Absence of a Significant Interaction of Two Common NOS1 and 5-HTT Polymorphisms on Sensorimotor Gating in Humans

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Summary

The neurotransmitter serotonin has been critically implicated in the pathogenesis of several mental disorders. The serotonin transporter (5-HTT) is a key regulator of serotonergic neurotransmission and its genetic variability is associated with increased risk of psychopathology. One well known polymorphic locus in the *5-HTT* gene affecting its expression is a tandem repeat in the promoter region (*5-HTTLPR*). It has been reported that 5-HTT is functionally coupled with the neuronal nitric oxide synthase (NOS1 or nNOS), an enzyme catalyzing the production of nitric oxide (NO). We have previously demonstrated that a tandem repeat polymorphism in the promoter of *NOS1* exon 1f (Ex1f-VNTR) is associated with sensorimotor gating, a marker of inhibitory processing and a well-established endophenotype of several neuropsychiatric disorders. Here we investigated the combined genetic effects of *NOS1* Ex1f-VNTR and *5-HTTLPR* on sensorimotor gating, measured by prepulse inhibition (PPI) of the acoustic startle reflex, in 164 healthy adults. We found no evidence for the interaction between *NOS1* Ex1f-VNTR and *5-HTTLPR* on PPI. PPI was associated with *NOS1* Ex1f-VNTR, but not *5-HTTLPR*. Our data suggest that while NOS1 plays a role in sensorimotor gating, the nitrergic pathway of gating regulation does not involve the action of 5-HTT.

Key words

Prepulse inhibition • Serotonin • Serotonin transporter • Nitric oxide • Endophenotype

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Introduction

Sensorimotor gating is an automatic neural process that enables humans and animals to suppress non-salient sensory input at the early stage of information processing. This process is thought to protect the brain from sensory overload and facilitate the efficient engagement of limited attentional resources (Light and Braff 1999). Sensorimotor gating is routinely examined by measuring prepulse inhibition (PPI) of the acoustic startle reflex. PPI is a reduction in the magnitude of the startle response to a loud auditory stimulus (pulse) when a weak non-startling stimulus (prepulse) is presented shortly before the loud stimulus (typically 30-500 ms). PPI deficit is one of the most reliable neurophysiological alterations associated with schizophrenia but also several other neuropsychiatric conditions, including obsessive-compulsive disorder (OCD), panic disorder, Tourette syndrome, or Huntington's disease (for reviews, see e.g. Swerdlow and Light 2018 and Kohl *et al.* 2013). Abnormalities of sensorimotor gating are present not only in psychiatric patients but also in their unaffected

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relatives, i.e. individuals with increased genetic risk for the disorders (Li *et al.* 2020). Hence, PPI is considered as a biomarker (endophenotype or intermediate phenotype) of these disorders and its heritability has been estimated to 29-58 % (Rovný *et al.* 2020).

There is increasing evidence for a role of nitric oxide (NO) in neuropsychiatric disorders (for a recent review, see e.g. Tripathi *et al.* 2020). Research on the involvement of NO in the regulation of PPI may thus contribute to a better understanding of the etiopathogenesis of mental illness. We have recently demonstrated that variation of the gene encoding the neuronal nitric oxide synthase (*NOS1*, also referred to as *nNOS*) is associated with PPI in healthy humans (Rovný *et al.* 2018). In particular, PPI was weaker in carriers of a short allele of a VNTR (variable number of tandem repeats) polymorphism in the promoter region of exon 1f (Ex1f-VNTR). This allele is associated with lower *NOS1* transcription and increased risk of psychopathology (for a review, see Freudenberg *et al.* 2015). In the present study, we have made a step further to explore a potential factor or mechanism moderating the effect of *NOS1* genotype on PPI and focused on the serotonin transporter (5-HTT), which is responsible for the reuptake of the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) into presynaptic neurons. It has been namely shown that NOS1 interacts with 5-HTT in two ways: first, NO (produced by NOS1) modulates the activity of 5-HTT through a cyclic guanosine monophosphate/protein kinase G-dependent mechanism; and second, *via* direct protein-protein interaction, NOS1 inhibits the trafficking of 5-HTT to the plasma membrane (Chanrion *et al.* 2007, for reviews, see Garthwaite 2007 and Bermingham and Blakely 2016). Thus, the activation of the nitrergic pathway may increase the concentration of 5-HT in the synaptic cleft through inhibition of 5-HTT action. Importantly, it has been demonstrated that mice lacking the *Nos1* gene show decreased 5-HT turnover as well as profound deficits in impulse control (Chiavegatto *et al.* 2001).

Several lines of evidence highlight the relevance of serotonergic neurotransmission, including 5-HTT, in the pathophysiology and treatment of mental disorders (for a review, see Spies *et al.* 2015). Moreover, particularly relevant for our study, genetic variants in 5-HTT and other proteins involved in serotonergic neurotransmission have been linked not only with impaired functioning, but also normal variability in mental functions and behavior (for a review, see Serretti *et al.* 2006). By far the most extensively studied polymorphism in the *5-HTT* gene (*SLC6A4*) is a tandem repeat in the promoter region, the so-called serotonin-transporter-linked polymorphic region (*5-HTTLPR*). The length of *5-HTTLPR* affects the level of expression of *SLC6A4* (Lesch *et al.* 1996) and has been linked with an increased risk of psychopathology, including affective and anxiety disorders, OCD or Tourette syndrome (Anguelova *et al.* 2003, Gressier *et al.* 2013, Moya *et al.* 2013, Taylor 2013). Considering the functional link between 5-HTT and NOS1, as well as the role of complex genetic architecture of mental functions and their disorders, we expected the possibility that the genotype of *5-HTTLPR* might moderate the effect of *NOS1* Ex1f-VNTR on PPI in humans.

Methods

Study participants

In the current study, we extended a previously described cohort of 96 healthy adults (Rovný *et al.* 2018) by including additional 68 individuals, resulting in a total sample of 164 subjects (113 males, mean age ± *SD*=23.6±3.2 years, 54 smokers). Study participants were recruited *via* advertisement from general population in Bratislava, Slovakia. Study inclusion/exclusion criteria have been reported previously in detail (Rovný *et al.* 2018). Only healthy subjects with no personal and family history of mental disorders in first-degree relatives, no current medication, no substance abuse (except tobacco) and normal hearing ability were included in this study. In total 57 of 221 subjects were excluded from the study participation. Three subjects were excluded due to family history of severe mental illness among first-degree relatives, 6 subjects had a positive toxicology screen, 15 subjects were startle non-responders, 2 subjects had severe neurological or metabolic disease and 30 subjects were excluded because their genotype could not be assessed. One subject with extreme values of PPI (values deviating more than three times the interquartile range from the upper or lower quartile) was excluded from the statistical analysis. The sample size was estimated based on previous research (e.g. Bräuer *et al.* 2009) and provided enough statistical power to detect effect sizes equal to or larger than $\eta_p^2 = 0.07$ (α=0.05, 1 – β = 0.80, G*Power (Faul *et al.* 2007). Effect sizes of a comparable magnitude were reported in previous genetic studies of PPI (e.g. Quednow *et al.* 2011).

Procedures

The experimental procedures were identical to those described in our previous paper (Rovný *et al.* 2018). In brief, upon arrival to the lab all participants got information about the procedures and gave their written informed consent. Thereafter, participants were interviewed to obtain information on medical history, health status and use of psychoactive substances. Subsequently, buccal mucosa samples were collected and urine multi-drug toxicology and cotinine tests were carried out. Before the acoustic startle response assessment, all participants underwent a screening test of hearing to verify that their hearing ability is within the normal range. The entire session lasted approximately two hours. Participants received financial reward for study participation. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and approved by the institutional review board (approval number EK/2/16).

Startle response measurement

Startle response was measured as described in our previous work (Rovný *et al.* 2018). The eye-blink component of the acoustic startle reflex was measured by recording electromyogram (EMG) of the orbicularis oculi muscles using surface electrodes. Startle was elicited by white noise bursts administered binaurally through headphones. The auditory stimulation comprised pulse alone trials (PA, 40 ms burst of white noise at 105 dB), prepulse-pulse trials (PP) in which pulse was preceded 30, 60 or 120 ms by a prepulse (20 ms burst of white noise at 75 dB). White noise of 55 dB was continuously presented in the background throughout the session. The stimulation paradigm involved 69 trials, which were divided into three blocks. The first and the third block contained only PA trials (5 and 4, respectively). The second block involved 10 PA trials and 10 PP trials for each PP interval presented in random order. The stimuli were separated by a random inter-trial interval of 10-20 s. PPI at PP intervals of 30, 60 and 120 ms (hereafter denoted as PPI30, PPI60 and PPI120) was calculated as (1-mPP/mPA)×100 %, where mPP and mPA denote mean startle amplitude in PP and PA trials respectively. Baseline startle reactivity was assessed as the mean startle amplitude in the first block and was used as a covariate in the statistical tests of PPI (Csomor *et al.* 2008).

Genotyping

Genomic DNA for genotyping was extracted from buccal swabs of participants using 300 µl of 5 % CHELEX (CHELEX 100 Resin, Biorad, USA) and incubated at 56 °C for 30 min and at 100 °C for 10 min. During the incubation, samples were repeatedly vortexed and then centrifuged 3 min at 12 000 g. For specific DNA region amplification, universal PCR mastermix containing 1× PCR mastermix (5× HOT FIREPOL Blend Mastermix (Solis BioDyne, Tartu, Estonia)), 5 pmol FAM-labelled assay specific primers (*NOS1* Ex1f-VNTR: 5′-CCCTGCGTGGCTACTACTACATT-3′ and 5′-CTGGGCTCCAAAGCATACAT-3′; *5-HTTLPR*: 5′-GGCGTTGCCGCTCTGAATGC-3′ and 5′-GAGGGACTGAGCTGGACAACCAC-3′; Heils *et al.* 2002, Hoogman *et al.* 2011), 5 µl DNA solution and nuclease free water to final volume 20 µl was used. The following PCR program was used: initial denaturation step at 95 °C for 5 min, followed by 30 cycles each consisting of denaturation step at 94 °C for 45 s, annealing step at 60 °C for 45 s, polymerization step at 72 °C for 45 s and then final polymerization step at 72 °C for 10 min. For detection of the genotype of the VNTR polymorphism, samples were mixed with solution of HiDi Formamide (8.5 µl; Life Technologies, USA) and LIZ 500 Size Standard (0.5 µl; Life Technologies, USA) in ratio 1:9, denatured at 95 °C for 5 min and genotyped using fragment analysis on ABI Prism 3130xl Genetic Analyzer and software GeneMapper v3.5 (Life Technologies, USA). Based on earlier studies (Nakamura *et al.* 2000, Freudenberg *et al.* 2015), alleles were classified as short (*NOS1* Ex1f-VNTR: 19-27, *5-HTTLPR*: 14 repeats, respectively) or long (*NOS1* Ex1f-VNTR: 28-36, *5-HTTLPR*: 16 repeats, respectively).

Statistical analysis

Data were analyzed using SPSS 22 (SPSS Inc., Chicago, IL USA) and JASP v0.13.1 software (JASP Team 2020). Prior to the statistical analyses all outcome variables were inspected for distributional properties and extreme values. Outliers identified by the standard interquartile range rule of 1.5 were treated using a one-sided winsorization at the 2.5th percentile for variables PPI30, PPI60 and PPI120 (Wilcox 2012). Categorical variables were described by absolute and relative frequencies, and continuous variables by means and standard deviations, respectively. Pearson product-moment correlation coefficients were computed between age and the startle measures (BSR, PPI30, PPI60, and

PPI120). The differences in relative frequencies in sex and smoking status were tested by χ^2 tests. The independent-samples Student's *t*-tests were used to test the differences in mean startle measures for sex, smoking status and genotypes (*NOS1* Ex1f-VNTR and *5-HTTLPR*). The Student's *t*-tests with Welch's approximation of degrees of freedom were used in case the assumption of equality of variances was violated. Pairwise linkage disequilibrium (LD) between *NOS1* Ex1f-VNTR and *5-HTTLPR* polymorphisms was estimated using Haploview version 4.2 (Barrett *et al.* 2005). The normalized *D'* statistic was used to calculate LD. The χ^2 goodness-of-fit tests were used to determine whether the genotype frequencies of *NOS1* Ex1f-VNTR and *5-HTTLPR* polymorphisms met the Hardy-Weinberg equilibrium. The differences between the groups defined by the presence of *NOS1* Ex1f-VNTR and *5-HTTLPR* risk alleles were tested by χ^2 tests or independent-samples *t*-tests. The effects of genotype on PPI were assessed by a 2×2×3 three-way repeated-measures analysis of covariance (ANCOVA) with two fixed between-subjects factors (*NOS1* Ex1f-VNTR and *5-HTTLPR* genotype, 2 levels each), one fixed within-subjects factor (prepulse-pulse interval, 3 levels) and BSR as a continuous fixed covariate. *Post hoc* comparisons were performed with Tukey's honestly significant difference *post hoc* tests. Age, sex and tobacco smoking status were included as fixed effects in next ANCOVA models to explore their effects on the PPI measures (Swerdlow *et al.* 1993, Kumari *et al.* 1996, Ludewig *et al.* 2003). All null hypotheses were tested against two-sided alternative hypotheses at a significance level α=0.05. Next, a Bayesian approach was implemented to further investigate the genetic interaction. A Bayesian 2×2×3 three-way repeated-measures ANCOVA was conducted in JASP. The default JASP priors were used (r scale prior width of 0.5 for fixed effects and 0.354 for covariates). A classification scheme proposed by Lee and Wagenmakers (2013) was employed for interpreting the magnitude of Bayes factors. Accordingly, BF_{10} more than 3 and less than 1/3 were classified as indicative of more than anecdotal evidence in favor of the H1 and H0, respectively.

Results

Genotype frequencies for the *NOS1* Ex1f-VNTR and *5-HTTLPR* polymorphisms (Table 1) were distributed according to Hardy-Weinberg equilibrium (*NOS1* Ex1f-VNTR: $\chi^2(1)=0.005$, $p=0.94$; *5-HTTLPR* $\chi^2(1)=0.034$, $p=0.85$). Linkage disequilibrium between the two polymorphisms was weak ($D'=0.014$). To ensure an adequate size of genotype groups for statistical analysis, homozygotes and heterozygotes for the rare alleles (short for both loci) were classified as minor allele carriers (Table 1). These genotype groups did not differ significantly in sex, age, smoking status, or baseline startle reactivity (χ^2 or *t*-tests as appropriate, all *p*-values >0.05).

To evaluate the experimental effects, a repeated-measures analysis of covariance (RM-ANCOVA) with baseline startle magnitude as a covariate was calculated (Table 2). The main effect of prepulse-pulse (PP) interval was significant ($p=0.002$), in agreement with the well known feature of PPI to increase with increasing PP interval duration (mean values: PPI30=65.1 %, PPI60=75.0 %, PPI120=76.2 %). Furthermore, a significant effect of *NOS1* Ex1f-VNTR genotype was revealed ($p=0.038$). Individuals carrying the short (risk) allele of Ex1f-VNTR had significantly weaker PPI compared with homozygotes for the long allele of Ex1f-VNTR (Fig. 1A). *5-HTTLPR* genotype showed no significant main effect or interaction with *NOS1* Ex1f-VNTR genotype (Fig. 1B, C). These results did not change when age, sex or smoking status were included as additional covariates into the model. Next, a Bayesian RM-ANCOVA was calculated to examine the plausibility of the genetic interaction between *NOS1* Ex1f-VNTR and *5-HTTLPR*. The estimated Bayes factor ($BF_{01}=3.25$) indicates that the likelihood of the observed PPI data is more than three times higher under the assumption of absence, rather than presence, of the interaction between *NOS1* Ex1f-VNTR and *5-HTTLPR*.

Table 1. Allelic distribution of the investigated NOS1 and 5-HTT polymorphisms.

	Genotype			
Polymorphism	**LL**	**LS**	**SS**	**S+**
NOS1 Ex1f-VNTR	44 (26.8 %)	84 (51.2 %)	36 (22.0 %)	120 (73.2 %)
5-HTTLPR	67 (40.9 %)	73 (44.5 %)	24 (14.6 %)	97 (59.1 %)

L: long allele; S: short allele, S+: carriers of short allele (LS+SS).

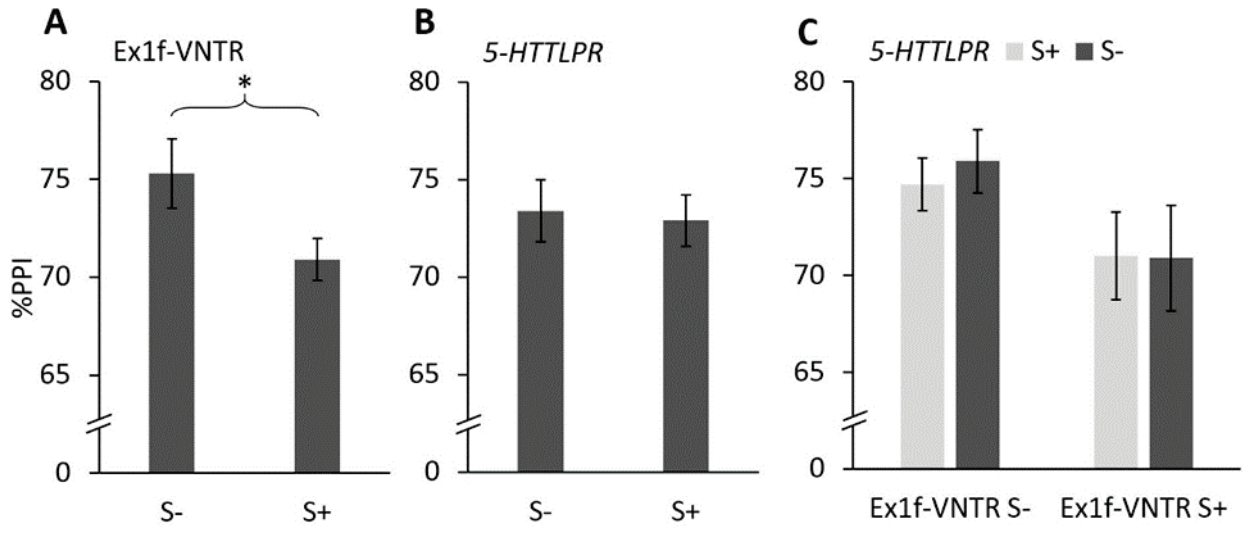

Fig. 1. Prepulse inhibition (estimated means across three prepulse-pulse intervals 30, 60, and 120 ms, adjusted for baseline startle magnitude) by genotype of (**A**) *NOS1* Ex1f-VNTR, (**B**) *5-HTTLPR* and (**C**) combined genotype of Ex1f-VNTR and *5-HTTLPR*. Error bars represent ± SEM. * $p<0.05$.

Table 2. Repeated-measures ANCOVA testing the effects of *NOS1* Ex1f-VNTR and *5-HTTLPR* on mean PPI with baseline startle response (BSR) as a covariate.

Effect	$F_{(1,159)}$	p	η_p^2
PP interval	10.27	0.002	0.043
BSR	40.95	<0.001	0.205
NOS1 Ex1f-VNTR	4.40	0.038	0.027
5-HTTLPR	0.07	0.787	0.000
PP interval * *NOS1* Ex1f-VNTR	0.00	0.960	0.000
PP interval * *5-HTTLPR*	0.02	0.902	0.000
NOS1 Ex1f-VNTR * *5-HTTLPR*	0.10	0.759	0.001
PP interval * *NOS1* Ex1f-VNTR * *5-HTTLPR*	0.04	0.834	0.000

Discussion

Numerous studies have linked variability of *NOS1* and *5-HTT* genes with human behavioral functions and their disturbance manifested in several neuropsychiatric disorders (Serretti *et al.* 2006, Freudenberg *et al.* 2015, Gatt *et al.* 2015, Topaloglu *et al.* 2017). We have previously demonstrated that *NOS1* Ex1f-VNTR polymorphism affects the efficiency of sensorimotor gating, impaired in many of these pathological conditions (Rovný *et al.* 2018). Given the physiological coupling between NOS1 and 5-HTT (Garthwaite 2007, Bermingham and Blakely 2016), we examined whether common functional genetic variants *NOS1* Ex1f-VNTR and *5-HTTLPR*, jointly affect sensorimotor gating. We found, however, moderate evidence against the interaction between these polymorphisms on PPI.

In agreement with our previous work (Rovný *et al.* 2018), the genotype of *NOS1* Ex1f-VNTR had a significant impact on PPI so that the carriers of short (risk) allele exhibited less efficient inhibition. Although this finding cannot be interpreted as an independent replication due to partial overlap between the samples, it provides cumulative evidence for the role of NO in the regulation of sensorimotor gating.

It also fits with our finding in rats, showing that PPI deficits are accompanied by altered NOS1 expression in the brain of animals reared in social isolation after weaning, used as a rodent model of schizophrenia pathology (Chmelova *et al.* 2019, Vrankova *et al.* 2021). Since the effect of *NOS1* Ex1f-VNTR was not significantly moderated by the genotype of *5-HTTLPR*, the nitrergic mechanisms of gating regulation seem not to

depend on 5-HTT. Furthermore, a simple effect of *5-HTTLPR* genotype on PPI was also insignificant. This indicates that the contribution of 5-HTT to psychopathology may not involve a disruption of gating efficiency, which is in line with the fact that a direct association of *5-HTTLPR* with PPI has not been reliably documented in previous genetic studies in humans (Rovný *et al.* 2020). However, we must emphasize that our finding is not in conflict with the abundant evidence for the role of serotonergic mechanisms in gating. For instance, depletion of 5-HT disrupts PPI (Mann *et al.* 2008), while an opposite effect results from an excessive release of 5-HT, such as following the administration of MDMA (3,4-methylene-dioxymethamphetamine) (Liechti *et al.* 2001). Human genetic studies of PPI have identified a reliable association with *HTR2A* gene encoding the 5-HT2A receptor, which has been mostly considered from the perspective of its role in schizophrenia (Baou *et al.* 2016, Quednow *et al.* 2020, Rovný *et al.* 2020). In particular, hallucinogens (such as psilocybin or LSD), which act as agonists at 5-HT2A receptor, disrupt PPI and the blockade of 5-HT2A receptor prevents this effect to occur (Quednow *et al.* 2012, Halberstadt and Nichols 2020). Nevertheless, 5-HT2A receptor function has also been linked with other disorders associated with gating deficit, such as OCD (Derksen *et al.* 2020). Moreover, signaling *via* other types of 5-HT receptors has also been implicated in gating (Sipes and Geyer 1994). For instance, it has been reported that availability of 5-HT1B receptors in the basal ganglia and thalamus positively correlated with PPI in healthy humans while such a relationship was absent in OCD patients (Pittenger *et al.* 2016). Our data thus suggest that other molecules involved in serotonergic neurotransmission, rather than 5-HTT, may be more probable candidates of nitrergic regulations playing a role in sensorimotor gating. In particular, 5-HT1A and 5-HT2C receptors, which mediate the inhibitory action of 5-HT on NO signaling, may represent promising candidates for future studies (Maura *et al.* 2000, Raymond *et al.* 2001).

To conclude, our data suggest that while NOS1 plays a role in sensorimotor gating, the nitrergic pathway of gating regulation does not involve the action of 5 HTT. Although these findings cannot be yet directly translated into clinical practice, they expand our knowledge of the genetic basis of gating, an important psychiatric endophenotype. Such knowledge is necessary to decompose the complex etiopathogenetic architecture of mental disorders and may provide new outlooks for their treatments in the future. Since both nitrergic and serotonergic signaling are importantly involved in many neuropsychiatric disorders, further research on the interactions of these pathways and their roles in the pathogenesis of psychopathological symptoms is warranted.

Conflict of Interest

There is no conflict of interest.

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