Reduced 3-O-methyl-dopa levels in OCD patients and their unaffected parents is associated with the low activity M158 COMT allele.

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Running Title: Reduced 3-O-methyl-dopa levels in OCD

Background: The catechol-O-methyltransferase *(COMT)* gene is considered as a candidate gene in obsessive-compulsive disorder (OCD). Specifically, the *COMT* low-activity M158 allele has been suggested to be associated with OCD. However, there is no study reporting that COMT activity is decreased in OCD patients and that the decrease is mediated by the V158M polymorphism. Therefore, the purpose of our study was to assess COMT activity in OCD by measuring plasma levels of 3-O-methyl-dopa (3-OMD), which result from the methylation of levodopa by COMT, and to investigate the relationship between 3-OMD levels and the V158M polymorphism. We also examined whether 3-OMD levels represented an endophenotype, associated with the genetic liability to OCD, by assessing unaffected relatives of OCD patients.

Method: We assessed plasma 3-OMD levels in a sample of drug-free OCD probands (n = 34) and their unaffected parents (n = 63), and compared them with controls (n = 85). The *COMT* V158M polymorphism was genotyped in all participants.

Results: Lower plasma 3-OMD levels were found in OCD probands and their unaffected parents compared to controls. The *COMT* M158 allele was associated with reduced plasma 3-OMD levels in a co-dominant manner, both in OCD probands and their relatives, but not in controls.

Conclusion: Our results suggest that COMT activity could act as a limiting factor for the production of 3-OMD in OCD patients and in their relatives. These findings further support a role of COMT in the susceptibility to OCD and provide evidence that 3-OMD levels could represent an endophenotype in OCD.

Key words: anxiety, gene, dopamine, phenotype, methyl-transferase

INTRODUCTION

Obsessive-compulsive disorder (OCD) is characterized by recurrent and intrusive thoughts and ritualistic behaviors or mental acts that a person feels compelled to perform. Although the etiology of the disorder remains largely unknown, evidence from familial, twin and segregation studies supports that OCD has a strong genetic component [Samuels et al., 2007].

The catechol-O-methyltransferase *(COMT)* gene is considered as a candidate gene in OCD. Obsessive-compulsive symptoms are frequently reported in patients with the 22q11.2 deletion syndrome (DiGeorge/velocardiofacial syndrome), up to 32% of patients meet criteria for OCD [Gothelf et al., 2004]. Among the genes located in the 3 Mb most frequent deletion is *COMT*, involved in the catabolism of norepinephrine and dopamine neurotransmitters [Guldberg and Marsden 1975, Kobrynski and Sullivan 2007]. In addition, dopamine abnormalities have been implicated in the pathophysiology of OCD on the basis of several lines of clinical evidence [Westenberg *et al.*, 2007]. These include the high incidence of OC behavior in a number of basal-ganglia-related disorders, such as Tourette syndrome [Grados et al., 2008], changes in OC symptoms following the administration of the dopamine reuptake inhibitor amphetamine to OCD patients [Insel et al., 1983], and the therapeutic benefits obtained by co-administration of dopaminergic blockers [Bloch et al., 2006].

The *COMT* gene contains a relatively frequent single nucleotide polymorphism (SNP) in codon 158, V158M. The enzyme coded by the M158 allele is unstable at 37°C and shows four times lower activity than the enzyme coded by the V158 allele [Lotta et al., 1995]. The alleles are co-dominant, as heterozygous individuals have enzyme activity that is midway between homozygous individuals [Lachman et al., 1996]. Thus, genetically determined variations in COMT activity might affect catabolic flux of synaptic dopamine specifically in the prefrontal cortex in contrast to the striatum [Yavich et al., 2007]. Whereas in the striatum dopamine overflow is controlled by reuptake by the dopamine transporter, in the prefrontal cortex the

density of the dopamine transporter is much lower and dopamine elimination is mainly regulated by COMT. Accordingly, a recent study reported that the load of the low-activity M158 allele predicted enhanced prefrontal activity, especially in subjects with temperamental inflexibility [Drabant et al., 2006]. Thus, the high medial frontal cortex activity observed in OCD patients [Yücel et al., 2007] might be partially linked to the association observed between OCD and the low-activity M158 allele [Pooley et al., 2007]. However, there is no study reporting that *COMT* gene activity, as measured by a peripheral dopamine metabolite, is decreased in OCD patients and that the decrease is mediated by the V158M polymorphism. Furthermore, the association between the M158 *COMT* allele and OCD remains uncertain and the results are complicated by possible gender differences. The association reported by Pooley et al. [2007] was observed only in males, and a meta-analysis of all published case-control data also revealed an association between *COMT* M158 and OCD in men but not in women [Pooley et al., 2007]. However, an earlier meta-analysis failed to support an association between *COMT* and OCD [Azzam and Mathews, 2003].

In this study, we first explored the relationship between plasma 3-O-methyl-dopa (3-OMD) levels, resulting from the methylation of levodopa by COMT, and OCD. We hypothesized that plasma 3-OMD levels, reflecting COMT activity, would be decreased in OCD patients when compared to healthy volunteers. We then tested if the decrease in plasma 3-OMD levels would also be observed in the parents of OCD probands. We hypothesized that if COMT activity is genetically mediated, plasma 3-OMD levels would also be decreased in the unaffected parents of OCD patients. Finally, we explored the relationship between plasma 3-OMD levels and the *COMT* V158M polymorphism. We hypothesized that the M158 allele would be associated in a co-dominant manner to diminished 3-OMD plasma levels as previously observed *in vitro* [Weinshilboum and Raymond 1977].

MATERIALS AND METHODS

Subjects

Thirty-four drug-free OCD probands meeting DSM-IV criteria were recruited from two university affiliated psychiatry departments for children (Robert Debré hospital, Paris, France) and adults (Mondor - Chenevier hospitals, Créteil, France). The clinical and demographic characteristics of OCD probands are shown in Table 1. Lifetime psychiatric history was evaluated during a direct interview, either with the Diagnostic Interview for Genetic Studies (DIGS) [Nurnberger et al., 1994] for patients over the age of 17 years, or with the Kiddie Schedule for Affective Disorders and Schizophrenia - Epidemiologic version (K-SADS-e) [Orvaschel et al., 1982] for those aged under 17 years. The severity of the OC symptoms at the time of inclusion was scored with the Yale-Brown Obsessive Compulsive Scale [Goodman et al., 1989] (Table 1). To be included in the study, patients had to have OCD as their main disorder, with no associated depression at the time of inclusion, and have been drug-free for at least eight weeks, currently admitted as a wash-out period

In addition, 63 parents of OCD probands were also included in the study (Table 1). All parents were directly interviewed with the DIGS. Parents found to have a lifetime history of OCD, a mood disorder at the time of the interview, or who had taken a psychotropic treatment during the past eight weeks, were excluded from the study.

Controls (n=85) were healthy volunteers recruited among blood donors at the Mondor Hospital in Creteil (Table 1). They were included after being interviewed with the French version of the DIGS and the Family Interview for Genetic Studies [Maxwell ME. 1992] to confirm the absence of both personal and family history of psychiatric disorders in first- and second-degree relatives. To minimize the possibility of psychiatric morbidity among subjects in the control group, only blood donors aged over 35 years were included. All patients, parents, and controls were Caucasian. The local Research Ethics Board approved the study

protocol. Written informed consent was obtained from all participating subjects. If the proband was under 18 years old, the proband's consent and written parental consent were obtained.

Blood sampling

All participants were asked to follow a diet poor in tryptophan and serotonin during two days before the blood sampling. Blood samples (7-10 ml) were collected between 8-10 a.m. into tubes containing EDTA. Plasma was prepared within two hours by centrifugation at 2000 *g* (4°C, 15 min) and stored at –80°C for a maximum of one month until used.

Biochemical measurement

The plasma concentration of 3-OMD was measured by reverse phase high performance liquid chromatography with electrochemical detection [Michotte et al., 1987].

Genotyping

Genomic DNA was extracted from lymphoblastoid cell lines by use of the Nucleospin Blood L kit (Macherney-Nagel extraction kit, Germany). The V158M polymorphism was genotyped by direct sequencing. PCR products were sequenced with the BigDye Terminator Cycle Sequencing Kit V3.1 (Applied Biosystems, Foster City, CA, USA) in an ABI 3100 sequencer (Applied Biosystems).

Statistical analysis

Results are expressed as the median (Interquartile Range) for continuous variables and N (%) for categorical variables. All tests were two-sided at a 0.05 significance level. Deviation from Hardy-Weinberg equilibrium was assessed using a Person chi-square test. The effect of the

two main variables 'phenotype' (probands, relatives, controls) and 'genotype' (V158M, V158V, M158M) on plasma 3-OMD levels were assessed in three steps. First, the effects of phenotype on plasma 3-OMD levels were made with an analysis of covariance (ANCOVA), with age at interview as a covariate (age at interview differed between probands, relatives and controls, and was correlated to plasma 3-OMD levels). Second, the effects of genotype on the plasma 3-OMD levels were compared using a one-way ANOVA. Finally, the genotype-by-phenotype interaction was assessed by using the Pearson chi-square test since 80% of the cells have an expected frequency of 5 or greater, and that no cell has an expected frequency smaller than 1.0 [Freeman and Halton, 1951].

To study the intra-familial correlations for plasma 3-OMD levels, we used the intraclass correlation method [Fisher, 1925], with 3-OMD levels as a continuous variable. All statistical analyses were performed with the aid of SPSS/PC+ computer software.

RESULTS

Plasma 3-OMD levels in OCD probands, their unaffected parents and controls

The groups differed in age but not in gender. The OCD probands were younger than the unaffected parents and controls (Table 1). Since age showed a weak but significant correlation with 3-OMD levels ($r=0.17$, $p=0.02$), age was used as a covariate in tests of group differences. A one-way ANCOVA comparing 3-OMD levels in the three groups with age as a covariate showed significant group differences ($F=21.1$, $df=2, 171$, $p<10^{-3}$) (Figure 1). The pair-wise comparisons revealed that probands had lower plasma 3-OMD levels than their unaffected parents ($F=3.9$, $df=1, 87$, $p=0.02$) and controls ($F= 30.1$, $df=1, 116$, $p<10^{-3}$), and that unaffected parents had also lower 3-OMD level than controls ($F=15.1$, $df=1, 138$, $p<10^{-3}$) (Figure 1). These differences were not driven by a sex effect. Levels of 3-OMD did not differ between males and females among OCD probands (10.8 ± 4.4 vs 10.4 ± 5.4 ng/ml,

respectively; t=0.3, df=32, p=0.8), their relatives (14.4 ± 5.6 vs 12.4 ± 5.2 ng/ml, respectively; t=1.4, df=61, p=0.2), or controls (25.2 ± 4.1 vs 28.3 ± 4.1 ng/ml, respectively; t=0.6, df=83, p=0.5). In addition, we tested if the peripheral 3-OMD abnormalities observed in OCD probands and their parents were aggregated in OCD families. The intra-familial correlation was calculated in the subgroup of unaffected relatives who had a proband in the sample (n = 32). No significant intra-familial correlation was observed (ICC = 0.19, F=1.48, p = 0.1).

Plasma 3-OMD levels and their relationship to the COMT V158M polymorphism

V158M genotypes were in Hardy-Weinberg equilibrium in the three groups (OCD probands: Pearson χ^2=2.9, df=2, p=0.2; unaffected parents: χ^2=0.1, df=2, p=0.9; and controls: χ^2=2.1, df=2, p=0.4) and did not differ significantly between groups (χ^2=8.7, df=4, p=0.08) (Table 2).

One-way ANOVA was used to compare mean plasma 3-OMD levels between genotypes across the sample. The 3-OMD level differed among genotypes (F=21.5, df=2, 179, $p<10^{-3}$). The pair-wise comparisons revealed that M158 homozygotes had significantly lower 3-OMD levels than V158M heterozygotes (t=-5.3, df= 133, $p<10^{-3}$) or V158 homozygotes (t=-6.0, df= 81, $p<10^{-3}$) (MM: 10.9 ± 6.0, MV: 15.1 ± 5.2 and VV: 16.3 ± 5.5 ng/ml).

Plasma 3-OMD levels, interaction between the phenotype and the genotype

Because plasma 3-OMD levels vary according to the phenotype (probands, relatives and controls) and the genotype (V158M, V158V, M158M), we looked for an interaction between phenotype and genotype using a general linear model. Despite the interaction between the two variables (F=7.6, df=4, $p<10^{-3}$), the main effects of the phenotype (F=27.8, df=2, $p<10^{-3}$) and the genotype (F=23.2, df=2, $p<10^{-3}$) remained significant. As hypothesized, the M158 allele was associated with reduced plasma 3-OMD levels in a co-dominant manner, both in

OCD probands ($F=34.1$, $df=1, 31$, $p<10^{-3}$) and in relatives ($F=30.9$, $df=1, 60$, $p<10^{-3}$) (Table 2). The 3-OMD concentration in OCD patients carrying the heterozygous genotype was midway between homozygotes (MM: 4.9 ± 2.8, MV: 12.5 ± 3.0 and VV: 16.8 ± 1.7 ng/ml). Surprisingly, the V158M polymorphism did not affect plasma 3-OMD levels in controls ($F=0.9$, $df=1, 81$, $p=0.4$) (Figure 1).

DISCUSSION

Despite numerous lines of evidence suggesting that COMT could be implicated in the pathophysiology of OCD, no previous studies tested if COMT activity was decreased in OCD patients and if this decrease was mediated by the *COMT* V158M polymorphism. The aim of our study was thus to measure plasma 3-OMD levels, which have long been used as an indirect index of COMT activity [Müller et al., 2002]. We report here for the first time reduced plasma 3-OMD levels in patients with OCD and in their unaffected parents compared to controls. Furthermore, we observed that the M158 allele was associated in a co-dominant manner with reduced plasma 3-OMD levels in OCD patients and in their parents but not in controls. This suggests that COMT activity could act as a limiting factor for the production of 3-OMD in OCD patients and in their relatives but not in controls.

3-OMD level, COMT activity and OCD

The decreased levels of plasma 3-OMD reported in OCD patients and in their parents reinforce previous clinical and non clinical studies supporting a dysfunction of the dopamine system in OCD [Denys et al., 2004]. Although the role of 3-OMD in the brain remains unclear [Lee et al., 2008], 3-OMD has been extensively measured in Parkinson patients to explore the effect of COMT inhibitors on the availability of L-dopa in the brain. For example, the peripheral COMT inhibitor Entacapone improves the availability of L-dopa by 30-40%

and decreases in the same range the peripheral formation of 3-OMD in L-dopa-treated patients with Parkinson's disease [Schrag, 2005]. Thus, the low levels of 3-OMD observed in OCD patients could reflect a decreased activity of COMT. As 50% of dopamine overflow in the prefrontal cortex results from COMT-mediated enzymatic degradation [Yavich et al., 2007], the high medio-frontal cortex activity reported in OCD might be partially linked to the decreased activity of COMT. The medio-frontal hyperactivation observed in OCD patients seems to be associated with specific dimensions of inhibitory control, such as response conflict detection and resolution and sensitivity to errors [Yücel et al., 2007]. Thus, pharmacological agents that could stimulate COMT activity might be helpful in the treatment of OCD patients.

3-OMD level, V158M polymorphism and OCD

The low plasma 3-OMD level observed in OCD patients and in their parents appears to be mainly explained by the effect of the functional V158M polymorphism of *COMT*. The M158 allele was associated in a co-dominant manner with decreased plasma 3-OMD levels in OCD patients and their parents, but not in controls. The reason why the V158M polymorphism limits 3-OMD synthesis in OCD but not in controls are unclear. The collection of the samples and the technical procedures were identical between groups and can not explain the differences observed. However, a bias in our control group can not be excluded since previous studies have reported that COMT activity directly measured on red blood cells is modulated by V158M in controls [Weinshilboum, 2006]. The possible effects of environmental factors or other regulatory factors acting at higher physiological levels can not be ruled out and might explain why COMT became limiting for the production of 3-OMD in OCD subjects and their parents but not in controls. The lack of intra-famialial correlation for 3-OMD level in OCD patients and their relatives also suggests that the inheritance of plasma 3-OMD levels may be

non Mendelian and influenced by epigenetic or multifactorial factors. The investigation of COMT activity in B lymphoblastoid cell lines in future studies could help to exclude the effects of environmental or other physiological factors in the regulation of 3-OMD levels. Interestingly, our results are very similar to those showing that another methyl-transferase implicated in the methylation of serotonin (acetylserotonin methyltransferase, ASMT) could be involved in the reduced plasma melatonin levels observed in individuals with autism spectrum disorders [Melke et al., 2008]. Reduced plasma melatonin concentration was correlated with a deficit in ASMT activity in patients with autism spectrum disorders and their parents but not in controls. The mechanisms through which these two methyltransferases – very similar in their functions – might be implicated in psychiatric disorders remain unknown. The similarity in the dysfunctions of these enzymes in OCD and autism spectrum disorders might point out to a common pathway, such as epigenetic regulators, that could be implicated in both disorders [Tsankova et al., 2007], which share some clinical behaviors (e.g. insistence on sameness and repetitive behaviors) [Russel et al., 2005].

A recent meta-analysis revealed that the low expression M158 allele was associated with OCD in males but not in females [Pooley et al., 2007]. However this meta-analysis did not include the study by Alsobrook et al., [2002], in which M158 was associated with OCD in females but not in males. Although we did not observe a major gender effect on 3-OMD levels, the size of our sample limits our ability to sub-stratify our results according to genotypes to further investigate the effect of gender on our results.

3-OMD: an endophenotype for OCD?

As we hypothesized, plasma 3-OMD levels were decreased in the unaffected parents of OCD patients. The over transmission of the *COMT* low activity allele from parents to children [Pooley et al., 2007] could explain the observed 3-OMD level abnormalities in OCD probands

and in their unaffected relatives. Thus, low plasma 3-OMD levels could represent an endophenotype of OCD: its level is correlated with the phenotype in patients, it is modified among the unaffected relatives of OCD patients at a higher rate than in the general population, and involves the *COMT* V158M polymorphism [Leboyer et al., 2003; Gottesman and Gould, 2003]. It is now established that COMT activity represents a risk factor for the dysfunction of the neostriatal-prefrontal circuit and is more related to the episodic and working memory dysfunction than to the psychiatric phenotype [Meyer-Lindenberg and Weinberger 2006]. This could explain the working memory deficits in the Tower of London test we reported recently in the unaffected parents of OCD probands [Delorme et al., 2007]. Unfortunately, due to the limited number of relatives that accepted to participate in all the biochemical, genetic and cognitive evaluations, we were unable to explore the relationship between genotypes, plasma 3-OMD level and Tower of London scores.

Limitations

Although non significant, differences in V158M genotype distribution between groups might affect our results. Specifically, the number of probands carrying the VV genotype is small. However, the global validity of our results is not likely to be influenced by this limited sample of VV probands since the effect of the V158M genotype on plasma 3-OMD levels was similar in probands and in relatives, and the V158M genotype distribution was comparable in unaffected parents and in healthy volunteers. Another limitation of our results is that we only genotyped the V158M polymorphism and it is now established that additional SNPs in the *COMT* gene modulate COMT activity by altering mRNA secondary structure [Nackley et al., 2006]. Two polymorphisms, one located in the P2 promoter region (rs2097603) and one in the 3'UTR region (rs165599), seem to affect prefrontal activity independently from the V158M polymorphism [Meyer-Lindenberg et al., 2006]. However, the V158M polymorphism

accounts for the larger effect on the overall functional state of the *COMT* gene. A further limitation is the study of a peripheral marker such as plasma 3-OMD levels, which may only partially reflect central nervous system processes. Finally, the use of 3-OMD as an index of COMT activity could be considered an additional limitation. A direct estimation of COMT activity needs to be performed in future works. Most studies use a radiometric assay for the determination of COMT activity on brain tissues or erythrocytes [Zurcher and Da Prada 1982]. Unfortunately, we did not collect the red blood cells of our patients in our study.

Conclusion

In conclusion, our results support the evidence that COMT may play a role in the susceptibility to OCD. Further efforts should be made to clarify the potential implication of COMT in the disorder. Plasma 3-OMD levels, easy to measure in patients and relatives, may help to explore the heterogeneity of the OCD phenotype and delineate more homogenous groups that could help to conclude to the unequivocal implication of the *COMT* gene in OCD.

DISCLOSURE/CONFLICT OF INTEREST

All authors have no financial interests or potential conflicts of interest to declare.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Relation between plasma 3-O-methyldopa levels in drug-free OCD patients (n=34), their unaffected parents (n=63) and controls (n=85) and the *COMT* V158M polymorphism. Data represent distribution and mean.