

## ASSOCIATION ANALYSIS OF *MAOA* AND *SLC6A4* GENE VARIATION IN SOUTH EAST EUROPEAN WAR RELATED POSTTRAUMATIC STRESS DISORDER

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### SUMMARY

**Background:** The aim of this study is to investigate the association of gene variations of the monoamine oxidase A (*MAOA*) and the serotonin transporter solute carrier family 6 member 4 (*SLC6A4*) gene with posttraumatic stress disorder (PTSD) severity and coping strategies in patients with war related PTSD.

**Subjects and methods:** The study included 747 individuals who had experienced war trauma in the South Eastern Europe conflicts between 1991 and 1999. Genotyping of the *MAOA* VNTR and *SLC6A4* tandem repeat polymorphism in combination with rs25531 was done in 719 participants: 232 females and 487 males. Among them, 369 have had current or lifetime PTSD and 350 have had no PTSD symptoms. For psychometric approach we used the Clinician Adminstrated PTSD Scale (CAPS), the Brief Symptom Inventory (BSI), the adapted Hoffman-Lazarus Coping scale and a basic socio-demographic data questionnaire.

**Results:** There were no significant intergroup (PTSD versus non PTSD) differences in the genotype distribution of *MAOA* and *SLC6A4* gene polymorphisms. The primary finding of our study was that the *MAOA* short allele (*MAOA-S*) was nominally significantly associated with the severity of PTSD symptoms in the total subgroup of participants with lifetime PTSD; males for symptoms of hyperarousal and females with symptoms of re-experience and hyperarousal. In our research the male subsample with current PTSD and *MAOA-S* genotype had nominally significantly higher scores for some positive coping strategies compared to those carrying the long allele genotype (*MAOA-L*). There was no significant association between the severity of PTSD symptoms, BSI phenotype, coping scores and the *SLC6A4* genotype.

**Conclusion:** The present results support the notion that *MAOA* VNTR gene variation modulates development and recovery of posttraumatic stress disorder in a war traumatised population, but did not support a connection between *SLC6A4* gene variations and war related PTSD.

**Key words:** *MAOA* - *SLC6A4* - posttraumatic stress disorder - single nucleotide polymorphisms - neurogenetics

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### INTRODUCTION

Wars at the end of the nineties of the 20<sup>th</sup> century in the region of ex-Yugoslavian countries brought all the cruelty of war vivid again on European ground. These populations were exposed to death, threatened death, actual or threatened serious injury, or actual or threate-

ned sexual violence, which fulfil Criterion A – stressor for development of PTSD (DSM-5) (American Psychiatric Association 2013). Up to 35% of the population exposed to traumatic war experiences developed PTSD symptoms (Priebe et al. 2010, Džubur Kulenović et al. 2016). One of the key questions in trauma related research is why some individuals exposed to a traumatic

event do develop PTSD and others do not (DiGangi et al. 2013), and what is the influence of genetic predisposition and gene-environment interaction in the etiology of PTSD (Domschke 2012). A twin study estimates the heritability of PTSD within the range from 23% in male twins (Wolf et al. 2014) to 72% in female twins (Sartor et al. 2011), and also documented that genetic susceptibilities for PTSD are shared with other mental disorders, particularly major depression (Wolf et al. 2010, Sartor et al. 2011, Sartor et al. 2012). In the last large genome-wide association study (GWAS), the molecular genetics heritability results are consistent with twin studies, and suggest that PTSD heritability among females is higher than among males (Duncan et al. 2017). Genetic studies have identified variations within single genes as potential risk factors for PTSD, and so far more than 25 candidate genes have been investigated (Ryan et al. 2016). Several recent reviews highlight that in the majority of these studies the candidate genes for PTSD were selected based on their known or assumed involvement in the etiology and pathogenesis of PTSD (Domschke 2012, Ryan et al. 2016, Smoller 2016). Gene-environment-interaction (GxE) studies indicated that environmental exposures such as childhood maltreatment, poverty, and low social support increase the risk of developing PTSD among trauma-exposed individuals (Ryan et al. 2016). Recent studies suggested methylation as an epigenetic mechanism to influence gene regulation and to mediate adaptation to environmental influences and point out their particular relevance for the pathogenesis of PTSD (Ziegler 2017, Domschke 2012).

Earlier studies showed correlations of the genetic variation in the monoamine oxidase A gene (MAOA) with panic disorder (Deckert et al. 1999), depression (Domschke et al. 2008), pathological grief in depression (Kersting et al. 2007), and aggressive and antisocial behaviour (Buades-Rotger & Gallardo-Pujol 2014). The MAOA gene codes the activity of the enzyme monoamine oxidase A which plays a key role in the catabolism of neurotransmitters, including dopamine, norepinephrine and serotonin. Expression of the enzyme correlates with a functional 30 bp variable number tandem repeat (VNTR) polymorphism which is located 1.2 kb upstream of the MAOA coding sequence. The two most common polymorphisms are the 3- and 4-repeat alleles, which are assumed to be associated with decreased and increased transcriptional activity, respectively (Sabol et al. 1998). The functionally more active longer (L) alleles (3a, 4 and 5) were found to be significantly associated with panic disorder in the female subgroups of German and Italian patients (Deckert et al. 1999) which was confirmed in a meta-analytic study conducted by Reif et al. (2012).

Located within the promoter region of the serotonin transporter solute carrier family 6 member 4 (SLC6A4) gene, the gene length polymorphism 5-HTTLPR (serotonin-transporter-6 disorders). However, findings have been inconsistent regarding the nature of the relation-

ship between the 5-HTTLPR, childhood maltreatment, and anxiety-related traits, with both the short (S) allele and the long (L) allele having been associated with a risk for developing depression (Caspi et al. 2003, Carli et al. 2011, Kaufman et al. 2004, Laucht et al. 2009), panic disorder (Choe et al. 2013), posttraumatic stress disorder (Grabe et al. 2009, Xie et al. 2009, Xie et al. 2012), social anxiety disorder (Reinelt et al. 2013), and in concern with childhood adversity. Several studies were unable to discern an interactive effect of the 5-HTTLPR and history of abuse on anxiety related phenotypes (Blaya et al. 2010, Cividanes et al. 2014, Zavos et al. 2012), mirroring the failure to identify a strong effect of SLC6A4 gene variations on categorical anxiety phenotypes (Schumacher & Deckert 2010). The relationship between environmental adversity and individual genetic vulnerability may be further moderated by dynamic processes as a result of positive influences counteracting a GxE risk profile, i.e., elements of successful coping with adversity, thus promoting resilient functioning (Schiele 2016).

Considering the previous findings of the impact of MAOA and SLC6A4 gene variations on panic and depression disorders, the aim of this study was to analyze how the level of posttraumatic stress symptoms, coping strategies and severity of psychological symptoms in a population who survived war trauma is related to the VNTR polymorphisms in MAOA and the SLC6A4 promoter region.

## **SUBJECTS AND METHODS**

### **Subjects**

This study is a part of the South Eastern Europe (SEE) – PTSD study about “molecular mechanisms of posttraumatic stress disorder” which was supported by the DAAD (Deutscher Akademischer Austauschdienst). Methods regarding the process of recruitment, study design, diagnostic assessment, inclusion and exclusion criteria and EDTA blood collection of the SEE-PTSD study were described more detailed described by Džubur Kulenović et al. (2016). Participants (N=719) were recruited in the period from 2013 to 2015 in the five psychiatric centres located in countries whose population had experienced war-related trauma between 1991 and 1999: Zagreb in Croatia (1991-1992), Sarajevo, Tuzla and Mostar in Bosnia and Herzegovina (1992-1995), and Prishtina in Kosovo (1999-1999). The inclusion criteria were that participants were at least 16 years of age at the time of traumatization and not older than 65 years of age at time of recruitment. Exclusion criteria were: intellectual disability (MMSE<25), organic and brain trauma related disorders, epilepsy, psychotic disorders, addiction disorders except smoking, oncological illnesses, medication known to affect methylation status, e.g. valproic acid, 1<sup>st</sup> and 2<sup>nd</sup> degree relation to an already recruited person. Interviews were performed by medical personnel (psychiatrists, psychologists or psychiatric residents) after training of the principal investigators.

After recruitment altogether 719 participants (mean age 49.4±7.9; 487 males) were divided into three experimental groups. The first group consisted of 218 patients (mean age 50.1±6.7; 157 males) with current PTSD, the second comprised 151 participants with lifetime PTSD (mean age 49.5±8.2; 98 males) and finally the last group included 350 healthy volunteers with no diagnosable PTSD (mean age 48.8±8.5; 232 males).

### Ethical Votes

Ethical votes at the participating clinical centers were obtained between 2011 and 2013 on the basis of local translations of an information and consent form designed by the Würzburg center. All participants thus were informed and gave written informed consent according to the principles of the declaration of Helsinki (WMA 2013).

### Psychometric Instruments

Participants' presence or absence symptoms of PTSD in screening stage were assessed using the Structured Clinical Interview M.I.N.I. (Mini International Neuropsychiatric Interview). The Clinician Administered PTSD Scale (CAPS) (Blake et al. 1995) was used to make a categorical PTSD diagnosis and to assess the severity of symptom of PTSD. For the assessment of psychological symptoms we used the Brief Symptom Inventory (BSI) (Derogatis & Melisaratos 1983). The BSI is a self-report symptom scale composed of nine primary symptom dimensions and includes three global indices of distress (Global Severity Index, Positive Symptom Distress Index, and Positive Symptom Total), which measure the overall psychological distress level, the intensity of symptoms, and the number of self-reported symptoms. The reliability of the BSI for the present sample was high (Cronbach  $\alpha=0.987$ ). For the assessment of ways of coping the adopted Hoffman-Lazarus Coping Scale (Arcel & Ljubotina 1995) with eight subscales was used. The subscales are: social support, confrontation, distancing, self control, positive reappraisal, planned problem solving, escape-avoidance, accepting responsibility. The reliability of the Coping scale for the present sample was high (Cronbach  $\alpha=0.835$ ). Also, socio-demographic data were collected.

### Molecular analyses

Molecular analyses were performed in the Laboratory of Functional Genomics at the Department of Psychiatry Psychosomatics and Psychotherapy, Würzburg. Prior genotyping, genomic DNA was isolated from frozen venous EDTA-blood using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and stored until use at -80°C.

The best-described common genetic variation of the MAOA gene is a 30bp variable number of tandem repeat

(VNTR) polymorphism in the promoter region termed MAOA-uVNTR. Within this study MAOA-uVNTR genotypes (2,3,3.5,4, and 5), which represent the amount of repeats in the distinct samples were determined according to previously published protocols (Reif et al. 2013). Because of its X chromosomal location (Xp11.4-p11.3), males have only one allele, while females have two alleles. According to their influence on MAOA enzyme activity (Reif 2014) all 'short' S alleles (2 and 3 repeats) were grouped to the „MAOA-S" group (females with two S alleles and males with one S allele) and in statistical analysis compared to the 'long' L (3.5, 4 and 5 repeats) alleles labelled as "MAOA-L" group (considered females with two L alleles, females with one S and one L allele, and males with one L allele) according to a dominant model. Because of the evidence that "high active" long alleles confer risk to develop panic disorder in females (Reif, 2014), we performed also sex-specific analysis, for males and females, respectively.

Genes engaged in the serotonergic pathways are regarded as candidate genes due to the documented role of serotonin in the etiopathogenesis of mood disorders. One of the most often investigated genes of this group is the SLC6A4, located on the human chromosome 17q11.2. 17q12. Within this study we examined the most extensively investigated serotonin transporter gene linked polymorphic region 5-HTTLPR, which is characterized by an insertion or deletion of a 44bp sequence in combination with rs25531. The short allele (S) with the 44bp deletion was found to have a three times lower transcriptional activity than the long allele (L) including a 44-bp insertion (Grochans 2015, Schiele 2016, Lesch 1996). Genotyping of the 5-HTTLPR was done following previously published protocols (Schiele 2016).

MAOA genotyping was successfully done in 704 participants, and SLC6A4 genotyping was successfully done in 678 participants, reaching a genotyping call rate of at least 94%. All polymorphisms did not deviate from Hardy-Weinberg equilibrium ( $p>0.3$ ). The MAOA and SLC6A4 allele and genotype distributions in PTSD groups and control sample are given in Table 1.

### Statistical analyses

Statistics were performed using R v. 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria) and SPSS Statistics, version 22. The  $\chi^2$  test was used for case-control analyses and the Mann-Whitney U test was carried out for CAPS and BSI analyses, and coping strategies. A significance level was Bonferroni adjusted for 23 variants that were analyzed in total in the entire project ( $\alpha=0.002$ ) (Džubur Kulenović et al. 2016). Within the two groups of patients, i.e. individuals with lifetime or current PTSD, linear regression was carried out individually for analyses on the dimensional CAPS and BSI scores. For MAOA, carriers of no other than the S allele (homozygous females, hemizygous males) were tested

**Table 1.** Genotype distribution of the MAOA and SLC6A4 gene in the sample of war traumatized individuals (N=704)

MAOA genotype	PTSD group n (%)	Non-PTSD group n (%)
Total	361 (100.0)	343 (100.0)
L/L*	230 (63.7)	202 (158.9)
S/L	50 (13.9)	53 (15.5)
S/S**	81 (22.4)	88 (25.7)
	$\chi^2=0.998, p=0.318$	
Female	114 (31.6)	116 (33.8)
L/L*	55 (15.2)	49 (14.3)
S/L	50 (13.9)	53 (15.5)
S/S**	9 (2.5)	14 (4.1)
	$p=0.404$	
Male	247 (68.4)	227 (66.2)
L	175 (48.5)	153 (44.6)
S	72 (19.4)	74 (21.6)
	$p=0.476$	
<b>SLC6A4 genotype</b>		
Total (N=678)	347 (100.0)	331 (100.0)
L/L***	117 (17.2)	106 (15.6)
S/L	164 (24.2)	157 (23.2)
S/S <sup>#</sup>	66 (9.7)	68 (10.0)
	$p=0.510$	

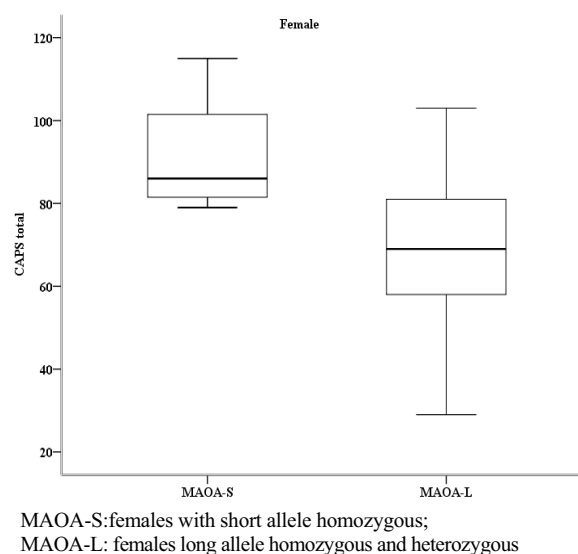
MAOA: monoamino oxidase A; VNTR - variable number of tandem repeat; SLC6A4 - serotonin transporter gene; \*L - long group belong all alleles with 3.5, 4, or 5 repeats of the VNTR; \*\*S - short group belong all alleles with 2 and 3 repeats of the VNTR; \*\*\*L - high-expressing long allele; <sup>#</sup>S - low-expressing short allele; PTSD - posttraumatic stress disorder

against individuals carrying any other genotype combination (i.e. carriers of at least one L allele). Males and females were analyzed separately. Concerning SLC6A4, homozygous individuals with the long-rs25531 (A) allelic combination (LALA) were tested against individuals with any other 5-HTTLPR genotype.

## RESULTS

### Monoamine Oxidase A (MAOA)

There were no significant differences between the PTSD and non-PTSD group in genotype distributions of the MAOA ( $\chi^2=0.998, p=0.318$ ) (Table 1). According to the severity of PTSD symptoms (CAPS) we found that in the lifetime PTSD group individuals of the MAOA-S group compared to those of the MAOA-L group showed nominally significant higher score of the CAPS total and symptoms of hyperarousal (Table 2). Also, females with lifetime PTSD and MAOA-S genotype had nominal significantly higher CAPS total scores, symptoms of re-experience and hyperarousal than females carrying the MAOA-L genotype (Figure 1, Table 2). Finally, also males with the MAOA-S genotype (short allele carriers) and lifetime PTSD had significantly higher score of CAPS total and symptoms of hyperarousal than males with MAOA-L genotype (long allele carriers) (Figure 2, Table 2) at a nominal level. There were no significant difference in severity of PTSD symptoms between MAOA-S and MAOA-L group in the subgroup of participants with current PTSD for the total sample, neither between males or females ( $p_{all}>0.05$ ).



**Figure 1.** The distribution of CAPS values according to MAOA VNTR lengths in female lifetime PTSD subgroup (N=53;  $p=0.018$ )

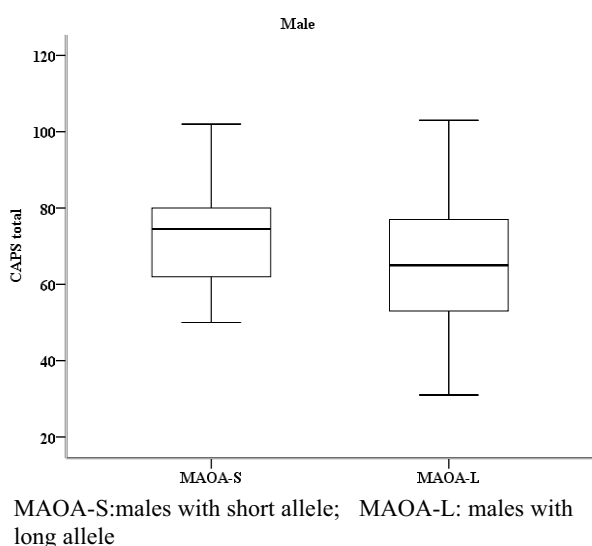
There were no differences in BSI scores between the MAOA genotype groups in either males or females with lifetime PTSD or current PTSD ( $p_{all}>0.05$ ). Regarding the severity of the psychological symptoms there were some differences in the total subsample of current PTSD patients where the MAOA-S group had slightly higher but not nominally significant BSI total scores than the MAOA-L group ( $Z=-1.929, p=0.054$ ), especially for symptom obsessive-compulsive ( $Z=-2.612, p=0.009$ ).

**Table 2.** Differences in severity of PTSD symptoms between the MAOA genotype groups

Sample	PTSD symptoms	MAOA genotype groups		Mann-Witney U test
		S M±SD (Median)	L M±SD (Median)	
<b>Lifetime PTSD subgroup</b>				
Male; n (%) (N=92)		26 (28.3)	66 (71.7)	
	CAPS total	70.5±16.79 (74.5)	63.59±17.42 (65.0)	Z=-1.99, p=0.046
	Re-experience	21.73±5.52 (22.0)	20.13±5.81 (20.0)	Z=-1.56, p=0.118
	Avoidance	26.19±7.94 (26.0)	23.87±7.64 (25.0)	Z=-1.36, p=0.174
	Hyperarousal	22.58±5.53 (23.5)	19.59±6.56 (19.5)	Z=-2.09, p=0.037
Female; n (%) (N=53)		4 (7.5)	49 (92.5)	
	CAPS total	91.50±16.09 (86.0)	67.98±17.27 (69.0)	Z=-2.37, p=0.018
	Re-experience	29.25±2.75 (29.5)	22.73±6.39 (22.0)	Z=-2.14, p=0.032
	Avoidance	33.50±9.68 (29.0)	25.82±8.02 (26.0)	Z=-1.53, p=0.125
	Hyperarousal	28.75±5.12 (27.5)	19.43±6.31 (20.0)	Z=-2.58, p=0.010

MAOA- monoamino oxidase A; M - mean; SD - standard deviation; CAPS – Clinically Administered PTSD Scale

S – group of genotype with short allele (males with short allele and females short allele homozygous); L – group of genotype with long allele (males with long allele; females long allele homozygous and heterozygous); PTSD - posttraumatic stress disorder



**Figure 2.** The distribution of CAPS values according to MAOA VNTR lengths in male lifetime PTSD subgroup (N=92; p=0.046)

Total score of positive strategies did not differ significantly between MAOA-S and MAOA-L group in females with PTSD, as well as with lifetime PTSD and current PTSD subgroups ( $p_{all}>0.05$ ). Also, there were no significant differences between MAOA genotype groups in total score of negative strategies neither in females with PTSD, nor in those suffering from lifetime PTSD and current PTSD ( $p_{all}>0.05$ ). However, in females with current PTSD we found that MAOA-L group showed significantly lower scores in escape avoidance strategies ( $Z=-2.441$ ,  $p=0.015$ ) compared with MAOA-S group at a nominal level. The total score of positive coping strategies did not differ significantly for males with PTSD, as well as in the lifetime PTSD subgroup ( $p_{all}>0.05$ ). Also, there was no significant difference between MAOA genotype groups in total score of negative coping strategies in males with PTSD,

lifetime PTSD and current PTSD ( $p_{all}>0.05$ ). In male subsample with current PTSD we found though, that carrier of the short allele (MAOA-S group) had a nominal significantly higher average score of positive distancing ( $Z=-2.611$ ,  $p=0.009$ ), accepting responsibility ( $Z=-2.401$ ,  $p=0.016$ ) and total score of positive coping strategies ( $Z=-2.142$ ,  $p=0.032$ ) compared to those with long allele (MAOA-L group).

#### Solute carrier family 6 member 4 (SLC6A4)

There were neither significant differences between the PTSD and non-PTSD group in genotype distributions of the SLC6A4 gene ( $\chi^2=0.348$ ,  $p=0.840$ ) (Table 1), nor any difference in CAPS, BSI and positive or negative coping scores between SLC6A4 genotype groups in the PTSD group, in the current PTSD subgroup and in the lifetime PTSD subgroup ( $p_{all}>0.05$ ).

## DISCUSSION

The primary finding of our study was that the MAOA short allele was nominally significantly associated with the severity of PTSD symptoms in the total subgroup of participants with lifetime PTSD, as well as in females and males when analyzed individually within this patient subgroup. Subgroup MAOA-S males with lifetime PTSD showed a nominally significant higher association for symptoms of hyperarousal, and subgroup MAOA-S female with lifetime PTSD was nominally significantly higher associated with symptoms of re-experience and hyperarousal. Our results are in accordance with previous researches that short MAOA VNTR alleles (MAOA-S) are associated with impulsive aggressive behaviour (Reif et al. 2014). Increased aggression might not be a baseline trait in short allele carriers, but rather may occur in response to provocation (McDermot et al. 2009), underscoring the role of threatening stimuli in emotional processing.

In our research the male subsample with current PTSD and short allele genotype (MAOA-S group) had a nominally significantly higher score of positive distancing, accepting responsibility and total score of positive coping strategies compared to those carrying the long allele genotype (MAOA-L group). That finding may correspond with previous research where MAOA-S genotypes significantly influenced the regulation of automatic approach-avoidance reactions (Ernst et al. 2013).

Long alleles of the MAOA VNTR promoter polymorphism are associated with panic disorder and correspond to an increased enzyme activity. Carriers of the risk allele had significantly worse outcomes of cognitive behavioural treatment (CBT) as measured by the Hamilton Anxiety scale (Reif et al. 2014). In women with current PTSD our findings showed that high MAOA activity is a risk factor of avoidance at the genetic or epigenetic level.

Previous research emphasised MAOA hypermethylation as a risk factor for the development of current PTSD in males (Ziegler et al. 2017) while reversibility of hypomethylation is a potential epigenetic correlate for psychotherapy success of panic disorder in females (Ziegler et al. 2016). Our findings are consistent with the hypothesis derived from methylation studies considering low MAOA activity as a risk factor for genetic predisposition to PTSD in males.

There are a number of studies showing that short repeats (MAOA-S) are associated with impulsive-aggressive behaviour in males (Kim-Cohen et al. 2006, Reif et al. 2007); in contrast, long alleles are associated with panic disorder in females (Deckert et al. 1999, Maron et al. 2005, Samochowiec et al. 2004). This phenomenon has been dubbed the 'warrior vs worrier-gene' dichotomy (Gibbons 2004) as different alleles of MAOA apparently are associated with aggression (low-activity, males) as well as anxiety (high-activity, females). Attempts to understand the neural mechanisms linking altered gene expression to neural systems are manifold and demonstrated MAOA to mediate environmental effects and a heightened sensitivity to aversive experiences in short allele carriers (McDermott et al. 2009).

A complex, but hypothetical model provides a framework of how genetic variation of MAOA might predispose to dysfunctional 'flight' behaviour - that is, panic attacks and PTSD symptoms of hyperarousal - by modulating serotonin and norepinephrine levels in neural networks. Carriers of the low-expressing compared to the high-expressing genetic variant (MAOA) showed increasing regulatory activity in the right dorsolateral prefrontal cortex (DLPFC) during incompatible conditions (approach negative, avoid positive) (Ernst et al. 2013). MAOA genotypes have been investigated with regard to personality traits, where carriers of the low-expressing genetic variant (MAOA) repeatedly, but not always (Haberstick et al. 2005), showed enhanced trait impulsivity (Huang et al. 2004) and even aggressive, criminal behaviour (Nilsson et al. 2006). On the other hand, female carriers of high-expressing alleles are more prone to develop panic disorder (Reif et al. 2012).

In our research we did not get a significant correlation of SLC6A4 variants and war related PTSD. We identified some nominal significance regarding the correlation of SLC6A4 and symptoms of psychoticism and slightly trending but not significant results concerning hostility. 5HTTLPR/rs25531 carriers of the more active LALA genotype consistently scored highest on all considered measures of anxiety if they had a history of childhood adversity, but only when general self-efficacy was low (Schiele et al. 2015). The 'differential susceptibility hypothesis' (Belsky & Pluess 2009, Belsky et al. 2009) proposing the term "plasticity genes" rather than "risk genes", implicates that a given genotype does not convey vulnerability for anxiety per se, but is subject to both positive and negative environmental influences. Genes seem to drive differential sensitivity to environmental conditions as a whole, and depending on the nature of these environmental influences, their contribution can be beneficial or harmful and manifest on a phenotypic level.

## CONCLUSIONS

Limitations of this study are the relatively small sample size and a low statistical power. After Bonferroni correction for multiple tests none of the nominally significant MAOA results remained significant and thus are to be considered exploratory and hypothesis generating. Further studies ought to be conducted to test MAOA polymorphisms for associations with more refined personality traits conferring vulnerability to anxiety, impulsivity and aggression as well as psychological strengths and capacity for recovery in accordance with different environmental factors, rather than with categorical psychiatric diagnosis.

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**Conflict of interest:** None to declare.

### Contribution of individual authors:

Each author has actively participated in the international research project (see Acknowledgments) and, therefore, has substantially contributed to the development and publication of this manuscript.

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