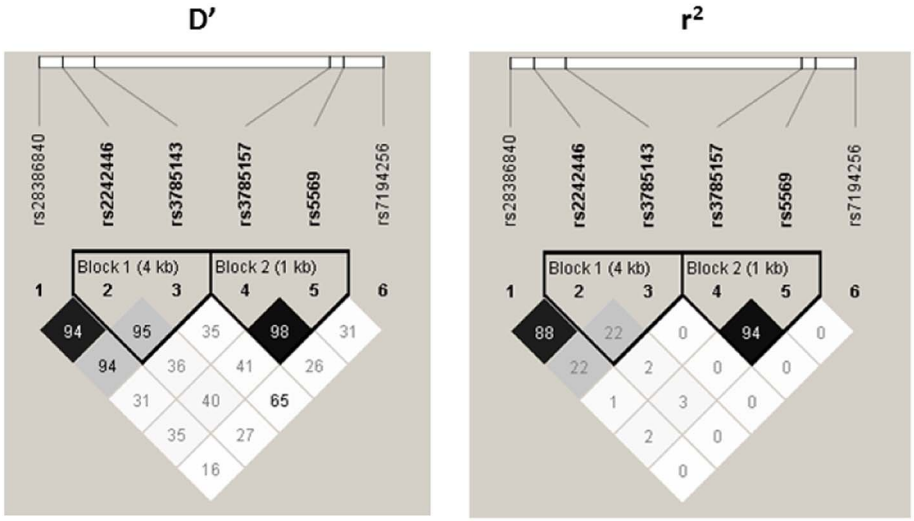

Fig. 2. Linkage disequilibrium data of the studied *NET* polymorphisms.
Statistics based on 649 individual genotype data (486 controls and 163 ADHD patients) are presented as Lewontin's D (D′) and r^2 values based on the Haploview program.

associations remained significant (i.e., none of the investigated 6 SNPs showed association with a *p*-value lower than 0.008). Using all 6 SNPs in the estimation of haplotype effect the T-C-T-C-G-C haplotype showed significantly lower inattention score compared to the most frequent A-T-C-C-G-C haplotype ($p = 0.003$), indicating that only the 5′ end polymorphisms make an impact on the inattention symptoms.

3.3. Pharmacogenetic analysis of NET polymorphisms with MPH-response

The drug-response analyses were conducted with 122 ADHD patients receiving MPH treatment. Using the categorical system, where 90 patients (73.8%) were described as responder and 32 (26.2%) were non-responder, we could detect only tendentious association ($0.05 < p < 0.1$) at rs28386840 (− 3081 A/T SNP) and rs5569 (1287 A/G SNP from exon 9), see Table 2. for the genotype frequencies.

Combining our categorical MPH-response data with previously published datasets for these two polymorphisms, the meta-analysis showed potential association at rs28386840, but not at rs5569 (Supplementary Fig. 1). Since the responder definition was varying in the different studies, this meta-analysis should be regarded only as indicative at the rs28386840 (for more information see Supplementary information).

In the dimensional approach repeated measures analyses of variance were carried out to test the effect of MPH on ADHD-RS subscales, and its possible interaction with these two *NET* SNPs. Symptom severity scores after the first month of MPH treatment were used to test MPH efficiency. MPH significantly reduced ADHD-RS scores (both Inattention and Hyperactivity-Impulsivity) after one month of treatment ($F(1,120) > 200$, $p < 0.001$). There was an interaction of MPH-effect and rs28386840 at the Hyperactivity-Impulsivity score ($F(1,120) = 4.26$, $p = 0.041$), but not at the Inattention score ($F(1,120) = 1.76$, $p = 0.187$), possibly because there was already a genetic influence of this SNP on the Inattention subscale ($F(1,120) = 2.84$, $p = 0.094$ within this subgroup of 122 patients). Again, this genetic difference could be seen at the total ADHD-RS score, but did not reach statistical significance ($p = 0.080$). For baseline and post-treatment scores of the *NET* rs28386840 genotypes within 122 ADHD patients see Table 3. The rs5569 did not show any significant effect on either ADHD symptom scales.

In the haplotype analysis using those 5′ end SNPs (rs28386840, rs2242446, rs3785143) showing association with ADHD symptoms and/or symptom improvement, the T-C-T haplotype showed significantly better inattention score improvement (difference score: -2.12, 95% CI: -0.33 & -3.90, $p = 0.020$) compared to the most frequent A-T-C haplotype (Fig. 4).

4. Discussion

Since the availability of dopamine transporter is low in the cortex, but norepinephrine transporter is relatively abundant and can take up extracellular dopamine in this brain region (Moron et al., 2002), we hypothesized that functional variants of the *NET* (*SLC6A2*) gene (e.g., either rs28386840 or rs2242446 from the promoter region or rs7194256 from the 3′ UTR) affecting its expression might contribute to attention problems due to suboptimal cortical mechanisms. In the present study we analyzed six SNPs of this candidate gene based on

SNP	Study	Odds Ratio	Lower limit	Upper limit	Z-Value	p-Value	Events / Total ADHD	Events / Total Control
rs2242446	present study	1.025	0.776	1.354	0.177	0.859	102 / 326	246 / 800
rs2242446	Lee 2011	0.939	0.689	1.279	-0.400	0.689	94 / 272	180 / 500
rs2242446	Oh 2017	0.956	0.655	1.396	-0.232	0.817	60 / 206	104 / 346
rs2242446	**risk: C-allele**	**0.976**	**0.748**	**1.273**	**-0.182**	**0.856**		
rs28386840	present study	0.924	0.697	1.224	-0.554	0.580	96 / 326	249 / 800
rs28386840	Cho 2008	0.801	0.588	1.092	-1.404	0.160	146 / 372	133 / 298
rs28386840	Eslami Amirabadi 2015	0.931	0.667	1.298	-0.424	0.672	106 / 302	111 / 302
rs28386840	Gomez-Sanchez 2016	1.212	0.954	1.541	1.574	0.115	191 / 580	196 / 680
rs28386840	Hohmann 2015	0.517	0.308	0.869	-2.490	0.013	20 / 106	170 / 548
rs28386840	Joung 2009	1.598	1.083	2.359	2.361	0.018	111 / 206	87 / 206
rs28386840	Kim 2006	2.145	1.268	3.627	2.846	0.004	70 / 188	26 / 120
rs28386840	Oh 2017	0.764	0.539	1.083	-1.511	0.131	84 / 206	164 / 346
rs28386840	**risk: T-allele**	**1.006**	**0.847**	**1.194**	**0.066**	**0.947**		
rs3785157	present study	1.098	0.828	1.457	0.651	0.515	98 / 326	225 / 800
rs3785157	Bobb 2005	0.936	0.563	1.554	-0.257	0.797	114 / 163	92 / 129
rs3785157	Hohmann 2015	1.613	1.054	2.467	2.202	0.028	45 / 106	172 / 548
rs3785157	**risk: T-allele**	**1.187**	**0.883**	**1.596**	**1.134**	**0.257**		
rs5569	present study	1.052	0.794	1.395	0.355	0.723	98 / 326	232 / 800
rs5569	Cho 2008	1.010	0.725	1.406	0.058	0.954	113 / 370	91 / 300
rs5569	Gomez-Sanchez 2016	0.978	0.768	1.245	-0.182	0.856	174 / 580	206 / 676
rs5569	Lee 2011	0.962	0.691	1.339	-0.229	0.819	74 / 272	141 / 504
rs5569	Oh 2017	0.823	0.567	1.196	-1.021	0.307	61 / 206	117 / 346
rs5569	**risk: A-allele**	**0.969**	**0.791**	**1.186**	**-0.308**	**0.758**		
rs998424	Bobb 2005	1.051	0.637	1.734	0.194	0.846	51 / 163	39 / 129
rs998424	Lee 2011	0.957	0.686	1.334	-0.259	0.795	73 / 274	137 / 498
rs998424	**risk: A-allele**	**0.992**	**0.685**	**1.436**	**-0.045**	**0.964**		

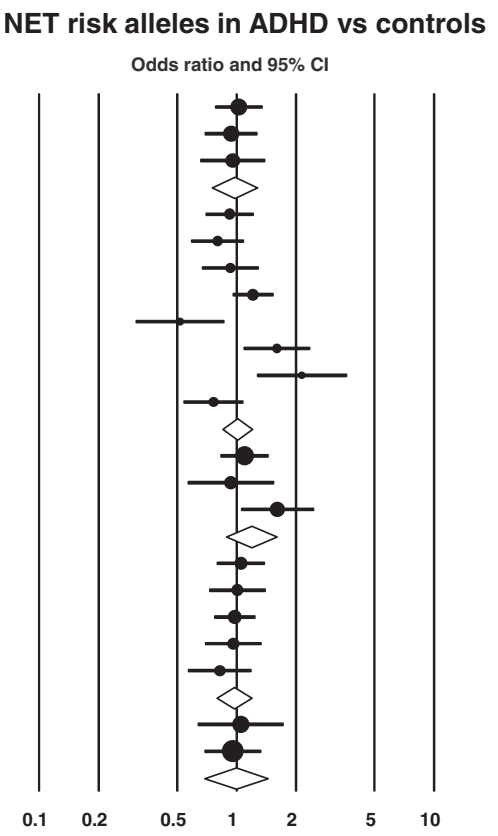

Fig. 3. Meta-analyses of *NET* SNPs in ADHD using allele-wise data of case-control analyses.

their previous association with ADHD or MPH-response. We selected SNPs from possibly different haplotype blocks reported in a Finnish Caucasian population by Belfer et al. (2004), with at least 5% of minor allele frequency. However, in our dataset the rs3785157 from intron 7 and rs5569 from exon 9 belonged to the same haploblock (see Fig. 2). Nonetheless, the chosen marker polymorphisms covered the two important regions of the *NET* gene previously indicated in ADHD (Hawi et al., 2013; Kim et al., 2006; Kim et al., 2008; Yang et al., 2004).

Our genetic association results did not support the involvement of any of the investigated *NET* polymorphisms in ADHD using a case-control design, this was further supported by meta-analyses (Fig. 3). Similarly, no significant association was reported in this candidate gene at the Psychiatric Genomics Consortium ADHD 2012 dataset (only one rare *NET* variant had $p = 0.048$). The limitations of our study include the relatively small sample size and the lack of allele transmission data. However, our study population consist of ethnically homogenous Caucasian groups, therefore, population stratification is unlikely to pose a problem in our analyses. Also, our control sample was not sex- and age-matched, the larger control sample selected from the general population was only sex-matched. Whereas we included all participants from a thoroughly screened child control sample who did not have ADHD diagnosis or indication of serious attention problems, independently of sex or age.

On the other hand, nominally significant differences were observed at MPH-response: ADHD patients with at least one rs28386840 (− 3081) T-allele showed better response, which is in agreement with previous Korean studies (Kim et al., 2010; Park et al., 2012b). Especially that the hyperactivity/impulsivity score reduction in our study was similar to that of reported by Kim et al. (2010), and also it presents a similar effect as the commission error reduction reported by Park et al. (2012b). It is important to mention, that the only functional study of this frequent *NET* gene variant showed that the rs28386840 T-allele had reduced transcriptional activity compared to the A-allele in a reporter gene system (Kim et al., 2006). In this study the T-allele was also associated with ADHD, which was supported by Korean and Canadian studies (Joung et al., 2010; Sengupta et al., 2012). However, not all studies using Caucasian populations could support this association with ADHD (Renner et al., 2011), and our meta-analysis did not show significant association of this *NET* promoter variant with ADHD diagnosis. Unfortunately, this SNP was not reported at the Psychiatric Genomics

Table 2
Genotype frequencies of *NET* polymorphisms within the ADHD patient group according to methylphenidate response. The rare homozygote and heterozygote genotypes were grouped together, because the cells with expected count were < 5, hence df = 1 in the chi-square analysis.

Polymorphic sites		Promoter rs28386840 (− 3081 A/T)			Promoter rs2242446 (− 182 C/T)			Intron 1 rs3785143 (intron 2 C/T)			Intron 7 rs3785157 (intron 8 C/T)			Exon 9 rs5569 (1287 A/G)			3′ UTR rs7194256 (3338 C/T)		
Genotype		AA	AT	TT	CC	CT	TT	CC	CT	TT	CC	CT	TT	AA	AG	GG	CC	CT	TT
Non-responder	N	21	6	5	5	8	19	26	5	1	20	10	2	2	10	20	21	10	1
	%	65.6	18.8	15.6	15.6	25.0	59.4	81.3	15.6	3.1	62.5	31.3	6.3	6.3	31.3	62.5	65.6	31.3	3.1
Responder	N	43	38	9	10	40	40	73	17	0	41	41	8	8	42	40	68	21	1
	%	47.8	42.2	10.0	11.1	44.4	44.4	81.1	18.9	0.0	45.6	45.6	8.9	8.9	46.7	44.4	75.6	23.3	1.1
p (df = 1)		0.082			0.147			0.986			0.100			0.079			0.277		

Table 3
Severity scores of ADHD-RS subscales in the different *NET* rs28386840 (− 3081 A/T SNP) genotype groups.

	AA (n = 64)	AT (n = 44)	TT (n = 14)	AT or TT (n = 58)
Total score (baseline)	33.78 ± 9.55	32.02 ± 9.37	31.57 ± 10.02	31.91 ± 9.44
Total score MPH 1 month	26.59 ± 10.73	22.41 ± 8.74	24.50 ± 10.01	22.91 ± 9.01
Inattention (baseline)	16.95 ± 4.81	15.36 ± 4.92	15.71 ± 5.62	15.45 ± 5.05
Inattention MPH 1 month	13.28 ± 5.50	10.77 ± 4.85	12.14 ± 4.77	11.10 ± 4.82
Hyperact.-Impul. (baseline)	16.67 ± 5.98	16.43 ± 5.25	15.86 ± 5.13	16.29 ± 5.18
Hyperact.-Impul. MPH 1 m.	13.33 ± 6.10	11.64 ± 4.71	12.50 ± 5.45	11.84 ± 4.86

The ADHD-RS scores (mean ± SD) are shown from the beginning of the study (baseline) and after one month of methylphenidate treatment (MPH) using 122 ADHD patients' data participating in the drug-response study.

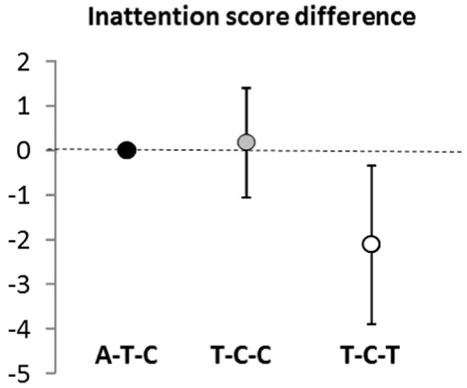

Fig. 4. Difference scores of inattention symptom reduction at the *NET* haplotype groups constructed from the 5′ end SNPs (rs28386840, rs2242446, rs3785143).
The differences of inattention score with 95% CI of the estimated rs28386840 T - rs2242446 C - rs3785143 T haplotype (open symbol, T-C-T) and T-C-C haplotype (grey symbol) groups compared to the most frequent A-T-C haplotype (black circle) are presented, based on THESIAS calculations.

Consortium ADHD 2012 dataset.

Clearly, SNPs from the 5′ end region of the *NET* gene have been repeatedly indicated in ADHD, and our study also points out a possible association with this gene region, especially with inattention symptoms (Fig. 4). ADHD patients with the 1st intron rs3785143 CC genotype and the rs28386840 A - rs2242446 T - rs3785143 C haplotype showed higher scores at attention problems. In addition, a genome-wide association study (Mick et al., 2008) using quantitative analysis of MPH-response showed suggestive association ($p \leq 0.01$) with *NET* SNPs from this same first intron region. This indicates the better sensitivity of using symptom severity scores instead of diagnostic categories at detecting small genetic effects.

Future studies should use more homogenous and/or specific ADHD subtypes as its usefulness was reported by a Canadian group (Sengupta et al., 2012; Thakur et al., 2012). Unfortunately, we did not have maternal smoking data on every ADHD patients, therefore, we could not carry out the subgroup specific analysis reported by Thakur et al. (2012). Further studies or meta-analyses using Caucasian and Asian populations separately are required to gain more detailed information about these indicated *NET* gene variants.

5. Conclusions

Our pharmacogenetic association analyses of the norepinephrine transporter (*NET*, *SLC6A2*) gene in methylphenidate response among Hungarian ADHD children pointed to the possible involvement of the promoter − 3081 A/T SNP (rs28386840), which was supported by a meta-analysis combining our results using Central-European ADHD population with published data of Korean ADHD samples. In addition, inattention symptoms (at baseline) were associated with a more downstream promoter variant − 182C/T SNP (rs2242446) and the intronic rs3785143. Haplotype analysis supported this association, indicating the importance of the 5′ region polymorphisms in the *NET* gene at developing attention problems.

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The sponsor did not have any influence on the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation of the manuscript.

Contributors

MS-S and ZN conceived and designed the study. ZT and EB managed the patient recruitment and data collection. KL was responsible for the child control, and MS-S for the adult control sample selection. NA, EZH, and ZN conducted the genetic analyses. MR and NA conducted the meta-analyses. NA and ZN drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest

None of the authors reported biomedical financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2018.01.013.

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