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ORIGINAL PAPER

# BDNF rs6265 (Val66Met) Polymorphism as a Risk Factor for Blepharospasm

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## Abstract

A few genetic variants are implicated in the development of blepharospasm (BSP). The precise role of the rs6265 on the brain-derived neurotrophic factor (BDNF) gene on BSP remains controversial. The effect of rs6265 on BSP was evaluated. 206 patients with BSP and 206 healthy controls were recruited and genotyped for the rs6265. We also performed a meta-analysis, by pooling our results with those from previous studies. A significant effect of rs6265 on the risk of BSP was found in the dominant model of inheritance [odds ratio (OR) (95% confidence interval (CI) 1.52 (1.01–2.29), $p=0.044$]. Mutational load analysis of rs6265 in the risk of BSP using the $OR_G$ revealed that higher load of the "A" allele of rs6265 denotes higher probability of a subject to develop BSP ($OR_G$ 1.48; 95% CI 1.00–2.19). Finally, pooled results from the meta-analysis revealed that the rs6265 is associated with an increased risk of BSP in the dominant model [OR 1.26; 95% CI 1.02–1.55, $p_z=0.03$]. Also, higher load of the "A" allele of rs6265 denotes higher probability of a subject to develop BSP ($OR_G$ 1.26; 95% CI 1.04–1.53). The present study provides additional evidence to the existing knowledge concerning the contribution of the rs6265 BDNF on the risk of developing BSP. While the pathophysiology and genetic susceptibility in BSP and focal dystonia are only partially understood, it seems that BDNF and rs6265 may constitute one essential risk factor that is heavily involved.

**Keywords** BDNF · Blepharospasm · Focal dystonia · Polymorphism · SNP

## Introduction

Dystonia is a neurological movement disorder, whose nature and etiology remain largely unknown (Charlesworth et al. 2013). Recently, the general definition, as well as the classification of dystonia, were updated by an international committee of dystonia experts (Albanese et al. 2013). The etiology and the clinical features are the two distinct axes of this dystonia's classification (Albanese et al. 2013).

Dystonia's prevalence is estimated to be around 430 per million (Defazio et al. 2004). Blepharospasm (BSP) and cervical dystonia (CD) are considered to be the most common forms of focal dystonia. The prevalence of BSP ranges from 16 to 133 per million (Defazio et al. 2004), whereas CD appears to have a prevalence of 0.28% in the United States (Jankovic et al. 2007). BSP is thought to be less common than CD, however, in Japan and in Italy, BSP is more prevalent when compared to CD (Valls-Sole and Defazio 2016).

BSP is a sub-phenotype of focal dystonia and stems from involuntary orbicularis oculi spasms (Defazio et al. 2017; Xiromerisiou et al. 2013). It is usually bilateral, symmetric

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and synchronous (Defazio et al. 2015). BSP is also twice more common in females compared to males, its age of onset is between the fifth and the seventh decade, and it has a greater tendency towards spreading to adjacent body parts compared to other phenotypes of focal dystonia (Albanese et al. 2013; Weiss et al. 2006; Defazio et al. 2009).

A few pathogenic genetic variants have been so far identified in monogenic cases of familial dystonia (Lohmann and Klein 2017; Xiromerisiou et al. 2012). Moreover, results from candidate gene association studies (CGASs) have revealed specific genetic loci that may confer susceptibility to dystonia (Siokas et al. 2017b). Increased plasticity is considered to be a possible dystonia pathophysiological mechanism, among others, as well (Kojovic et al. 2013; Quartarone et al. 2008).

Synaptic plasticity is influenced by the brain-derived neurotrophic factor (BDNF) (You et al. 2016; Belviranli and Okudan 2018). The human BDNF gene is located within chromosome 11, in region p13–14, and it spans ~70 kb (Cattaneo et al. 2016). The BDNF gene consists of 11 exons in the 5′ end and of 9 functional promoters as well (Pruunsild et al. 2007). The rs6265 (G/A) is a common SNP across the prodomain region of the BDNF gene (Liu et al. 2014). It results in the substitution of Val in amino acid position 66 with Met (Val → Met), a substitution which may influence synaptic plasticity (Hempstead 2015; Notaras et al. 2015; Anastasia and Hempstead 2014) and possibly predispose to dystonia and BSP (Chen et al. 2013).

Studies that have been conducted so far, regarding the role of rs6265 in dystonia (in general) and BSP, have yielded conflicting results (Sako et al. 2015; Gomez-Garre et al. 2014). Moreover, two meta-analyses have, up to this date, evaluated the effects of the rs6265 variant on dystonia (Gomez-Garre et al. 2014; Sako et al. 2015). No significant association was found in the overall dystonia group, as well as in CD and BSP sub-phenotypes in the first one (Gomez-Garre et al. 2014), whereas in the second, a statistically significant effect of the AA genotype on the idiopathic dystonia risk was reported (Sako et al. 2015).

In view of the former speculation, during the current study, we wish to expand our understanding on the role of rs6265 as a risk factor in BSP. First, we aimed to assess the association of rs6265 in a Greek BSP cohort. Then we additionally performed a supplementary meta-analysis that pooled our data with the respective ones of previously published studies, in order for validate the effect of rs6265 on the risk BSP.

## Methods

### Case–Control Study

#### Study Population

A total of 206 patients with BSP and 206 healthy individuals as controls were recruited during this study. All participants were Caucasians. None of the participants reported a positive family history. BSP patients had been examined at the Neurology and Ophthalmology outpatient clinics of the University Hospital located in Larissa, Greece. BSP was diagnosed by a specialist neurologist and a specialist ophthalmologist. The study was approved by the University of Thessaly Ethics Committee and informed consent was received from all the participants.

#### Isolation of DNA and Genotyping

Genomic DNA was extracted from peripheral blood samples using a salting out method as previously described (Siokas et al. 2017a). The genotyping of the SNP was performed with a TaqMan allele-specific discrimination assays method on an ABI PRISM 7900 Sequence Detection System and analyzed with the SDS software (Applied Biosystems, Foster City, CA, USA) (Siokas et al. 2017c), by laboratory personnel blinded to the clinical status. The genotyping call rate was ≥98.5%. In order for the genotyping reproducibility to be assessed, a proportion equal to 10% of randomly selected DNA samples, were genotyped for a second time, without any inconsistency detected.

#### Statistical Analysis

The statistical power was estimated with the use of the CaTS Power Calculator (http://csg.sph.umich.edu//abecasis/cats/gas_power_calculator/index.html) (Skol et al. 2006). Our study had 80.0% power to detect an association of a SNP with a genetic relative risk of 1.48, under the assumption of a multiplicative model, minor allele frequency of 23% in BSP cases, type I error level of 0.05, in a sample consisting of 206 healthy controls and 206 BSP cases. Using the exact test, Hardy–Weinberg equilibrium (HWE) was calculated. The differences of allelic and genotype frequencies between the BSP patients and controls in the case–control cohort were calculated with Fisher's exact test and Pearson's Chi-square test.

Using the SNPStats software (http://bioinfo.iconcologia.net/SNPstats/) (Sole et al. 2006) and assuming five genetic inheritance modes (co-dominant, over-dominant, dominant, recessive, and additive), odds ratios (ORs) along with the

respective 95% confidence intervals (CIs) were calculated. In the co-dominant model, an overall *p*-value with 2 degrees of freedom and two ORs were estimated for the SNP [one for the heterozygosity for the mutant allele, compared to the homozygosity for the wild allele (mt/wt vs. wt/wt) and one for the homozygosity for the mutant allele compared to the homozygosity for the wild allele (mt/mt vs. wt/wt)]. In the dominant mode, the sum of homozygosity for the mutant allele and of the heterozygosity is compared with the homozygosity for the wild allele (mt/mt + mt/wt vs. wt/wt). In the recessive one, homozygosity for the mutant allele is compared with the sum of homozygosity for the wild allele and heterozygosity (mt/mt vs. mt/wt + wt/wt). In the over-dominant mode, heterozygosity is compared with the sum of homozygosities (mt/wt vs. mt/mt + wt/wt). Finally, under the additive model, each copy of the mutant allele modifies the risk in an additively so the homozygous for mutant allele (mt/mt) has double risk compared with heterozygous (mt/wt).

In order for the risk of BSP development to be estimated, we used the generalized odds ratio ($OR_G$) (Zintzaras 2010, 2012), making use of the ORGGASMA software (http://www.biomath.uth.gr), among healthy controls and BSP cases. ORGGASMA expresses the probability of being diseased compared to the probability of being non-diseased, given that the mutational load is higher in diseased than in the non-diseased (Zintzaras 2010, 2012).

A *p*-value lower of 0.05 was set as the significance threshold. Statistical analysis was carried out with SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

## Supplementary Analysis

### Meta-analysis

#### Literature Search

Eligible case–control CGAS were identified by searching the PubMed database from its inception until the 4th of June 2018. The following search terms were included: (a) "dystonia" or (b) "blepharospasm" in combination with the terms "BDNF" and "polymorphism" as free words. The detailed search algorithm is presented in the Supplementary File 1. Titles and abstracts were reviewed to determine relevance. Only articles written in English were included. The final literature search was performed on June 4, 2018. Finally, the bibliographies of the resulting full texts were searched for other relevant citations. We additionally scanned the reference lists of the initially obtained articles, in an attempt to identify any study that had probably failed to be identified.

We applied the following inclusion criteria: (a) studies with case–control design, with BSP cases and with neurologically healthy controls, (b) rs6265 variant of the BDNF gene must have been genotyped, (c) genotypic frequencies of BSP had to be available in either original protocols or in the previous meta-analyses. The participants in each study were classified based on the initial phenotypic classifications.

#### Data Extraction

Author, year of publication, populations' ethnicities, number of cases and controls, mean age and gender distribution, family history, age at disease onset, TOR1A ΔGAG mutation status, HWE, as well as examined dystonia phenotypes were extracted from each study, when available. The procedure of the selection of the included studies is available as flowchart in Supplementary File 2.

#### Statistical Analysis

The association between the rs6265 and BSP was estimated by calculating the pooled ORs with the respective 95% CIs for the: (a) mt/mt genotype, (b) mt/wt genotype, and (c) dominant model (mt/mt + wt/mt vs. wt/wt). With the *Z* test, the significance of the OR was determined ($p < 0.05$ was considered statistically significant). Moreover, the association between genotype distribution and phenotypic traits was quantified with the terms of $OR_G$ and the respective 95% CIs ORs. Furthermore, $OR_G$ and 95% CIs were also estimated in each included case–control CGAS. An overall analysis, including studies regardless of the participants' ethnicity, and a subgroup analysis including studies with European and Caucasian populations were performed.

The Cochran's $Q$ and $I^2$ index were used to calculate the statistical heterogeneity of the studies. When substantial heterogeneity was present ($p_Q < 0.10$ and/or $I^2 > 75\%$), the random-effects model (the DerSimonian and Laird method) was used (Theuns et al. 2014; DerSimonian and Laird 1986). Otherwise, we applied the fixed-effects model (the Mantel–Haenszel method) (Mantel and Haenszel 1959). Publication bias was graphically assessed with the funnel plots. Linear regression asymmetry, in terms of test by Egger (Egger et al. 1997), with $p < 0.10$ set as the threshold of publication bias, was also applied.

Statistical analyses were assessed using the Review Manager (RevMan) Version 5.3 software (http://tech.cochrane.org/revman). The $OR_G$s were estimated with the ORGGASMA software (http://www.biomath.uth.gr). At Supplementary File 3, the PRISMA guidelines, for the present meta-analysis, are available.

## Results

### Case–Control Study

A total of 206 BSP patients (54.9% female) and 206 healthy controls (matched for age and sex) were recruited during this study. The mean age of blood selection was $67.32 \pm 12.02$ years and the mean age of onset was $61.15 \pm 12.03$ years. No deviation from the HWE ($p > 0.05$) was observed. There were no statistically significant differences of allelic ($p = 0.057$) and genotype ($p = 0.13$) frequencies between the BSP patients and controls based on Fisher's exact test and Pearson's Chi-square test. Allele and genotype frequencies in BSP cases and in healthy controls are shown in Table 1.

A statistically significant effect of rs6265 on the risk of BSP emerged in the dominant mode [OR (95% CI): 1.52 (1.01–2.29), $p = 0.044$]. No significant effect of rs6265 on the risk of BSP was found in any of the co-dominant, recessive, over-dominant and log-additive modes of inheritance ($p > 0.05$). ORs, CIs, and $p$-values, for all modes, are available in Table 2. Lastly, analysis, with the $OR_G$, of the effect of the mutational load of rs6265 in the risk of BSP, using the $OR_G$, revealed that higher load of the "A" allele of rs6265 denotes higher probability of a subject to develop BSP ($OR_G$ 1.48; 95% CI 1.00–2.19).

**Table 2** Single locus analysis for association between rs6265 (BDNF) and BSP, in co-dominant, dominant, recessive, over-dominant and log-additive modes

| Mode | Genotype | OR (95% CI) | $p$-value |
|---|---|---|---|
| Codominant | G/G | 1.00 | 0.13 |
| | G/A | 1.53 (1.00–2.34) | |
| | A/A | 1.48 (0.57–3.87) | |
| Dominant | G/G | 1.00 | **0.044** |
| | G/A–A/A | **1.52 (1.01–2.29)** | |
| Recessive | G/G–G/A | 1.00 | 0.6 |
| | A/A | 1.29 (0.50–3.34) | |
| Overdominant | G/G–A/A | 1.00 | 0.064 |
| | G/A | 1.49 (0.98–2.27) | |
| Log-additive | – | 1.39 (0.98–1.96) | 0.061 |

*BDNF* brain-derived neurotrophic factor, *BSP* blepharospasm, *CI* confidence interval, *OR* odds ratio

Statistical significant values are given in bold

## Supplementary Results

### Meta-analysis

#### Study Selection Procedure and Characteristics of the Included Studies

The search of the PubMed database (after removal of the duplicates) yielded ten studies published between December 2008 and March 2015. Two independent reviewers (VS and ED) screened titles and abstracts. Eight studies, eligible for the current meta-analysis, were preserved. However, the study of Sako et al. (2015) was excluded (Sako et al. 2015), as it was a meta-analysis. From the seven remaining studies (Chen et al. 2013; Cramer et al. 2010; Gomez-Garre et al. 2014; Ma et al. 2013; Martino et al. 2009; Svetel et al. 2013; Groen et al. 2012), it was not possible to retrieve the genotypic frequencies for the BSP patients in three of those (Groen et al. 2012; Ma et al. 2013; Cramer et al. 2010). Therefore, four studies (Chen et al. 2013; Gomez-Garre et al. 2014; Martino et al. 2009; Svetel et al. 2013), along with the current one, were finally included in the quantitative meta-analysis, with a total of 572 BSP cases and 1745 healthy controls. Detailed characteristics of the included case–control CGAS studies are presented in Supplementary Table 1.

#### Tests of Heterogeneity

No significant heterogeneity has been observed in any of the analyses ($I^2 \leq 42.28\%$ and $p_Q \geq 0.13$). Therefore, the fixed-effects models were preferred, as extensively described in "Methods" section.

**Table 1** Allelic and genotype frequencies for BDNF rs6265 in healthy controls, in BSP cases and whole sample

| SNP | Genotypes/ alleles | Healthy controls $n = 206$ | BSP $n = 206$ | Whole sample $n = 412$ | $p$-value* |
|---|---|---|---|---|---|
| rs6265 | | $n$ (%) | $n$ (%) | $n$ (%) | |
| Genotype | G/G | 142 (0.60) | 120 (0.6) | 262 (0.65) | 0.13 |
| | G/A | 55 (0.27) | 71 (0.35) | 126 (0.31) | |
| | A/A | 8 (0.04) | 10 (0.05) | 18 (0.04) | |
| Allele | G | 339 (0.83) | 311 (0.77) | 650 (0.8) | 0.057 |
| | A | 71 (0.17) | 91 (0.23) | 162 (0.2) | |

*SNP* single-nucleotide polymorphism, *BDNF* brain-derived neurotrophic factor, *BSP* blepharospasm

*Comparison of the genotype distribution and of the allele frequency between BSP patients and HCs

### Publication Bias

Funnel plots (Supplementary Figs. 1 and 2) did not provide indication of any significant asymmetry, in any comparison. No indication of publication bias was presented either, based on the results from Egger's test ($p = 0.812$ for the overall analysis, and $p = 0.778$ for the analysis of the Caucasian/European subgroup).

### Results

The main results are depicted with forest plots in Supplementary Fig. 3 for the overall analysis, and in Supplementary Fig. 4, for the subgroup analysis of Caucasian/European cohorts. A marginal association for the rs6265 in dominant model was found. More precisely, the rs6265 is associated with an increased risk of BSP in the dominant model (GA + AA vs. GG) [OR 1.26; 95% CI 1.02–1.55, $p_z = 0.03$]. The genotypic frequencies and the respective results with the model-free approach ($OR_G$s) are shown in Supplementary Table 2. Overall, in the model-free approach, increased mutational load of the "A" allele of rs6265 denotes higher probability of a subject to develop BSP ($OR_G$ 1.26; 95% CI 1.04–1.53).

## Discussion

In the current study, we carried out a case–control study, including a notable number of participants, and we aimed to explore the effect of rs6265 BNDF on the risk of BSP. Based on our results, a marginally statistically significant implication of the rs6265 on the risk of BSP was detected. These results are also validated, after a pooled meta-analysis, with the results yielded from previous studies. Our meta-analysis vastly increases the number of meta-analyses currently available for dystonia (Siokas et al. 2017b; Gomez-Garre et al. 2014; Groen et al. 2013; Newman et al. 2012; Sako et al. 2015). However, it is only the third regarding the role of rs6265 on dystonia (Gomez-Garre et al. 2014; Sako et al. 2015), and only the second which targets the BSP focal-dystonia sub-phenotype (Gomez-Garre et al. 2014).

Previous CGASs rarely did analyze genetic variants after stratification to BSP phenotype. Consequently, only a few genetic variants have been associated with BSP. More precisely, allele 2 of the DRD5 and the D1.1 of D1 receptor gene have been associated with BSP (Misbahuddin et al. 2002). Furthermore, the minor allele rs1182 of the TOR1A gene has been found to influence the spread of BSP to adjacent body regions (Defazio et al. 2009). The most widely tested variant regarding BSP seems to be rs6265 of BDNF. Apart from the association with BSP (Chen et al. 2013), rs6265 has been reported to be associated with CD (Cramer et al. 2010) and with bilateral postural arm tremor in CD patients as well (Groen et al. 2012).

To the best of our knowledge, until today, a genome-wide association study (GWAS) in a BSP cohort has not been conducted. However, two GWASs regarding other focal dystonias have been materialized (Lohmann et al. 2014; Mok et al. 2014). According to these GWASs, there is a preliminary indication that variants across arylsulfatase G (ARSG) may confer susceptibility towards musician's dystonia, and across sodium leak channel (NALCN) towards focal CD (Lohmann et al. 2014; Mok et al. 2014). Recently, a few deleterious variants in CACNA1A, DNAH17, TRPV4, CAPN11, VPS13C, UNC13B, SPTBN4, MYOD1, and MRPL15 have been detected through whole-exome sequencing (WES) on 31 subjects from 21 independent pedigrees with BSP (Tian et al. 2018).

Absence of the rs6265 results in normal BDNF, with valine (Val) at codon 66 within the prodomain region of BNDF, leading to normal BDNF–TRKB signaling, which in turn leads to normal neuronal plasticity (Anastasia and Hempstead 2014; Hempstead 2015; Notaras et al. 2015). On the contrary, the rs6265 leads to Val66Met substitution and impairs the release of BDNF from neurons, while also changing the structure of the BDNF prodomain (Met prodomain) (Anastasia and Hempstead 2014; Hempstead 2015; Notaras et al. 2015). The decreased BDNF release leads to decreased BDNF–TRKB signaling, which leads to impaired neuronal plasticity (Anastasia and Hempstead 2014; Hempstead 2015; Notaras et al. 2015).

The current study carries also some limitations. Firstly, the analysis was materialized irrespective of the ΔGAG mutation status of the BSP subjects. Moreover, the inclusion of additional risk environmental factors in the regression models (Dardiotis et al. 2018) would have given more robust results.

In conclusion, our results provide additional evidence to the existing literature concerning the contribution of the rs6265 BDNF on the risk of developing BSP. While the underlying pathophysiology and genetic susceptibility in BSP and focal dystonia are only partially understood, it seems that BDNF may constitute one essential risk factor that is heavily involved. Replication collaborative studies and GWAS in other, preferably larger, multiethnic BSP samples are needed in order for the matter to be further elucidated.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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