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RESEARCH ARTICLE

Polymorphism in the Serotonin Receptor 2a (*HTR2A*) Gene as Possible Predisposal Factor for Aggressive Traits

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Abstract

Aggressive manifestations and their consequences are a major issue of mankind, highlighting the need for understanding the contributory factors. Still, aggression-related genetic analyses have so far mainly been conducted on small population subsets such as individuals suffering from a certain psychiatric disorder or a narrow-range age cohort, but no data on the general population is yet available. In the present study, our aim was to identify polymorphisms in genes affecting neurobiological processes that might explain some of the inter-individual variation between aggression levels in the non-clinical Caucasian adult population. 55 single nucleotide polymorphisms (SNP) were simultaneously determined in 887 subjects who also filled out the self-report Buss-Perry Aggression Questionnaire (BPAQ). Single marker association analyses between genotypes and aggression scores indicated a significant role of rs7322347 located in the *HTR2A* gene encoding serotonin receptor 2a following Bonferroni correction for multiple testing ($p = 0.0007$) both for males and females. Taking the four BPAQ subscales individually, scores for Hostility, Anger and Physical Aggression showed significant association with rs7322347 T allele in themselves, while no association was found with Verbal Aggression. Of the subscales, relationship with rs7322347 was strongest in the case of Hostility, where statistical significance virtually equaled that observed with the whole BPAQ. In conclusion, this is the first study to our knowledge analyzing SNPs in a wide variety of genes in terms of aggression in a large sample-size non-clinical adult population, also describing a novel candidate polymorphism as predisposal to aggressive traits.

Introduction

Aggression, defined as any behavior intended to be destructive, lies at the root of numerous major ills of humanity ranging from verbal abuse through both interpersonal and self-directed violence to mass criminal acts. Consequences of aggression-driven acts pose an enormous burden on society and economics, rendering it important to understand the biological basis behind [1,2].

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Increased levels of aggression are characteristic to patients with a variety of neurodegenerative and psychiatric disorders as well as to alcoholics and drug addicts [3–7], but can also often be observed among the normal human population, even conferring certain privileges to the aggressor under certain circumstances e.g. by means of social dominance [8,9]. From the evolutionary point of view, some degree of aggression is indeed necessary for gaining adequate fitness (through an improved access of food supplies and other resources) and reproductive success; however, these benefits are compensated for by an increased risk of injury and social isolation. Hence, optimal levels of aggression are presumably shaped by a fine balance between effects of positive and negative selection pressure, implying a strong genetic background next to the role of environment [10,11]. This assumption is further underpinned by the fact that aggression proved to be heritable in several twin studies, with an estimated genetic contribution to the risk of aggressiveness of above 40% [12–17].

Experimental evidence suggest that aggressive manifestations and the accompanying emotions (anger, anxiety, fear) can be strongly related to highly conserved brain regions, chiefly to the amygdala and its linked neural circuits, but also to the anterior cingulated cortex and the prefrontal cortex [18,19]. In terms of biochemistry, it is principally the monoaminergic neurotransmitter systems (e.g. dopamine, noradrenaline and serotonin pathways) that are believed to play a major role in aggressive behavior, though possible effects of sexual hormones, the hypothalamic-pituitary-adrenal (HPA) axis and blood sugar levels have also been implicated [20,21].

Great efforts have been made to decipher the possible genetic background behind predisposition to aggression, describing novel polymorphisms in a variety of genes with a role in neuropsychiatry, and also identifying promising candidates for aggressive behavior and the related mental states (impulsivity, hostility). However, most of these association studies were carried out in small samples, raising the possibility of committing statistical errors (Pavlov 2012). Besides, the vast majority of aggression-related genetic investigations either were based on comparisons between healthy individuals and patients suffering from personality disorders etc., or concentrated on restricted samples not representative of the general population (e.g. [22–28]). These factors render data evaluation challenging, and often lead to controversial results.

Our aim was to simultaneously examine the effect of a set of putatively functional single nucleotide polymorphisms (SNP) on aggressive tendencies of the general Hungarian adult population using a microarray system, with a principal focus on monoaminergic pathways and its close interactors. Selected SNPs are located in genes encoding monoaminergic neurotransmitter transporters and receptors, their associated proteins and other signal transduction molecules, enzymes involved in the biosynthesis or degradation of neurotransmitters, neurotrophic factors and regulators of circadian rhythm as well as of neuronal death, all with an implicated role in emotional responses and behavioral traits [20,29–32].

Materials and Methods

Individuals involved

Non-related individuals of Caucasian Hungarian origin without any known psychiatric disorder were recruited for this study on a voluntary basis at the Institute of Psychology, Eotvos Lorand University (Budapest). Buccal samples and self-filled out aggression questionnaires were obtained from 887 subjects (45.8% males and 54.2% females). The sample comprised of 495 psychology and law enforcement students studying in the Budapest area and 392 random volunteers recruited at academic institutions and events popularizing this survey. All participants belonged to the middle socioeconomic status. Mean age was 23.2 (±7.55) years within the range from 18 to 75 years. All participants gave written informed consent and the study was

approved by the Scientific and Research Ethics Committee of the Medical Research Council ("ETT TUKEB"—Ministry of Health, Medical Research Council, Budapest, H-1051 Hungary).

Phenotypic measure

The original 29-item version of the self-report Buss-Perry Aggression Questionnaire (BPAQ) [33] was used to assess aggressive tendencies. This instrument comprises four subscales: Verbal Aggression (5 items), Physical Aggression (9 items), Anger (7 items) and Hostility (8 items). Individual items are rated from one ('extremely uncharacteristic of me') to five ('extremely characteristic of me'). Total score for aggression was calculated as the sum of ratings for all the items, with a possible range between 29 and 145. Hungarian version of the original English language questionnaire was obtained by the "forward-backward" translation method and was pilot tested prior to the present study [34].

Sample collection

Buccal cells were collected by gently scraping the inner cheek with cotton-tipped collection swabs. Genomic DNA preparation was performed by a traditional, salting-out procedure [35]. Briefly, collection swabs were incubated overnight in 450 μl cell lysis buffer (0.2 g/l Proteinase K, 0.1 M NaCl, 0.5% SDS, 0.01 M Tris buffer pH = 8.0) at 56°C, followed by RNase treatment at room temperature. Proteins were precipitated with saturated NaCl (6 M) and removed by centrifugation. DNA was precipitated with isopropanol, purified with 70% ethanol and resuspended in 100 μl of Tris-EDTA pH = 8.0 (containing 0.5 M EDTA). DNA concentrations were measured by a fluorometry based intercalation assay (AccuBlue Broad Range dsDNA Quantification Kit, Biotium). Concentration of samples analyzed in this study ranged between 15 and 200 ng/μl. Isolated DNA samples were kept at −20°C until used.

Marker selection

Common SNPs with a higher than 5% minor allele frequency (MAF) were selected from the dbSNP database of NCBI [36]. Priority was given to polymorphisms referred to in various association studies in connection with personality or mood disorders as well as aggression or impulsivity in psychiatric disorders, and to putative functional variants, either causing an amino acid change or with an implicated gene regulatory role.

Genotyping

Genotyping was performed in 384-well plates on an Open Array real-time PCR platform (Applied Biosystems) based on allele-specific, fluorescent (TaqMan) probes and pre-designed, validated primers immobilized to a solid surface obtained from the manufacturer. Approximately 100 ng DNA per sample was used in each measurement. DNA amplification was carried out in the GeneAmp PCR System 9700 (Applied Biosystems) according to the manufacturer's instructions, using the master mix, containing each dNTP and AmpliTaq Gold DNA-polymerase, provided by the manufacturer. Endpoint detection of signal intensities of allele specific fluorescent dyes was conducted by the OpenArray NT Imager, and genotypes were called by the TaqMan Genotyper v1.2 software. Call rate for individual SNPs is shown in Table 1 (mean: 77.9%).

Statistical analysis

Statistical analyses were performed by the SPSS 22.0 (SPSS Inc.) software. Allele and genotype frequency distributions were determined by the χ^2 test. Independent samples t-test was used to assess gender differences, and relationship with age was tested by Pearson correlation. Genetic

Table 1. Genotype distribution of the studied SNPs.

	SNP	Gene	N	Genotype						HWE*	Call rate
				MM		Mm		mm			
1.	rs1048101	*ADRA1A*	763	218	28,6%	384	50,3%	161	21,1%	0.945	86%
2.	rs3808585	*ADRA1A*	722	396	54,8%	277	38,4%	49	6,8%	0.998	81%
3.	rs2236554	*ADRA1D*	757	293	38,7%	346	45,7%	118	15,6%	0.641	85%
4.	rs553668	*ADRA2A*	692	519	75,0%	158	22,8%	15	2,2%	0.770	78%
5.	rs11030104	*BDNF*	702	393	56,0%	264	37,6%	45	6,4%	0.997	79%
6.	rs2049045	*BDNF*	690	419	60,7%	241	34,9%	30	4,3%	0.820	78%
7.	rs6265	*BDNF*	601	362	60,2%	212	35,3%	27	4,5%	0.847	68%
8.	rs7103411	*BDNF*	715	393	55,0%	276	38,6%	46	6,4%	0.966	81%
9.	rs7094179	*CDNF*	687	305	44,4%	302	44,0%	80	11,6%	0.924	77%
10.	rs7900873	*CDNF*	696	384	55,2%	273	39,2%	39	5,6%	0.573	78%
11.	rs1051730	*CHRNA3*	753	320	42,5%	345	45,8%	88	11,7%	0.943	85%
12.	rs16969968	*CHRNA5*	663	279	42,1%	307	46,3%	77	11,6%	0.866	75%
13.	rs4680	*COMT*	603	177	29,4%	295	48,9%	131	21,7%	0.927	68%
14.	rs135745	*CSNK1E*	718	187	26,0%	375	52,2%	156	21,7%	0.460	81%
15.	rs1997644	*CSNK1E*	688	176	25,6%	364	52,9%	148	21,5%	0.291	78%
16.	rs1611115	*DBH*	761	443	58,2%	283	37,2%	35	4,6%	0.482	86%
17.	rs6271	*DBH*	780	657	84,2%	116	14,9%	7	0,9%	0.759	88%
18.	rs4532	*DRD1*	761	286	37,6%	357	46,9%	118	15,5%	0.931	86%
19.	rs6277	*DRD2*	579	169	29,2%	284	49,1%	126	21,8%	0.948	65%
20.	rs1800497	*DRD2*	605	399	66,0%	192	31,7%	14	2,3%	0.261	68%
21.	rs1079597	*DRD2*	608	443	72,9%	158	26,0%	7	1,2%	0.226	69%
22.	rs1800498	*DRD2*	595	215	36,1%	280	47,1%	100	16,8%	0.862	67%
23.	rs2134655	*DRD3*	760	410	53,9%	295	38,8%	55	7,2%	0.981	86%
24.	rs3732790	*DRD3*	734	243	33,1%	365	49,7%	126	17,2%	0.857	83%
25.	rs6280	*DRD3*	749	354	47,3%	326	43,5%	69	9,2%	0.887	84%
26.	rs963468	*DRD3*	736	246	33,4%	364	49,5%	126	17,1%	0.909	83%
27.	rs11246226	*DRD4*	685	173	25,3%	347	50,7%	165	24,1%	0.941	77%
28.	rs3758653	*DRD4*	714	486	68,1%	208	29,1%	20	2,8%	0.923	80%
29.	rs916455	*DRD4*	702	644	91,7%	56	8,0%	2	0,3%	0.803	79%
30.	rs936460	*DRD4*	697	344	49,4%	284	40,7%	69	9,9%	0.655	79%
31.	rs3733829	*EGLN2*	683	263	38,5%	321	47,0%	99	14,5%	0.998	77%
32.	rs222843	*GABARAP*	683	307	44,9%	293	42,9%	83	12,2%	0.601	77%
33.	rs11111	*GDNF*	719	540	75,1%	160	22,3%	19	2,6%	0.241	81%
34.	rs1549250	*GDNF*	710	231	32,5%	353	49,7%	126	17,7%	0.907	80%
35.	rs1981844	*GDNF*	576	320	55,6%	223	38,7%	33	5,7%	0.771	65%
36.	rs2910702	*GDNF*	705	387	54,9%	269	38,2%	49	7,0%	0.971	79%
37.	rs2973041	*GDNF*	695	492	70,8%	182	26,2%	21	3,0%	0.710	78%
38.	rs2973050	*GDNF*	582	242	41,6%	275	47,3%	65	11,2%	0.608	66%
39.	rs3096140	*GDNF*	671	320	47,7%	287	42,8%	64	9,5%	1.000	76%
40.	rs3812047	*GDNF*	679	521	76,7%	144	21,2%	14	2,1%	0.559	77%
41.	rs6925	*HTR1A*	607	167	27,5%	289	47,6%	151	24,9%	0.510	68%
42.	rs1228814	*HTR1B*	599	432	72,1%	153	25,5%	14	2,3%	0.995	68%
43.	rs130058	*HTR1B*	595	330	55,5%	232	39,0%	33	5,5%	0.642	67%
44.	rs13212041	*HTR1B*	606	376	62,0%	209	34,5%	21	3,5%	0.467	68%
45.	rs11568817	*HTR1B*	600	187	31,2%	292	48,7%	121	20,2%	0.937	68%

(*Continued*)

Table 1. (*Continued*)

	SNP	Gene	N	Genotype						HWE*	Call rate
				MM		Mm		mm			
46.	rs6296	*HTR1B*	607	325	53,5%	233	38,4%	49	8,1%	0.730	68%
47.	rs6311	*HTR2A*	777	243	31,3%	391	50,3%	143	18,4%	0.809	88%
48.	rs6313	*HTR2A*	769	240	31,2%	385	50,1%	144	18,7%	0.893	87%
49.	rs6314	*HTR2A*	773	640	82,8%	130	16,8%	3	0,4%	0.409	87%
50.	rs7322347	*HTR2A*	765	242	31,6%	370	48,4%	153	20,0%	0.866	86%
51.	rs7984966	*HTR2A*	758	411	54,2%	293	38,7%	54	7,1%	0.984	85%
52.	rs3813929	*HTR2C*	744	555	74,6%	117	15,7%	72	9,7%	0.975	84%
53.	rs518147	*HTR2C*	717	379	52,9%	166	23,2%	172	24,0%	0.237	81%
54.	rs6318	*HTR2C*	769	570	74,1%	127	16,5%	72	9,4%	0.737	87%
55.	rs907094	*PPP1R1B*	705	409	58,0%	246	34,9%	50	7,1%	0.308	79%

M: major allele, m: minor allele

*Hardy Weinberg Equilibrium.

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associations were tested by one way analysis of covariance (ANCOVA) assuming a dominant model of inheritance with sex and age as covariates. Bonferroni correction for multiple testing was applied for the total number of SNPs in this study when assessing relationship between BPAQ scores and individual SNPs (the corrected level of significance was $p = 0.05 / 55 = 0.0009$). In all other cases, $p < 0.05$ values were regarded as significant. Effect of prior associations in males and females was analyzed by two-way ANCOVA with age as covariate. All tests were two-tailed. Lewontin's *D'* and r^2 values of linkage disequilibrium were calculated using HaploView 4.2. [37]. Haplotypes were determined by the PHASE software [38,39].

Results

Reliability of the markers analyzed

Internal consistency of the self-report BPAQ was assessed by Chronbach's alpha, which had a value of 0.895 for total scores ensuring reliability of the study. Coefficients for Verbal Aggression, Physical Aggression, Anger and Hostility were 0.640, 0.842, 0.831 and 0.792, respectively.

Alleles of all the SNPs studied were in Hardy-Weinberg equilibrium (Table 1).

Potential confounders

Gender differences on the BPAQ scale were evaluated by Independent samples t-test. Males presented significantly higher scores than females (68.52±17.14 compared to 64.49±15.09; $p<0.001$). Relationship between BPAQ scores and age was tested by Pearson correlation coefficient and was found to be significant ($p = 0.008$). Thus, both gender and age were used as covariates in all association analyses.

Significant association of the HTR2A rs7322347 T/A intronic SNP with aggression

Table 2 summarizes results of phenotypic data as a function of each SNP analyzed. Association with aggression reached nominal level of significance $p<0.05$ in the case of two SNPs, rs916455 located in the promoter region of the *DRD4* gene and rs7322347 in intron 2 of

Table 2. Association of the 55 polymorphisms studied with aggression levels.

	SNP	Gene	Aggression (total score)			p[#]
			MM	Mm	mm	
1.	rs1048101	*ADRA1A*	66.66	66.50	66.46	0.9684
2.	rs3808585	*ADRA1A*	66.15	68.19	65.93	0.2294
3.	rs2236554	*ADRA1D*	65.31	67.18	68.29	0.0840
4.	rs553668	*ADRA2A*	66.52	66.61	70.47	0.8682
5.	rs11030104	*BDNF*	66.56	66.60	67.73	0.8735
6.	rs2049045	*BDNF*	66.34	67.15	66.73	0.5703
7.	rs6265	*BDNF*	66.94	66.80	65.98	0.9220
8.	rs7103411	*BDNF*	66.55	66.53	67.34	0.9163
9.	rs7094179	*CDNF*	65.81	66.46	68.32	0.6485
10.	rs7900873	*CDNF*	67.03	66.49	64.68	0.3912
11.	rs1051730	*CHRNA3*	67.53	65.51	66.58	0.1190
12.	rs16969968	*CHRNA5*	67.45	65.77	66.61	0.2138
13.	rs4680	*COMT*	67.07	66.58	67.62	0.8569
14.	rs135745	*CSNK1E*	65.99	66.63	66.24	0.7121
15.	rs1997644	*CSNK1E*	66.83	66.31	65.68	0.7781
16.	rs1611115	*DBH*	65.74	67.36	70.89	0.0941
17.	rs6271	*DBH*	66.59	66.68	61.00	0.8731
18.	rs4532	*DRD1*	66.55	65.94	67.91	0.9000
19.	rs6277	*DRD2*	66.81	67.06	66.39	0.9148
20.	rs1800497	*DRD2*	66.72	67.70	61.29	0.7106
21.	rs1079597	*DRD2*	67.20	66.38	57.95	0.4397
22.	rs1800498	*DRD2*	67.07	66.46	67.34	0.7979
23.	rs2134655	*DRD3*	65.71	67.81	66.12	0.1250
24.	rs3732790	*DRD3*	67.03	66.42	65.90	0.5267
25.	rs6280	*DRD3*	66.98	66.60	64.46	0.4667
26.	rs963468	*DRD3*	67.25	67.01	65.06	0.5779
27.	rs11246226	*DRD4*	67.31	66.33	66.41	0.4831
28.	rs3758653	*DRD4*	66.51	66.48	69.93	0.9091
29.	***rs916455***	***DRD4***	***66.93***	***62.82***	***46.67***	***0.0275***
30.	rs936460	*DRD4*	66.60	66.39	67.70	0.9890
31.	rs3733829	*EGLN2*	66.96	66.76	65.38	0.6238
32.	rs222843	*GABARAP*	66.50	66.09	68.77	0.9562
33.	rs11111	*GDNF*	66.56	65.90	73.85	0.9972
34.	rs1549250	*GDNF*	66.75	65.38	70.34	0.8604
35.	rs1981844	*GDNF*	66.48	66.85	72.22	0.4727
36.	rs2910702	*GDNF*	66.27	66.40	68.79	0.5293
37.	rs2973041	*GDNF*	66.68	66.24	71.95	0.9268
38.	rs2973050	*GDNF*	66.30	66.49	68.89	0.5259
39.	rs3096140	*GDNF*	65.79	66.97	68.52	0.1457
40.	rs3812047	*GDNF*	66.45	67.46	70.87	0.3422
41.	rs6925	*HTR1A*	66.55	67.63	65.63	0.9441
42.	rs1228814	*HTR1B*	67.20	66.47	63.95	0.5336
43.	rs130058	*HTR1B*	67.22	65.55	70.74	0.3419
44.	rs13212041	*HTR1B*	67.09	66.21	66.87	0.5259
45.	rs11568817	*HTR1B*	68.70	65.76	67.12	0.0605

(*Continued*)

Table 2. (*Continued*)

	SNP	Gene	Aggression (total score)			p[#]
			MM	Mm	mm	
46.	rs6296	*HTR1B*	66.21	67.33	68.60	0.2601
47.	rs6311	*HTR2A*	66.78	66.32	67.41	0.9130
48.	rs6313	*HTR2A*	66.44	66.09	67.44	0.9969
49.	rs6314	*HTR2A*	67.02	63.98	73.67	0.0765
50.	***rs7322347***	***HTR2A***	***69.21***	***64.92***	***66.35***	***0.0007****
51.	rs7984966	*HTR2A*	67.45	65.65	65.58	0.1356
52.	rs3813929	*HTR2C*	67.01	63.67	66.43	0.2163
53.	rs518147	*HTR2C*	66.73	66.44	67.18	0.3203
54.	rs6318	*HTR2C*	66.43	66.46	67.63	0.2277
55.	rs907094	*PPP1R1B*	67.04	65.18	68.84	0.2994

Nominally significant associations are indicated by bold, italics.

* Significant after Bonferroni correction

[#] Dominant model (MM vs. Mm and mm).

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HTR2A. Corresponding statistical values for these were [F = 4.878, p = 0.0275, η^2 = 0.007, power = 0.597] and [F = 11.617, p = 0.0007 η^2 = 0.015, power = 0.926], respectively. In order to reduce the likelihood of a type I error, Bonferroni adjustment on the target alpha level was performed to correct for multiple testing. Effect of the rs7322347 polymorphism remained significant after Bonferroni-correction, labeled by an asterisk in Table 2. Individuals homozygous for the wild type allele (T) of rs7322347 had significantly higher aggression scores (69.21±17.00) compared to those carrying at least one minor allele (A) of this polymorphism (65.34±15.69). The corresponding Cohen's d effect size for rs7322347 was d = 0.24.

In order to gain a more detailed insight into the nature of the observed association, *post hoc* analyses were performed testing for possible relationship between rs7322347 and each of the four individual BPAQ subscales (Fig. 1). With the exception of Verbal Aggression, where mean scores did not differ in non-carriers compared to carriers of allele A (15.09±3.47 vs. 14.65± 3.24; p = 0.1076), scores of all subscales showed significant association with rs7322347. Differences in mean scores between those homozygous for rs7322347 T and those with at least one

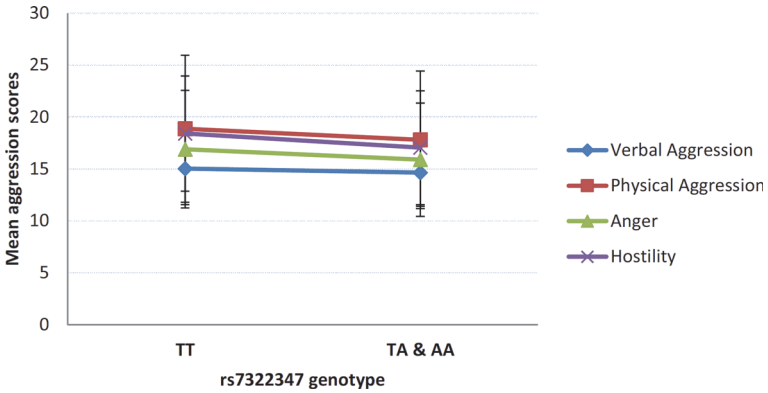

Fig 1. Relationship of each of the Buss-Perry Aggression Questionnaire subscales with rs7322347 A allele carrier status. Mean scores of each the Buss-Perry Aggression Questionnaire subscales according to rs7322347 genotypes. Error bars represent standard errors of the mean.

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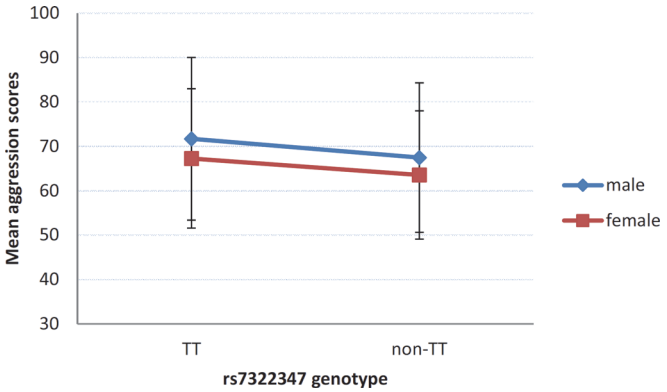

Fig 2. Effect of the HTR2A rs7322347 polymorphism on male and female aggression. Mean scores of the Buss-Perry Aggression Questionnaire in males and females according to rs7322347 genotypes. Error bars represent standard errors of the mean.

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copy of rs7322347 A was most remarkable in the case of Hostility (18.41±5.55 vs. 17.05±5.48), with statistical difference between groups virtually equaling that observed with the overall BPAQ scale [F = 11.535, p = 0.0007, η^2 = 0.015, power = 0.924]. Mean scores for both Physical Aggression and Anger were also higher in the absence of rs7322347 A than in its presence (18.86±7.08 vs. 17.80±6.63) [F = 7.419, p = 0.0066, η^2 = 0.010, power = 0.776] and (16.91±5.67 vs. 15.89±5.46) [F = 5.858, p = 0.0157, η^2 = 0.008, power = 0.676], respectively.

Effect of the HTR2A rs7322347 polymorphism on male and female aggression

As significant gender effect was observed in the BPAQ scores, male vs. female differences were also tested in terms of rs7322347 genotype and aggression using two-way ANCOVA with age as covariate. Although interaction between gender and aggression scores was highly significant [F = 10.991, p = 0.0010, η^2 = 0.014, power = 0.912], no gene-sex interaction was found (p = 0.8834). Both males and females carrying the minor (A) allele of rs7322347 showed lower levels of aggression (Fig. 2).

Linkage disequilibrium (LD) and haplotype analyses within the HTR2A gene

Taken that four other SNPs than rs7322347 (rs6311 C/T, rs6313 G/A, rs6314 G/A and rs7984966 T/C) within the *HTR2A* gene were also genotyped in this study, LD and haplotype analyses were performed as well to explore possible further contribution of loci in nearby regions to higher aggression levels. The associating polymorphism rs7322347 was found to be in complete linkage disequilibrium (D' = 1) with rs6314 located 1069 bp upstream from rs7322347 (Fig. 3), due to the fact that allele A of rs6314 could only be observed in subjects also carrying rs7322347 A and that all individuals homozygous for rs6314 A were homozygous for rs7322347 A as well. However, this was accompanied by a relatively low r^2 value as there was a marked difference in MAFs for these two SNPs (8.8% for rs6314 vs. 44.2% for rs7322347). The polymorphism rs7322347 was in strong LD with rs7984966 as well (chromosomal distance: 19343 bp), although to a lesser extent than with rs6314. In addition, prominently high LD was also observed between rs6313 and rs6311 spaced 1538 bp apart, where in the majority of cases allele A of rs6313 was linked to rs6311 T (662/665 chromosomes; 99.6%) and allele G of rs6313 to rs6311 C (850/853 chromosomes; 99.7%) (Fig. 3).

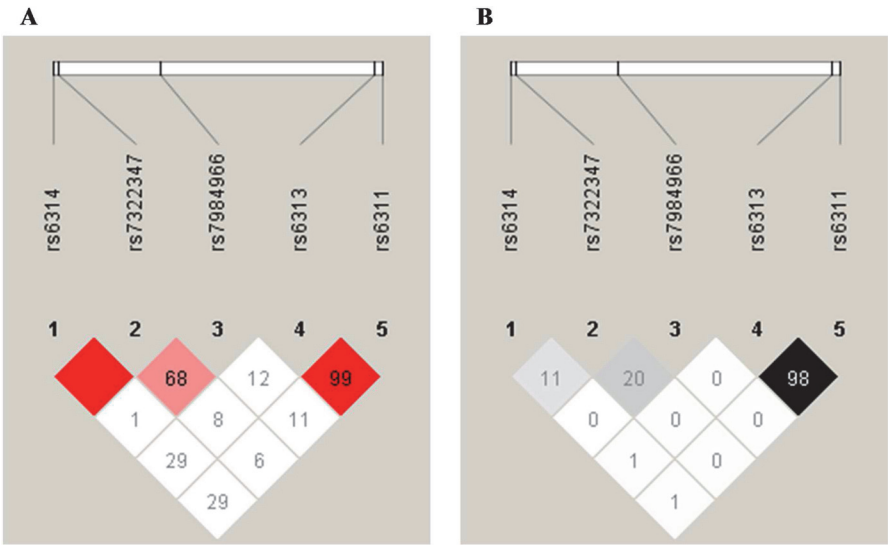

Fig 3. Linkage disequilibrium patterns between SNPs studies within the HTR2A gene. A: Lewontin's D' (%) and B: r2 (%) values of linkage disequilibrium between each SNP pairs, as determined by HaploView (version 4.2.). Higher values and darker colors indicate stronger LD between loci pairs. Red square indicates 100% LD.

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One-way ANCOVAs were applied on the overall BPAQ scale scores with 2-SNP haplotypes (comprising rs7322347 and each of the other four *HTR2A* variants genotyped) as the grouping variable and gender and age as covariates (Table 3). In a dominant model (haplotypes containing only major alleles of the constituting SNPs), haplotypes rs6314/ rs7322347 and rs7322347/ rs7984966 showed a significant effect [$F = 11.128$, $p = 0.0009$, $\eta^2 = 0.014$, power = 0.915 and $F = 7.352$, $p = 0.0068$, $\eta^2 = 0.009$, power = 0.773, respectively], while no significant differences in the mean scores of aggression were observed with regard to the other two haplotypes analyzed ($p = 0.1875$ and $p = 0.1232$, respectively). Subjects homozygous for haplotype rs6314 G/ rs7322347 T had higher aggression scores as compared to the rest of the population (69.05±17.07 vs. 65.33± 15.77). Similarly, individuals carrying haplotype rs7322347 T/ rs7984966 T on both chromosomes presented with higher mean BPAQ scores than those with other haplotype combinations (68.83 ±17.19 vs. 65.64±15.85). Haplotype-wise analyses also indicated significant association of haplotype rs6314/ rs7322347, but to a lesser extent than in the dominant model [$F = 3.205$, $p = 0.0408$, $\eta^2 = 0.004$, power = 0.614] (Table 4).

Table 3. Association of rs7322347 comprising 2-SNP within-*HTR2A* haplotypes with aggression scores.

	Aggression score		p
	HH	Hh & hh	
rs6314/ rs7322347	69.05±17.07	65.33±15.77	***0.0009***
rs7322347/ rs7984966	68.83±17.19	65.64±15.85	***0.0068***
rs7322347/ rs6313	68.58±18.92	66.23±15.89	0.1875
rs7322347/ rs6311	68.95±18.69	66.19±15.92	0.1232

H: Haplotype containing major alleles of the constituting SNPs;
h: haplotype containing minor allele of at least of the two constituting SNPs
Significant associations are indicated by bold, italics.

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Table 4. Haplotype-wise analysis of rs7322347 and each of the other *HTR2A* SNPs studied.

	N	Haplotype frequency	Aggression score	p
rs6314G-rs7322347T	862	0,56	67.28±16.53	***0.041***
rs6314G-rs7322347A	547	0,35	65.78±16.04	
rs6314A-rs7322347A	135	0,09	64.46±15.28	
rs6314A-rs7322347T	0	0	-	
rs7322347T-rs7984966T	809	0,52	67.34±16.54	0.115
rs7322347A-rs7984966C	347	0,22	65.45±15.63	
rs7322347A-rs7984966T	335	0,22	65.59±16.18	
rs7322347T-rs7984966C	53	0,03	66.37±16.38	
rs7322347T-rs6313G	592	0,38	67.03±16.50	0.072
rs7322347A-rs6313A	405	0,26	65.88±16.40	
rs7322347A-rs6313G	277	0,18	64.99±15.14	
rs7322347T-rs6313A	270	0,17	67.83±16.60	
rs7322347T-rs6311C	590	0,38	67.07±16.48	0.074
rs7322347A-rs6311T	403	0,26	65.92±16.42	
rs7322347A-rs6311C	279	0,18	64.94±15.10	
rs7322347T-rs6311T	272	0,18	67.74±16.66	

Significant p value is indicated by bold, italics.

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Discussion

In this study, we examined possible contribution of 55 SNPs to aggressive tendencies measured by the BPAQ in the general adult Hungarian population [33,34]. Only two of these SNPs showed association reaching nominal significance, and merely rs7322347 of the *HTR2A* gene retained significant effect after Bonferroni adjustment.

These findings underpin the long-suspected key role of the serotonin neurotransmitter system in aggression and the related disorders [40,41]. There is convergent evidence that low or impaired serotonergic function underlies aggression and impulsivity [42–44]. As within the central nervous system (CNS) serotonin is synthesized solely in neurons of the raphe nuclei innervating virtually the entire neuraxis, this neurotransmitter is believed to exert a global effect on the brain with a holistically general role, even though local specialized functions are achieved by a variety of receptors [45,46]. It has been proposed that the principal role of serotonin might be the withdrawal from dangerous and aversive situations; consequently, serotonergic hypofunction could lead to impaired avoidance of undesirable stimuli, which in turn could provoke aggressive responses [47]. Strong experimental evidence supports this concept. The inverse correlation of aggression, impulsivity and antisocial behavior with serotonin metabolite 5-hydroxyindoleacetic acid levels in the cerebrospinal fluid was already known decades ago [40,48–50]. Later on, numerous studies confirmed these early observations regarding the relationship between dysregulation of the serotonergic system and aggressive-impulsive traits both in human and animals [51–54]. Behavioral functions of serotonin and also the effect of drugs influencing serotonergic mechanisms shows a marked conservation even between evolutionarily remote species [55]. This enables utilization of animal models for different types of aggression, e.g. affective (or defensive) and predatory (referred to as impulsive and premediated in humans, respectively) [56]. Data especially on rodents and felines provide valuable insight into underlying molecular mechanisms, shedding light for example on the interplay of proinflammatory cytokines and serotonin receptors in defensive rage and also on differential modulation

of aggression by distinct types of serotonin receptors [57–59]. Administration of selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine, citalopram or paroxetine usually reduces aggression [60–69], though contradictory results have also been reported, especially in juvenile humans and animals [70–72]. Reduced levels of serotonin caused by depletion of its precursor tryptophan have been linked to aggressive behavior [73–76], and disrupted function of enzymes involved in serotonin metabolism, such as tryptophan hydroxylase or monoamine oxidase, are also related to aggressive traits [77–79]. Observations on the link between life history of aggression and platelet serotonin content as well as platelet serotonin receptor and transporter binding further underpin the constantly growing body of evidence referring to a close relationship between the serotonergic system and aggression [80–82].

Serotonin (5-hydroxytryptamine) receptor 2a, encoded by the gene *HTR2A*, is a G-protein coupled excitatory receptor exerting its influence through the activation of secondary messengers phospholipase C and D [83]. Among others it is expressed in high levels on pyramidal cells of the prefrontal cortex, where it is ideally positioned to modulate both cognitive functions such as working memory or executive control and also emotions through dynamic interactions with the amygdala [84,85]. Serotonin receptors are also distributed along the midbrain periaqueductal grey (PAG) and the hypothalamus [56], brain areas that both have a direct connection with the prefrontal cortex and amygdala and long have been proved to control components of aggression including vocalization [86,87]. In accordance, mice with inherited aberrations in development and function of serotonergic neurons in the CNS exhibit increased levels of aggression which can be ameliorated by SSRIs [88]. Functional polymorphisms of the *HTR2A* gene are thus expected to influence neuronal networks regulating all the above mentioned features, providing a physiological basis for associations between *HTR2A* genetic variations and different mental states. During the last decade, several groups investigated SNPs of the *HTR2A* gene in connection with psychiatric and personality disorders [89–95]. Noteworthy observations have been made with regard to a number of variations located mainly in the promoter or the coding region; however, though scarce, literature data also indicate that intronic variant rs7322347 might as well be of interest from behavioral aspects, as it showed marked association with the combined subtype of childhood attention-deficit hyperactivity disorder (ADHD) and with suicide attempt in females subjected to physical assault in younger age [96,97].

Interestingly, according to our findings the missense polymorphism rs6314 is in complete LD with rs7322347, and the haplotype defined by these two SNPs has a similarly high impact on aggression levels as rs7322347 alone, despite the great difference observed between their MAFs. This might reflect that a complex background lies behind the robust association observed in the case of rs7322347, possibly consisting of several minor factors. Intrinsically, marked physiological effect of a single genetic variation with a MAF nearing 50% is generally improbable, simply based on the consideration that the spread of newly arisen alleles with functional relevance is most probably controlled by either positive or negative selection, hardly allowing quasi equal allele frequencies to evolve. Although in the present case it is plausible that a fine evolutionary balance has been struck between avoiding fights thus injury and gaining access to better resources, it cannot be excluded that other, linked polymorphic loci also contribute to the overall observed effect, even though similarly high D' values as seen for rs6314 are unlikely for any such sites. Indeed, full linkage disequilibrium can only be expected when no crossing over event between the linked loci has yet occurred, which is mainly characteristic to the situation when at least one of the polymorphic sites is evolutionarily young. It is, though, noteworthy that immensely strong LD has been identified elsewhere within the *HTR2A* gene as well (between rs6311 and rs6313), both in this study and before [98–100].

As the linked polymorphism rs6314 causes a histidine to tyrosine change, thus the substitution of a basic amino acid residue to an uncharged one, this SNP could potentially affect both

protein structure and function [101]. *In vitro* studies implicate that its rare allele causes slower receptor response, decreased activation levels of phospholipases C and D, reduced calcium ion mobilization and thus a general hypofunctioning of the whole signaling cascade [102,103]. Recent findings imply that rs6314 also interferes with adequate splicing of pre-mRNA, with defective transcript forms triggering the RNA surveillance machinery, leading to a lower expression of the variant allele both on RNA and protein level [104].

Another possible explanation for the observed relationship between rs7322347 and aggression lies in gene regulation. Over the last few years, growing number of disease-associating polymorphisms in intergenic and intronic regions identified especially in GWA studies, combined with the fact that the more complex an organism is, the larger proportion of its genome will consist of non-coding sequences, has drawn attention of the scientific community towards the significance of expression regulation. By now, light has been thrown on several molecular mechanisms modifying gene expression, mostly with the involvement of non-coding sequences. Polymorphic intronic sites can lead to splicing efficiency bias or modified pre-mRNA stability, or they might affect long-distance gene regulation, for instance as part of an enhancer or an insulator, or through the RNAi pathway. In fact, according to the miRBase registry, T allele of rs7322347 disrupts a potential miRNA binding site [105,106]. It has recently been demonstrated by our group that differences in transcriptional regulation caused by a miRNA binding site disrupting SNP can indeed contribute to elevated aggression levels [107]. Though functional relevance of intronic miRNA target sites is obscure, recent evidence suggests that at least in plants miRNA interaction with intronic sequences is indeed involved in gene regulation processes [108]. In addition, expression quantitative trait loci (eQTL) data (http://genenetwork.nl/bloodeqtlbrowser) indicate that minor allele (A) of rs7322347 negatively affects (Z-score: -8.06) transcription of the *ESD* gene located 34 kb downstream of *HTR2A* [109]. *ESD* encodes esterase D, a poorly characterized protein with a suggested role in the recycling of sialic acids and also in detoxification [110,111]. Thus, it would be intriguing to explore possible interaction of *ESD* with neurobiological aspects and behavioral traits, especially as it is expressed all across the brain in considerable amounts according to AceView and TiGER databases [112,113].

In the present report, we demonstrate a robust contribution of the rs7322347 variation within the gene encoding serotonin receptor 2a to aggressive traits. As the study was conducted on a large sample of 887 normal individuals and the effect of this polymorphism was strong enough to endure Bonferroni correction for multiple tests, it can be assumed that the observed association has a substantive biological basis. This might provide us with a better insight into the driving forces underlying aggression, hopefully facilitating early identification of individuals at risk, hereby also improving prevention of negative consequences derived from aggressive manifestations. Nevertheless, care must be taken not to overestimate the impact of these findings. Psychological and behavioral processes are complex traits comprising a not at all negligible environmental component, and deciphering all gene-environment (G×E) as well as epistatic interactions is more than challenging. There is far more to mental states than simply biochemical processes; thus, even though anatomical structure of the brain, neurophysiological functioning and gene expression regulation mechanisms are essentially identical in all human beings, socialization and culture also largely influences our acts and behavior [114,115]. Presumably, the role of genes in terms of human behavior is neither less nor more than establishing a reaction spectrum; within the available range, however, former experiences, belief-systems and social atmosphere are supposed to serve as key determinants of the actual behavior [46].

In conclusion, this study adds on the growing evidence that the serotoninergic system greatly influences aggressive tendencies. To our best knowledge, this is the first report demonstrating a direct relationship between the *HTR2A* gene and aggression. However, confirmation of the present findings by independent replication would inevitably be necessary before drawing

any further conclusions from these results. Functional studies should also be performed in order to explore the exact biochemical background of the association described, and to elicit possible contribution of rs7322347 to psychiatric and personality disorders. By no means forgetting about the significance of environmental exposure, our findings will hopefully provide help to elucidate the genetic basis behind increased predisposition to aggression.

Author Contributions

Conceived and designed the experiments: ZR MSS. Performed the experiments: ZE AS. Analyzed the data: TN ZN. Wrote the paper: ZB ZR MSS.

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