

The influence of rs53576 and rs2254298 oxytocin receptor gene polymorphisms on plasma oxytocin levels and measures of empathy

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Abstract: Oxytocin achieves its effects after binding the oxytocin receptor (OXTR). Oxytocin plays an important role in empathy. The aim of this study was to examine the influence of two single nucleotide polymorphisms (SNPs) of the *OXTR* gene (rs53576 and rs2254298) on empathy measures and plasma oxytocin levels. Seventy-four university students were screened for the *OXTR* rs53576 and rs2254298 SNPs using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The level of oxytocin in the plasma was measured by the enzyme linked immunosorbent assay (ELISA). All subjects were assessed with the empathy quotient (EQ) questionnaire and Reading the Mind in the Eyes Test (RMET). Subjects carrying the rs53576 GG genotype had a higher EQ score, compared to GA/AA genotype carriers. By gender analysis, significance was reached only in females. Considering the influence of both examined polymorphisms on empathy, participants with the GGGG diplotype had a higher EQ in comparison with GAGG/AAGG carriers. These hormone effects were not correlated with plasma oxytocin levels. This is the first study implying that higher empathy in rs53576 GG genotype carriers may not depend on plasma oxytocin levels, but possibly on the number and function of OXTRs in the brain.

Keywords: empathy; oxytocin; oxytocin receptor; rs53576 SNP; rs2254298 SNP

INTRODUCTION

Oxytocin is a nonapeptide produced as a prohormone in the supraoptic and paraventricular nuclei of the hypothalamus, from where it is transported by axonal transport to the posterior pituitary gland. The inactive precursor is hydrolyzed into smaller fragments, one of which is neurophysin I. Peptidyl glycine α -amidating monooxygenase (PAM) enables the release of active oxytocin into the circulation [1]. Oxytocin achieves its effects after binding to the oxytocin receptor (OXTR), a polypeptide consisting of 389 amino acids that belongs to the class I G-protein-coupled receptor family. The OXTR is synthesized in the uterus, mammary gland, placenta, ovaries, testes, heart muscle and kidneys. In the brain, this receptor is expressed

in the hypothalamus, amygdala and hippocampus. Oxytocin plays an important physiological role in childbirth by initiating the contraction of the uterus during labor and by facilitating the lactation process. However, a large number of studies point to the additional effects of this hormone, including the role in establishing mother-infant relationships, partner relationships and in prosocial behavior [2]. In recent years, authors have attached great importance to the examination of the role of oxytocin in empathy, defined as the ability to understand (cognitive empathy) and experience (affective or emotional empathy) other people's emotions [3]. Both of these types of empathy have a hereditary basis, while environmental factors influence the development of only cognitive, but not of emotional empathy [4]. The study of the biological

basis of empathy and biomarkers that could play an important role in this process is the subject of interest of an increasing number of authors. There are some data that genetic variations in the *OXTR* gene could influence receptor function, the binding of oxytocin and the effects of the hormone. The *OXTR* gene is found on chromosome 3p25 and it consists of 4 exons and 3 introns [5]. The two most commonly studied single nucleotide polymorphisms (SNPs) are rs53576 (6930G>A) and rs2254298 (9073G>A) located in intron 3 of the *OXTR* gene. Previous studies indicate the association of both SNPs with autism [6,7], unipolar depression and separation anxiety [8]. Studies that examined the effect of the *OXTR* rs53576 SNP on empathy provided contradictory results [9,10]. Feldman et al. [11] showed that the *OXTR* rs2254298 risk allele was associated with lower plasma oxytocin levels and was related to less parental touch, indicating that future studies should include the rs53576 SNP and its effects. However, to the authors' knowledge, no data is available on how the diplotypes of both SNPs (*OXTR* rs53576 and rs2254298) affect empathy, or whether the influence of both SNPs on empathy depends on plasma oxytocin levels. The aim of this study was to examine the distribution of the rs53576 and rs2254298 genetic variations in the *OXTR* gene in university students, and to examine the impact of these SNPs on empathy measures and plasma oxytocin levels.

MATERIALS AND METHODS

Ethics statement

All participants voluntarily agreed and signed an informed consent to participate in the research. The Ethical Committee of the Medical Faculty, University of Niš, provided consent for conducting this study. There was no compensation for participation in the study, and the participants were treated in accordance with the Helsinki Declaration.

Participants

The study involved 74 students at the Medical Faculty, University of Niš, Serbia. The participants included 32 men ($M_{\text{age}}=21.06$, $SD=1.26$) and 42 women ($M_{\text{age}}=21.05$, $SD=1.51$), without self-reported histories of chronic

disease, psychiatric disorders, drug addiction, pregnancy (for females), and none were parents. The research was conducted in the Laboratory for Functional Genomics and Proteomics at the Medical Faculty, University of Niš, Serbia.

Blood sample preparation and genotyping

Blood samples were taken between 9:00 and 10:00 am. From all blood samples (with EDTA as the anticoagulant), 200 μL of blood were separated and used for DNA isolation. The blood samples were then centrifuged at 2000 $\times g$ for 10 min at +4°C, after which the plasma was separated and frozen at -80°C.

The isolation of DNA was performed using a commercial kit for DNA isolation (QIAamp DNA Blood Mini Kit, Qiagen GmbH, Hilden, Germany). We examined the following SNPs in the *OXTR* gene: rs53576 (6930G>A) and rs2254298 (9073G>A), by the PCR-RFLP method. The fragments (340 base pairs (bp) (rs53576) and 307 bp (rs2254298)) were amplified using forward and reverse primers (F: 5'-GCCAC-CATGCTCTCCACATC-3' and R: 5'-GCTGGACT-CAGGAGGAATAGGGAC-3' for the rs53576 SNP and F: 5'-TGAAAGCAGAGGTTGTGTGGACAGG-3' and R: 5'-AACGCCACCCCAGTTTCTTC-3' for the rs2254298 SNP). The PCR reaction mixture in a volume of 25 μL contained: 12.5 μL of KAPA 2G Fast HS Ready-Mix PCR kit solution (KAPA Biosystems, Germany), 0.5 μL of primer (10 pmol/ μL) (Fermentas GmbH, St. Leon-Rot, Germany) and 20 ng of DNA. The PCR conditions were as follows: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, elongation at 72°C for 15 s and termination at 72°C for 30 s. The amplified PCR products were visualized under UV light after agarose gel (2%) electrophoresis.

The PCR products were cut into smaller fragments by BamHI (rs53576 SNP) and BsrI (rs2254298 SNP) restriction enzymes (Fermentas GmbH, St. Leon-Rot, Germany) at 37°C overnight and analyzed by vertical polyacrylamide gel (8%) electrophoresis and interpreted according to the obtained restriction fragments as follows: rs53576 SNP: the G allele was detected as one fragment (340 bp), while the polymorphic A allele was shown as two fragments of 230 and 110 bp; rs2254298 SNP: the G allele was detected as four fragments of

164, 101, 34 and 8 bp, while the polymorphic A allele was confirmed by the presence of three fragments on the gel (164, 135 and 8 bp).

Measurement of oxytocin concentration

Plasma oxytocin concentration was measured by ELISA (ADI-900-153A, Enzo Life Sciences, Lausanne, Switzerland) according to the manufacturer's instructions. Samples were diluted 1:8 and assayed without extraction. Oxytocin concentration was expressed as pg/mL. The minimum detectable dose (MDD) was 15.6 pg/ml. Intra- and interassay coefficients were <10%.

Empathy Quotient (EQ) questionnaire

To measure empathy, each participant filled out the Serbian version of the self-report questionnaire, Empathy Quotient (EQ) [12]. The EQ questionnaire is based on a definition of empathy that includes all empathy components (cognition and affect). Participants rated each item from 1 (strongly agree) to 4 (strongly disagree). The results ranged from 0 to 80, with a higher score indicating a higher level of empathy.

Reading the Mind in the Eyes Test (RMET)

The RMET test is a behavioral measure of empathic accuracy which assesses individual differences in the ability to infer the affective mental states of strangers [13]. Participants were shown 36 black-and-white photographs where only the eye region of the face of different individuals was shown. The individual in each photograph displays a particular emotional or cognitive state. Each photo is paired with four affective-state adjectives as response options (e.g., "terrified", "upset", "arrogant" and "annoyed"). Participants select the adjective that according to their judgment best describes what the individual in the photo is feeling or thinking. The result is expressed as a total score, where the minimum score is 0 and the maximum score is 36.

Statistical analysis

The frequency of alleles and genotypes in the males and females was analyzed and compared using the χ^2 test or Fisher's exact test; possible deviation from the

expected values of the Hardy-Weinberg equilibrium test for both studied polymorphisms was determined. The plasma oxytocin level, EQ score and RMET score were expressed as the mean (M)±standard deviation (SD). There was normality-assumption for all studied parameters. Statistically significant differences in values between males and females, as well as between different genotypes or diplotypes, were determined by the t-test for two independent samples or by analysis of variance (ANOVA). Pearson's bivariate correlation analysis was performed to examine associations between plasma oxytocin levels and EQ. $P<0.05$ value was considered statistically significant. Statistical analysis was conducted using the SPSS software package version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Genotype and allele frequencies of the rs53576 OXTR gene polymorphism

Genotype frequencies of the OXTR rs53576 and rs2254298 gene polymorphisms did not deviate from the normal distribution of the Hardy-Weinberg equilibrium ($p>0.05$). The results shown in Table 1 indicate that the rs53576 GG (wild-type) genotype was present in 37 (50%) subjects, while only 4 (5.4%) subjects had the AA genotype. Thirty-three (44.6%) subjects were heterozygous. The genotype frequency distributions of the rs53576 SNP in males were not significantly different from those in females ($\chi^2=1.985$, $df=2$, $p=0.371$). The difference was not detected in the frequency of the A allele in males compared to females ($\chi^2=1.470$, $df=1$, $p=0.225$; Fisher's exact test $p=0.267$).

Table 1. Genotype and allele frequencies of the rs53576 OXTR gene polymorphism.

Genotype (rs53576)	All* n=74	Male* n=32	Female* n=42	Male vs Female p value
GG	37 (50.0%)	13 (40.6%)	24 (57.1%)	0.371
GA	33 (44.6%)	17 (53.1%)	16 (38.1%)	
AA	4 (5.4%)	2 (6.3%)	2 (4.8%)	
Allele	All 2n=148	Male 2n=64	Female 2n=84	p value
G	107 (72.3%)	43 (67.2%)	64 (76.2%)	0.225
A	41 (27.7%)	21 (32.8%)	20 (23.8%)	

*Hardy-Weinberg equilibrium $p>0.05$

Genotype and allele frequencies of the rs2254298 OXTR gene polymorphism

The results of rs2254298 SNP testing showed that the GG (wild-type) genotype was present in 65 (87.8%) subjects, GA in 8 (10.8%) subjects, and AA only in 1 subject (1.4%). Neither the distribution of genotypes nor the A allele frequency of the rs2254298 polymorphism in males showed a statistically significant difference compared to females ($\chi^2=0.919$, $df=2$, $p=0.632$; $\chi^2=0.766$, $df=1$, $p=0.381$; Fisher's Exact test $p=0.515$ respectively; Table 2).

Table 2. Genotype and allele frequencies of the rs2254298 OXTR gene polymorphism.

Genotype (rs2254298)	All* n=74	Male* n=32	Female* n=42	Male vs Female p value
GG	65 (87.8%)	29 (90.6%)	36 (85.7%)	0.919
GA	8 (10.8%)	3 (9.4%)	5 (11.9%)	
AA	1 (1.4%)	0 (0.0%)	1 (2.4%)	
Allele	All 2n=148	Male 2n=64	Female 2n=84	p value
G	138 (93.2%)	61 (95.3%)	77 (91.7%)	0.515
A	10 (6.8%)	3 (4.7%)	7 (8.3%)	

*Hardy-Weinberg equilibrium $p>0.05$

The influence of gender on the EQ score, RMET score and plasma oxytocin levels

The results for the EQ score, RMET score and plasma oxytocin levels did not show a statistically significant difference between the tested parameters in male and female participants (EQ, $p=0.115$; RMET, $p=0.573$; oxytocin, $p=0.502$).

The influence of the rs53576 and rs2254298 OXTR SNPs on the tested parameters

Bearing in mind that the rs53576 AA genotype was detected in only 4 subjects and the rs2254298 AA genotype in only 1 subject, we compared the EQ scores, RMET scores and oxytocin levels between GG genotype carriers (non A allele carriers) and GA/AA genotype carriers (A allele carriers) for both examined polymorphisms. Subjects carrying the rs53576 GG genotype had a significantly higher EQ score compared to carriers of the polymorphic A allele ($t=3.234$, $df=72$, $p=0.002$). Analysis of RMET scores and oxytocin levels revealed no significant differences between different genotypes

of the rs53576 SNP ($p=0.753$; $p=0.937$ respectively). The results of the study of the rs2254298 SNP effect on the tested parameters showed that there were no significant differences between GG genotype carriers and GA/AA genotype carriers in the EQ score ($p=0.803$), RMET score ($p=0.581$) and oxytocin levels ($p=0.207$, Table 3).

Table 3. The influence of rs53576 and rs2254298 OXTR gene polymorphisms on the EQ score, RMET score and plasma oxytocin levels.

Measure	Genotypes			
	rs53576		rs2254298	
	GG (n=37) M (SD)	GA/AA (n=37) M (SD)	GG (n=65) M (SD)	GA/AA (n=9) M (SD)
EQ	46.86 (7.99) *	41.27 (6.84)	44.15 (8.08)	43.44 (6.82)
RMET	25.02 (3.45)	24.78 (3.15)	24.98 (3.27)	24.33 (3.50)
Oxytocin (pg/ml)	354.85 (131.33)	352.51 (122.19)	360.60 (119.35)	303.73 (166.35)

* $p=0.002$ GG vs rs53576 GA/AA

The influence of the OXTR SNPs on the tested parameters by gender

Taking into account the gender of the participants, the results of the study of the rs53576 SNP influence on the tested parameters showed that females carrying the GG genotype had a higher EQ score than female GA/AA genotype carriers ($t=3.101$, $df=40$, $p=0.004$). There was no difference in EQ score, RMET score or oxytocin levels between female and male participants, whether GG genotype ($p=0.120$, $p=0.670$, $p=0.304$) or GA/AA genotype carriers were analyzed ($p=0.995$, $p=0.768$, $p=0.914$ respectively, Table 4).

Table 4. The influence of rs53576 OXTR gene polymorphism on the EQ score, RMET score and plasma oxytocin levels by gender.

Measure	Genotypes (rs53576 polymorphism)			
	GG		GA/AA	
	Male (n=13) M (SD)	Female (n=24) M (SD)	Male (n=19) M (SD)	Female (n=18) M (SD)
EQ	44.07 (8.04)	48.37 (7.70) *	41.26 (7.06)	41.27 (6.81)
RMET	24.69 (3.14)	25.20 (3.65)	24.63 (2.89)	24.94 (3.48)
Oxytocin (pg/ml)	324.26 (142.15)	371.41 (125.05)	354.66 (133.85)	350.25 (112.43)

* $p=0.004$ vs Female GA/AA genotype carriers

The influence of gender on the EQ score ($p=0.153$), RMET score ($p=0.210$) and oxytocin levels (0.747) was not significant in rs2254298 GG genotype carriers. A significant difference was not detected in the EQ score ($p=0.484$), RMET score ($p=0.108$) and oxytocin levels ($p=0.301$) for rs2254298 GA/AA genotype carriers either.

The influence of diplotypes of OXTR SNPs on the tested parameters

As regards diplotypes, there were statistically significant differences in the EQ score ($F=3.626$, $p=0.017$, $\eta^2=0.135$), while the RMET score and plasma oxytocin levels did not show significant differences between the groups ($F=1.464$, $df=3$, $p=0.232$; $F=1.275$, $df=3$, $p=0.290$ respectively). A *post hoc* analysis showed that GGGG diplotype carriers (non A allele carriers for both polymorphisms) had a higher EQ score than GAGG/AAGG diplotype carriers (non A allele carriers for rs2254298 polymorphism, mean difference MD=5.996, $p=0.012$, Table 5).

Table 5. The influence of diplotypes on the EQ score, RMET score and plasma oxytocin levels.

Measure	Diplotypes			
	GGGG (n=31) M (SD)	GAGG/ AAGG (n=34) M (SD)	GGGA/ GGAA (n=6) M (SD)	GAGA (n=3) M (SD)
EQ*	47.29 (8.27)	41.29 (6.86)	44.66 (6.50)	41.00 (8.18)
RMET	25.41 (3.37)	24.58 (3.18)	23.00 (3.40)	27.00 (2.00)
Oxytocin (pg/ml)	356.40 (128.93)	364.42 (111.75)	346.83 (156.06)	217.53 (181.88)

* $p = 0.017$ between groups, $p=0.012$ GGGG vs GAGG/AAGG

Correlations between the tested parameters

Correlation analyses between the EQ scores and oxytocin levels did not reveal significant associations in either the total sample of participants ($r=0.12$, $p=0.922$) or within the groups of rs53576 GG genotype carriers ($r=0.164$, $p=0.332$), of female rs53576 GG genotype carriers ($r=0.020$, $p=0.931$), and of GGGG diplotype carriers of the examined polymorphisms ($r=0.265$, $p=0.149$).

DISCUSSION

Although two polymorphisms (rs53576 and rs2254298) of the OXTR gene are often studied, to the authors' best knowledge and based on a literature search, this is the first study to examine the impact of the two polymorphisms on both empathy measures and oxytocin levels. In the present study, subjects carrying the GG genotype of OXTR rs53576 showed a significantly higher EQ score than A allele carriers. By gender analysis, significance was reached only in females. No difference was detected in the RMET score, as a measure of cognitive empathy, between different genotypes. The results of a meta-analysis of 24 studies [14] showed association between rs53576 GG genotype carriers and various forms of prosocial behavior. However, the results of the studies examining the effect of these SNPs on empathy are contradictory. Some studies did not find the effect of rs53576 SNP on empathy [10,15] when using self-rated Interpersonal Reactivity Index (IRI) scores. The results of other studies showed that subjects with the GG genotype exhibited higher trait empathy [9,16], the emotional component of empathy [4,16,17] and cognitive empathy [9], than carriers of the A allele; however, cognitive empathy was not found to be higher in wild-type carriers in the study of Uzefovsky et al. [4], with which our findings are in agreement. Our study did not show a difference in EQ and RMET scores between GG genotype carriers and A allele carriers of the OXTR rs2254298 SNP. Conversely, the results of two studies [10,15] showed higher cognitive empathy in rs2254298 GG genotype carriers compared to A allele carriers. Differences in results could stem from the different questionnaires and tests used, as the abovementioned studies used the IRI, while we used the EQ questionnaire and RMET. Our results were not consistent with the results of the study in which the RMET score was used [9]. It should be kept in mind that the distribution of polymorphisms varies depending on the sample size, genetic heterogeneity and ethnicity. In the study carried out by Rodrigues et al. [9], subjects had different ethnic origins, while our study included only Caucasian subjects. On the other hand, as an epigenetic factor, the cultural factor in different ethnic groups could affect behavior and cognitive empathy.

Plasma oxytocin levels of the subjects in this study carrying rs53576 or rs2254298 GG genotypes did not

show statistically significant differences compared to the values detected in the carriers of GA/AA genotypes of both studied SNPs. The results of our study showed that there was no correlation between the EQ score and plasma oxytocin levels in any of the examined groups. In the study by Perry et al. [18], where subjects were administered intranasal oxytocin, in people with an EQ over 40 (highly empathic) this application stimulated the establishment of close interpersonal relationships, while the effect of oxytocin on people with low empathy was absent. The study by Hurlemann et al. [19] showed that the administration of oxytocin enhances emotional but not cognitive empathy. It was also shown that in autistic individuals carrying the G allele of *OXTR* rs53576 and *OXTR* rs2254298 polymorphisms, the application of oxytocin was more effective compared to carriers of a polymorphic allele [20].

By examining the effect of the diplotypes of both of the tested polymorphisms on empathy and oxytocin levels, a significant difference in EQ scores between groups emerged, where GGGG diplotype carriers (non A allele carriers for both polymorphisms) had a significantly higher EQ score than GAGG/AAGG diplotype carriers (non A allele carriers for rs2254298 polymorphism), without a difference in plasma oxytocin levels.

Given the above, it is possible that the higher EQ score observed in this study in rs53576 GG genotype carriers (significantly in females), as well as in non A allele carriers for both polymorphisms (GGGG diplotype), was the result of hormone action that was not effected by oxytocin levels but by the number and function of the expressed receptors in the brain. Estrogen has been known to play an important role in *OXTR* expression [21]. We speculate that rs53576 GG genotype carriers, especially females, have higher expression of *OXTR* and a more efficient overall response to oxytocin binding in comparison to carriers of the A allele. However, this needs to be studied in more detail. Furthermore, *OXTR* rs53576 SNP could possibly influence the function of empathy-related brain areas, as neuroradiological methods used in the studies by Tost et al. [22] and Wang et al. [23] have shown that rs53576 G allele carriers have a larger amygdala and hypothalamus gray-matter volume and better functional association with the prefrontal cortex. Further studies of other variations in the *OXTR* gene in a larger

sample, as well as examination of the effects of SNP-SNP interactions on empathy, which we carried out in individuals for different SNPs [24,25], will clarify individual differences in the effects of this hormone.

The limitations of our study are related to the relatively small sample size. Plasma oxytocin levels show large daily variations, which we tried to eliminate by taking all the samples at the same time of day. Finally, even though our study was focused on oxytocin-related genetic effects on empathy, further investigation of this topic could include the effects of early environmental experiences as well.

In conclusion, higher empathy (EQ) in rs53576 GG genotype carriers and GGGG diplotype carriers in comparison with A allele carriers and GAGG/AAGG carriers, was not correlated with plasma oxytocin levels. These findings suggest that hormone effects might be due to an increased number and/or better functioning of the *OXTRs* in the brain in non A allele carriers. However, this requires further and more detailed investigation in a larger sample of subjects.

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Author contributions: Jelena Bašić and Vuk Milošević designed the experiment, performed the experiment, analyzed the data, contributed the reagents/materials/analysis tools, wrote the paper, prepared the tables and reviewed the drafts of the paper. Miloš Stanković designed the experiment, performed the experiment, analyzed the data, wrote the paper and reviewed the drafts of the paper. Tatjana Jevtović-Stoimenov designed the experiment, contributed the reagents/materials/analysis tools, performed the experiment and wrote the paper. Tatjana Cvetković contributed the reagents/materials/analysis tools, performed the experiment and wrote the paper. Milena Despotović performed the experiment, prepared the tables and wrote the paper. Dušica Pavlović designed the experiment and reviewed the drafts of the paper.

Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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