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## Epistasis Between Polymorphisms in COMT, ESR1, and GCH1 Influences COMT Enzyme Activity and Pain

Shad B. Smith<sup>1</sup>, Ilkka Reenilä<sup>2</sup>, Pekka T. Männistö<sup>2</sup>, Gary D. Slade<sup>1</sup>, William Maixner<sup>1</sup>, Luda Diatchenko<sup>1,3</sup>, and Andrea G. Nackley<sup>1,\*</sup>

<sup>1</sup>Center for Pain Research and Innovation, School of Dentistry, University of North Carolina, Chapel Hill, NC, 27599 USA <sup>2</sup>Division of Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki, Helsinki, FIN-00014 Finland <sup>3</sup>Alan Edwards Pain Centre For Research on Pain, McGill University, Montreal, Canada H3A 0C7

### Abstract

Abnormalities in the enzymatic activity of catechol-*O*-methyltransferase (COMT) contribute to chronic pain conditions, such as temporomandibular disorders (TMD). Thus, we sought to determine the effects of polymorphisms in *COMT* and functionally-related pain genes in the COMT pathway (estrogen receptor 1: *ESR1*, guanosine-5-triphosphate cyclohydrolase 1: *GCHI*, methylenetetrahydrofolate reductase: *MTHFR*) on COMT enzymatic activity, musculoskeletal pain, and pain-related intermediate phenotypes among TMD cases and healthy controls. Results demonstrate that the *COMT* rs4680 (*val*<sup>158</sup>*met*) polymorphism is most strongly associated with outcome measures, such that individuals with the minor A allele (*met*) exhibit reduced COMT activity, increased TMD risk, and increased musculoskeletal pain. Epistatic interactions were observed between the *COMT* rs4680 polymorphism and polymorphisms in *GCHI* and *ESR1*. Among individuals with the *COMT met* allele, those with two copies of the *GCHI* rs10483639 minor G allele exhibit normalized COMT activity and increased mechanical pain thresholds. Among individuals with the *COMT val* allele, those with two copies of the *ESR1* rs3020377 minor A allele exhibit reduced COMT activity, increased bodily pain, and poorer self-reported health. These data reveal that the *GCHI* minor G allele confers a protective advantage among *met* carriers, while the *ESR1* minor A allele is disadvantageous among *val* carriers. Furthermore, these data suggest that the ability to predict the downstream effects of genetic variation on COMT activity is critically important to understanding the molecular basis of chronic pain conditions.

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Corresponding Author: Andrea G. Nackley, Center for Pain Research and Innovation, School of Dentistry, CB 7450, University of North Carolina, Chapel Hill, NC 27599-7450, Phone: (919) 537-3204, Fax: (919) 966-5339, andrea\_nackley@dentistry.unc.edu.

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## Keywords

catecholamines; gene-gene interaction; gene expression; temporomandibular disorders (TMD); fibromyalgia

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## Introduction

Catechol-O-methyltransferase (COMT) is a ubiquitously expressed enzyme that regulates the bioavailability of catecholamines, playing a key role in maintaining basic physiologic functions such as sympathetic tone, mood, and inflammation, that influence pain. Through alternative promoters and translation initiation sites, the *COMT* gene yields two distinct proteins: soluble COMT (S-COMT) and membrane-bound COMT (MB-COMT) [42,62]. The *COMT* gene locus is rich with single nucleotide polymorphisms (SNPs), several of which have been associated with chronic pain and pain-related phenotypes.

Most *COMT* genetic association studies have focused on the common rs4680 SNP. The rs4680 A allele has been associated with increased susceptibility to post-surgical pain [1,24,32], fibromyalgia [3,15,29,44], and arthritis [65]. Additionally, rs4680 has been associated with clinically relevant intermediate phenotypes such as experimental pain [72], anxiety [23], depression, and attention [67]. The A allele produces a nonsynonymous change from valine to methionine at codon 158 (*val*<sup>158</sup>*met*), resulting in a less thermostable enzyme with reduced activity [41]. Studies have consistently shown that the *met* allele codes for a 25–40% reduction in enzyme activity measured in human red blood cells [53,59,69] and tissues [7] as well as in lysates from transfected cells [41,46,56]. Thus, the *val*<sup>158</sup>*met* allele represents a major source of individual variation in COMT activity.

More recently, investigators have identified additional polymorphisms in the *COMT* gene locus that, while not independently associated with pain, interact with the *val*<sup>158</sup>*met* allele. Haplotypes consisting of one SNP in the S-COMT promoter (rs6269 G/A) and two SNPs in the coding region (rs4633 C/T and rs4818 C/G) together with rs4680 G/A have been associated with clinical and experimental pain. Specifically, the ATCA and ACCG haplotypes have been associated with susceptibility to post-surgical pain [26], fibromyalgia [66], and temporomandibular disorders (TMD) [19] as well as experimental pain sensitivity [19] and treatment response [60]. *In vitro* studies have shown that the ACCG haplotype codes for an 80% reduction in enzymatic activity due to formation of a stable mRNA secondary structure that is more resistant to translation [46]. Thus, SNPs in distinct *COMT* gene loci can act alone or in concert to influence enzyme activity and pain.

Emerging evidence suggests that the size and direction of *COMT* polymorphic effects can be modified by epistatic interactions with genes in convergent molecular pathways. For example, the effects of the *met* allele on disease risk have been shown to be augmented by a low activity variant of the methylenetetrahydrofolate reductase (*MTHFR*) gene, which regulates the bioavailability of S-adenosyl-L-homocysteine (SAH; a strong, noncompetitive inhibitor of COMT) [45]. Thus, we hypothesize that polymorphisms in *MTHFR* and functionally-related genes implicated in pain, including estrogen receptor 1 (*ESR1*; which regulates *COMT* gene expression [35,70]) and guanosine-5-triphosphate cyclohydrolase 1

(*GCHI*; a rate-limiting enzyme for a cofactor for catecholamine synthesis [47]), interact with polymorphisms in *COMT* to influence *COMT* enzymatic activity and pain.

In order to test this hypothesis, the present study characterized the relationship between SNPs located in genes of the *COMT* pathway (*COMT*, *MTHFR*, *ESR1*, and *GCHI*), *COMT* enzymatic activity, musculoskeletal pain, and related intermediate phenotypes.

## Materials and Methods

### Subjects

Subjects were 398 white females aged 18–60 years enrolled in a case-control study of TMD. These subjects were recruited between 2005–2009 in Chapel Hill, NC, using advertisements placed in local newspapers and flyers posted in and around the UNC Health Center, including tertiary care clinics that provide treatment for people with chronic orofacial pain. All subjects signed written informed consent for experimental protocols and for genotyping and biomarker analysis, and gave a blood sample. A detailed overview of recruitment protocols including case definition and exclusion criteria has been described previously [57]. Briefly, cases (n=200) had to report facial pain for at least five days during the previous two weeks and be diagnosed with TMD arthralgia or myalgia following the Research Diagnostic Criteria (RDC) for TMD [21]. Digital palpation of muscle and joint cranial sites was performed using a force of 0.45 kg. Controls (n=198) had no self-reported history of orofacial pain within the preceding six months and no diagnosis for TMD either prior to or during the study examination. Enrolment was limited to females due to the higher prevalence of TMD in women than men [10], and to whites to avoid problems of population stratification in assessing genetic associations.

### Assessment of Bodily Pain and Experimental Pain

At the time of the clinical examination, all study subjects were evaluated for the presence of bodily pain. Three pounds of digital palpation pressure were applied bilaterally for 2 seconds to each of the following predefined locations modeled after ACR's 1990 criteria for fibromyalgia tender points examination [71]: occiput, trapezius, supraspinatus, lower cervical, second rib, lateral epicondyle, knee, gluteal, and greater trochanter. At each location, a response of pain to palpation was recorded.

Following the clinical examination, study subjects completed a battery of quantitative sensory testing (QST) assessments. First, thermal pain was assessed by applying a thermode to the forearm that increased in temperature at a rate of 0.5°C/sec. Thermal pain threshold was defined as the temperature at which pain was first reported, while tolerance was defined as the highest temperature that could be tolerated (with an upper limit of 50°C). Second, central sensitization was assessed using repeated thermal stimuli of 50°C applied to the right hand.[49] Pain was rated on a 0–100 numerical rating scale (NRS) after each of 10 pulses, of 0.5 sec duration repeated once every three seconds. Plots of each subject's pattern of response at each temperature were used to derive two summary measures of temporal "windup" (highest NRS response minus lowest NRS response and slope of a linear regression line fitted for the first five NRS responses) and two summary measures of

thermal sensitivity (first NRS and sum of all NRS responses). Third, pressure pain thresholds in response to a hand-held pressure algometer [34] were assessed bilaterally at two extracranial sites: trapezius muscles and the lateral epicondyle. Pressure threshold at each site was recorded as the algometer loading (kg) at which pain was first detected. Descriptive characteristics of bodily pain and experimental pain phenotypes measured in TMD cases and controls enrolled in this study have been published previously [11,12,57].

### Assessment of Self-reported Pain, Health, and Psychological Mood

Subjects completed several questionnaires to assess the severity of their pain and pain-related traits. Pain intensity and unpleasantness was measured by the Gracely Pain Scale, which asks subjects to report their lowest, average, and maximal pain on a scale of 0 to 100 [28]. Self-ratings of physical and mental health were measured by the Short Form 12 version 2 (SF-12v2), which asks questions in six domains: global health rating, physical functioning, physical roles, emotional functioning, emotional roles, and pain interference. Published scoring algorithms [68] were used to compute scores for each domain, ranging from 0 to 100, with higher values signifying better function and health. Stress was measured by the Perceived Stress Scale (PSS), which contains 10 questions about frequency of daily stressors on a five-point scale ranging from “never” to “very often” [16]. Responses were summed for an overall score. Additional domains of stress were measured by the Symptoms Checklist-90 Revised (SCL-90-R), which consists of 90 items that describe a feeling or thought, scored on a 5 point scale ranging from “not at all distressed” to “extremely distressed” [17]. It provides ratings of psychological distress in nine symptom areas: somatization, obsessive-compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. Psychological mood was measured by the Profile of Mood States- Bi-Polar (POMS), which consists of 72 questions used to compute six subscales of mood-related states (e.g., elated-depressed and energetic-tired) [39]. Descriptive characteristics of self-reported pain, health, and psychological mood phenotypes measured in TMD cases and controls enrolled in this study have been published previously [11,12,57].

### Genotyping

DNA extracted from whole blood donated by study subjects was genotyped by Beckman-Coulter Genomics (Morrisville, NC) using the Algnomics Pain Research Panel v.1.0 (<http://algnomics.com/pain-research-panel.html>). This platform is a dedicated array for the targeted assessment of genes involved in acute and chronic pain conditions, utilizing the Affymetrix MegAllele platform. The panel genotypes 3295 SNPs in 358 candidate genes whose protein products are linked to biological pathways that influence pain transmission, inflammatory response, or psychological state. Genotyping calls were checked for quality using utilities implemented in PLINK version 1.07. [50] All 398 samples exhibited Caucasian ancestry and an overall genotype call rate < 95%. All SNPs used for this study exhibited a call rate >95%, minor allele frequency > 1% in either case or control groups, and agreement across repeated samples >98%. The potential for population stratification to influence association statistics was assessed by calculating the lambda statistic [18]; no evidence was found for inflation of test statistics ( $\lambda < 1.0$ ).

This analysis was limited to four genes directly or indirectly involved in regulating COMT activity: *COMT*, *ESR1*, *GCHI*, and *MTHFR*. Within the *COMT* gene locus, 9 SNPs passed quality filters (Supplemental Table 1: table of COMT activity associations). The rs4633 SNP also passed quality filters but was in very strong ( $r^2=1.0$ ) LD with rs4680 and so was not included in further analysis. Within the *ESR1*, *GCHI*, and *MTHFR* loci, 27, 10, and 7 SNPs, respectively, passed quality filters. For the complete list of *ESR1*, *GCHI*, and *MTHFR* SNPs, please refer to Supplemental Table 4.

### COMT Activity Assay

S-COMT activity was measured in red blood cells (RBCs) following the method of Schultz et al. [55] with some modifications [64]. RBCs were hemolyzed in four volumes of 1 mM sodium phosphate buffer (instead of water), enzyme incubation was performed in pH 7.4 (instead of 7.8) for 60 min, using 500  $\mu$ M of 3,4-dihydroxy benzoic acid (DHBAC; instead of 400  $\mu$ M) as a substrate, and the samples were filtered with 0.45  $\mu$ M pore size membranes using syringe before HPLC. In principle, vanillic acid generated from the DHBAC substrate was quantified with a HPLC system. It consisted of a sample autoinjector (Shimadzu, Prominence SIL-20AC, Kyoto, Japan), a pump (Jasco pu- 2080, Tokyo, Japan), an RP-18 column (3  $\mu$ m, 4.6  $\times$  100 mm; Waters Spherisorb, Milford, MA, USA) with precolumn, column heater (Croco-Cil, Bordeaux, France), a coulometric detector (ESA model Coulochem III detector and a model 5011A cell; ESA Inc., Chelmsford, MA, USA; E1 detector potential was  $-0$  V and that of E2 analytical detector  $+0.5$  V) and Azur 5.0 software (Datalys, France). The mobile phase consisted of 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 3.3), 0.15 mM EDTA and 25% methanol; the flow-rate was 1.0 ml/min. The protein levels of the samples were analyzed using the Bradford assay [8]. COMT activity was expressed as pmol of vanillic acid formed per mg of protein in one minute.

This RBC-COMT assay has been validated with human samples containing either normal or low COMT levels. Intra-assay coefficient of variation (CV%) was typically <5% and in inter-assay <15%. Assay was highly linear for vanillic acid ( $r = 0.9996-0.9998$ ) and precision CV% for vanillic acid was 0.3% at 2  $\mu$ M and 4.8% at 0.2  $\mu$ M.

### Statistical Analysis

Genetic association analyses were performed using PLINK and JMP, Version 7 (SAS Institute Inc., Cary, NC) software packages. Haplotype phasing was performed in PLINK, using *COMT* SNPs rs6269 and rs4633 to construct haplotypes Low Pain Sensitivity (LPS), Average Pain Sensitivity (APS), and High Pain Sensitivity (HPS), as described previously. [19] The phasing algorithm succeeded in phasing all haplotypes and diplotypes with no ambiguity. Association tests were conducted by logistic (for binary case-status variables) or linear (for quantitative variables) regression, to test the null hypothesis that outcome measures were equal between genotype groups. Regression models included age as a covariate for variables that were associated with age in a univariate analysis.

Primary analyses were conducted to explore the relationship between *COMT*, *ESR1*, *GCHI*, and *MTHFR* genotype with COMT activity and case status. We first assessed the effects of all genotyped *COMT* polymorphisms and haplotypes of *COMT* on erythrocyte COMT

activity, using a codominant model. For subsequent analyses of *COMT* genetic effects and epistasis, we used the SNP rs4680 (*val*<sup>158</sup>*met*), which results in high activity (valine) and low activity (methionine) variants of *COMT*. To follow the usual convention regarding variant effect direction, statistical analyses on this SNP considered the *val* allele as the referent and the *met* allele as the variant, although in this sample the *met* allele was actually more frequent. We also analyzed SNPs in three *COMT* pathway genes, *MTHFR*, *ESR1*, and *GCHI*, using univariate tests of association. For these SNPs, we screened codominant genetic models and compared our results with previous reports in the literature showing evidence for alternate models of inheritance. We then constructed 2-way interaction models between rs4680 and SNPs within *MTHFR*, *ESR1*, and *GCHI* to identify SNPs in these genes that modulate the effects of *val*<sup>158</sup>*met* on *COMT* activity and TMD risk, and selected the SNP in each gene with the strongest evidence for interaction. For clarity of presentation, we modeled interaction effects by testing genotype differences in pathway gene SNPs within carriers of either *val* (for *ESR1*) or *met* (for *GCHI*).

Secondary analyses were also conducted to explore the relationship between *COMT* pathway genotypes and intermediate phenotypes relevant to TMD. We tested the effects of SNPs in *COMT*, *ESR1*, *GCHI*, and *MTHFR* on intermediate variables of pain sensitivity and psychological distress. Finally, exploratory analyses were conducted to test for epistatic effects of *val*<sup>158</sup>*met* and selected SNPs in *ESR1* and *GCHI* on *COMT* activity and TMD risk. We constructed a 3-way interaction model that contained three main effect terms for *val*<sup>158</sup>*met*, rs3020377 (dominant), and *GCHI* (recessive); three 2-way interaction terms for each pairwise combination of the three loci; and one 3-way interaction term.

For analyses of genetic association, we set the threshold for significance at an uncorrected p-value of  $p < 0.05$ . Although this does not account for the number of tests performed, the purpose of the study was to characterize the interactive effects of multiple genes on an array of nociceptive and affective phenotypes in order to detect consistent patterns among genotype groups. The analyses were designed to reproduce previous significant findings and extend them by investigating more complex epistatic interactions in a broad set of outcome measures. However, given the increased risk of false positives, novel results should be considered exploratory until confirmed in independent cohorts.

## Results

Primary analyses were focused on exploring the relationship between *COMT*, *ESR1*, *GCHI*, and *MTHFR* genotype with *COMT* activity and case status. Secondary analyses were focused on exploring the relationship between these *COMT* pathway genotypes and intermediate phenotypes (e.g., pain and mood) relevant to TMD.

### Effect of *COMT* genotype on *COMT* activity

*COMT* activity level was strongly associated ( $p < 0.001$ ) with almost all of the *COMT* SNPs, including rs4680 *val*<sup>158</sup>*met*, (Supplementary Table 1) as shown previously [7,41,46,53,56,59,69]. Each additional copy of the *met* allele resulted in a 0.31 pmol/min/mg protein decrease in measured *COMT* activity (Fig. 1A). Haplotype analysis likewise showed that the subjects homozygous for the APS haplotype (which contains *met* at rs4680) had the



lowest COMT activity, while subjects with two copies of LPS or HPS (which contain *val* at rs4680) had the highest activity (Supplementary Fig. 1).

The independent effects of the individual SNPs are confounded by linkage disequilibrium with *val*<sup>158</sup>*met*, the SNP with the largest effect on COMT activity (Fig. 1B), so each SNP was tested in a regression model that included terms for a main effect of rs4680 and an interaction between rs4680 and the respective SNP. After adjustment for *val*<sup>158</sup>*met*, most of the SNPs failed to show an independent effect on COMT (Fig. 1C and Supplementary Table 1), although the direction of effect of several SNPs that appeared to increase COMT activity showed a smaller effect in the opposite direction. For the representative SNP rs737865, the presence of one or two copies of the minor G allele decreased COMT activity across all genotypes of *val*<sup>158</sup>*met* (Fig. 1D), with an overall effect of  $-0.07$  pmol/min/mg protein per copy of the G allele ( $p < 0.001$ ). No *COMT* SNPs showed a significant interaction with *val*<sup>158</sup>*met*.

### Effect of *val*<sup>158</sup>*met* on pain phenotypes

In addition to COMT activity, we tested association between rs4680 and the primary outcome measure of TMD case status (Supplementary Table 2). The SNP was associated with TMD after adjustment for age, with the *val* allele being protective (OR = 0.72,  $p = 0.032$ ). We also examined the effects of this SNP on a battery of QST and psychosocial profile measures, as a secondary descriptive analysis. Of these intermediate variables, only tests of sensitivity to mechanical pressure were significantly associated, including pressure point thresholds taken at temporalis (age-adjusted beta =  $-0.13$ ,  $p = 0.027$ ), masseter (age-adjusted beta =  $-0.14$ ,  $p = 0.026$ ), TMJ (beta =  $-0.13$ ,  $p = 0.037$ ) and lateral epicondyle (beta =  $-0.24$ ,  $p = 0.007$ ) sites.

### Effect of *ESR1*, *GCH1*, and *MTHFR* genotype on COMT activity

Three genes contained in the genotyping panel in addition to *COMT* were selected for analysis due to their known involvement in COMT regulation and pain: *ESR1*, *GCH1*, and *MTHFR*. We examined 43 SNPs from these genes for genotype effects on COMT enzymatic activity (Supplementary Table 3). Three SNPs from the *ESR1* gene were associated with COMT activity, including rs3020377 (beta =  $-0.05$ ,  $p = 0.04$ ), rs726281 (beta =  $-0.06$ ,  $p = 0.02$ ) and rs3020407 (beta =  $-0.06$ ,  $p = 0.02$ ) (Fig. 2). These SNPs are correlated due to moderate LD between them (Supplementary Fig. 2), and likely represent a single effect.

### 2-way interactions on COMT activity: *COMT* × *ESR1*/*GCH1*/*MTHFR*

The *val*<sup>158</sup>*met* polymorphism exerts the greatest regulatory effect on COMT enzymatic activity, but the magnitude of this effect may be modified by other genes in convergent pathways. In order to identify variants that modulate the effect of *val*<sup>158</sup>*met* on COMT activity, 2-way interaction models were constructed between this SNP and the SNPs in the selected COMT pathway genes (Supplementary Table 3). In *MTHFR*, two SNPs showed interaction ( $p < 0.01$ ): rs2274976, an *arg*<sup>594</sup>*gln* non-synonymous change, and rs2066470, a *pro*<sup>39</sup>*pro* synonymous variant. There was no significant interaction with rs1801133 ( $p = 0.48$ ), which has been previously shown to interact with *val*<sup>158</sup>*met* to influence schizophrenia risk [45]. Two SNPs in *ESR1* exceeded the  $p < 0.05$  threshold for the

interaction, including rs6912184 ( $p=0.049$ ) and rs1884052 ( $p=0.035$ ). The SNPs associated with COMT activity in the previous main effect analysis did not show interaction with *val*<sup>L58</sup>*met*. However, further analysis of the SNP rs3020377 using a dominant model showed that carriers of the minor G allele had significantly lower levels of COMT activity in *val* carriers ( $p=0.0021$ ), but no difference in *met* carriers. In *GCHI*, rs10483639 was the most strongly associated ( $p=0.003$ ) in a locus of three neighboring SNPs (including rs752688, rs4411417, rs8007201) in the same haploblock (Supplementary Fig. 1b).

We selected one SNP from each of the three genes to carry forward to analysis of pain and pain-related intermediate phenotypes. In this selection step, in deciding between SNPs showing strong association test  $p$ -values, we prioritized SNPs with existing evidence for biological relevance to COMT activity and nociceptive pathways. For *MTHFR*, none of the associated SNPs were previously shown to interact with COMT; rs2066470 was chosen because it was the SNP with the highest MAF among those with strong interaction test  $p$ -values, in order to maximize power to see effects in subjects carrying the minor allele. The *ESR1* SNP rs3020377 was selected due to a strong effect on COMT activity in the high-activity *val* group alone, as well as a previous report that this SNP interacts with *val*<sup>L58</sup>*met* to influence endocrine-dependent mood disorders [33]. For *GCHI*, rs10483639 was selected as it showed a strong interaction with *val*<sup>L58</sup>*met*, and because it is a marker of a risk haplotype identified previously [43]. This haplotype was reported to have a recessive inheritance, so for consistency with these findings we used this model for subsequent association tests with rs10483639.

### **Effect of ESR1 genotype on COMT activity and pain phenotypes among COMT (*val*) carriers**

The significant difference between *ESR1* rs3020377 genotypes within *val* carriers suggests that variation in the estrogen receptor gene modulates COMT enzymatic activity in subjects with a genetic predisposition toward high activity due to the rs4680 *val* allele, but does not affect COMT activity in subjects with overall low activity (Fig. 3A). We investigated this interaction model in the panel of pain-related phenotypes. We observed similar interactions, with effects in carriers of the *val* allele, in several clinical measures of pain. Relative to *val* carriers with no copies of the rs3020377 minor G allele, subjects with 1 or 2 copies of the G allele reported higher scores on the SF12 physical functioning subscale ( $p = 0.017$ , Fig. 3B), as well as higher pain intensity ( $p = 0.018$ , Fig. 3C) and a trend toward higher pain unpleasantness ratings ( $p = 0.07$ , Fig. 3D) using the Gracely Scale. The number of body sites with pain upon palpation was also higher in *val* carriers with 1 or 2 copies of the G allele, although this trend was nonsignificant ( $p=0.08$ , Fig. 3E).

### **Effect of GCHI genotype on COMT activity and pain phenotypes among COMT (*met*) carriers**

In contrast with the *ESR1* modulatory effect observed in *val* carriers, modulatory effects of the *GCHI* SNP were observed within carriers of the rs4680 *met* allele. Subjects with 0 or 1 copy of the minor C allele of rs10483639 had significantly lower levels of COMT activity ( $p = 0.0030$ ) than homozygous carriers of the C allele (Fig. 4A). These subjects also had a reduced threshold for algometer pressure pain across all tested sites, although only the



association with the temporalis site was significant ( $p = 0.031$ , Fig. 4B). However, subjects that were homozygous for C were more likely to score negatively on measures of mood and affect, including the depression ( $p = 0.025$ , Fig. 4C) and hostility ( $p = 0.017$ , Fig. 4D) subscales of the SCL90, the elated-depressed ( $p = 0.016$ , Fig. 4E) and energetic ( $p = 0.025$ , Fig. 4F) axes of the POMS, and the distress domain on the PSS ( $p = 0.038$ , Fig. 4G). These subjects also reported a decreased ability to function in daily life due to emotional problems on the SF12 ( $p = 0.049$ , Fig. 4H).

### Effect of MTHFR genotype on COMT activity and pain phenotypes among COMT (val/met) carriers

Interaction tests were also performed between the *MTHFR* SNP rs2066470 and *val*<sup>158</sup>*met* as with the *ESR1* and *GCHI* SNPs, with strong effects observed in *met* carriers between rs2066470 genotypes on multiple clinical, affective, and molecular phenotypes. However, despite selecting this SNP due to its relatively greater MAF within the gene locus, there were still not enough subjects to make statistical comparisons between genotype groups (data not shown).

### Three gene interaction on COMT activity and TMD risk

Finally, we constructed 3-way genetic interaction models to evaluate the main effects and interactions of *COMT val*<sup>158</sup>*met*, *ESR1* rs3020377 (using a dominant model), and *GCHI* rs10483639 (recessive) on COMT enzymatic activity and TMD risk. We did not include *MTHFR* in further tests of epistasis due to low frequency of the minor allele of the selected SNP. Significant effects were observed on COMT activity (Fig. 5A) for the main effects of the *COMT* ( $p < 0.001$ ) and *ESR1* ( $p=0.013$ ) SNPs, and the interaction term for *COMT* × *GCHI* ( $p = 0.029$ ); a trend for an association for *COMT* × *ESR1* approached significance ( $p = 0.058$ ). The 3-way interaction term was not significant ( $p = 0.75$ ). For the TMD variable (Fig. 5B), a pattern was observed of coinciding higher COMT activity and lower TMD risk due to variation in *GCHI*, in the *met* carriers only. In *val* carriers, there was an apparent relationship between *ESR1* and *GCHI* on COMT activity, such that the *GCHI* major allele increased COMT activity in *ESR1* major allele homozygotes only, although the *GCHI* minor allele coincided with decreased TMD frequency in both *ESR1* groups. However, none of the main effect or interaction terms were significantly associated with TMD, so these preliminary findings require further investigation in larger cohorts to confirm.

## Discussion

COMT plays a critical role in the development and maintenance of pain. Understanding how variability at the genetic level maps onto variability at the functional level of this enzyme, will provide insight into the molecular mechanisms that drive maladaptive pain conditions, such as TMD. Here, we identify SNPs in *COMT* and *ESR1* that are independently associated with COMT enzymatic activity. Consistent with our hypothesis, we also identify interactions between SNPs in *COMT* and those in functionally-related genes that influence COMT enzymatic activity and pain.

## Polymorphisms in *COMT* and *ESR1* Genes are Independently Associated with *COMT* Enzymatic Activity

Our results demonstrate that the major source of individual variation in *COMT* activity is driven by the *val*<sup>158</sup>*met* allele. The number of *met* alleles was dose-dependently associated with *COMT* activity, such that individuals with one allele exhibited a 27% reduction and those with two alleles exhibited a 55% reduction. These results are consistent with those from other studies, demonstrating that the *met* allele codes for a more thermolabile *COMT* enzyme [7,41,46,53,56,59,69].

Of the remaining 8 *COMT* SNPs genotyped, only the rs737865 A/G allele independently accounted for additional variation in *COMT* activity. After adjusting for the effects of *val/met*, a modest association between rs737865 and *COMT* activity was revealed, such that individuals with two copies of the G allele exhibited a 18.4% reduction. The rs737865 SNP is positioned in intron 1 of *MB-COMT* and, while direct evidence for potential functional effects of rs737865 is lacking, the G allele has been associated with decreased *COMT* expression in human brain [9]. Additionally, a clinical study of pain following motor vehicle collision demonstrated that a haplotype containing the rs737865 G allele was predictive of higher pain interference.

Common *COMT* haplotypes were also tested for association with *COMT* activity. While previous studies by our group showed that cells expressing the ACCG *COMT* haplotype (constructed from rs6269, rs4633, rs4618, and rs4680) exhibited an 80% reduction in *COMT* activity, here we found no link between *COMT* haplotype and circulating *COMT* activity. This discrepancy between *in vitro* and *in vivo* effects of ACCG haplotype on *COMT* activity is likely due to methodological differences in the two studies. The initial *in vitro* studies established a clear link between ACCG and *COMT* activity in isolated cell lines overexpressing the construct [46]. However, subsequent *in vitro* studies found that the extent by which the ACCG haplotype down-regulated *COMT* expression was cell line-dependent [63]. *COMT* activity as determined in the present *in vivo* study reflects cumulative regulation by a chorus of genetic variants expressed in red blood cells.

Finally, we evaluated SNPs in genes whose products regulate the expression or activity of *COMT*. Of the 43 SNPs in *MTHFR*, *ESR1*, and *GCHI*, we identified 3 SNPs in intron 4 of *ESR1* that were associated with *COMT* enzymatic activity. These included the rs3020377 A/G allele, the rs726281 A/G allele, and the rs3020407 A/G allele. Individuals with one or two copies of the rs3020377 minor G allele exhibited an 8% reduction in *COMT* activity. Individuals with the rs726281 or rs3020407 minor G allele exhibited a dose-dependent decrease in *COMT* activity, such that those with one copy had an 8% reduction and those with two copies had a 12% reduction. As all three *ESR1* SNPs are in close proximity and moderate LD, their effects are unlikely to be independent. *ESR1* can bind to estrogen response elements (EREs) in the promoter region of *COMT* to downregulate its transcription [35,70]. Thus, we speculate that rs3020377 G, rs726281 G, rs3020407 G, or another SNP in high linkage with those tested codes for increased levels of *ESR1*, resulting in the subsequent downregulation of *COMT*.

## Polymorphisms in Genes of the COMT Pathway Interact to Influence COMT Enzymatic Activity, Musculoskeletal Pain, and Mood

While several distinct alleles in *COMT* and *ESR1* were independently associated with COMT activity, the interaction analyses were focused on *val*<sup>158</sup>*met* because it accounted for the greatest degree of individual variation. Our results demonstrate that the net size and direction of *val/met* effects are modified by epistatic interactions with SNPs in convergent molecular pathways. We identified a significant interaction between the *COMT val*<sup>158</sup>*met* allele and the *ESR1* rs3020377 A/G allele. Among individuals with the *COMT val* allele, those with two copies of the *ESR1* minor G allele exhibited reduced COMT activity, increased bodily pain, and poorer self-reported health. While this is the first study to demonstrate an interaction between *COMT val* and *ESR1* rs3020377 with respect to COMT activity and pain, our findings are in line with a previous report showing that rs3020377 interacts with *val/val* (but not *val/met* or *met/met*) to influence risk of premenstrual dysphoric disorder [33].

We also identified a significant interaction between the *COMT val*<sup>158</sup>*met* allele and the rs10483639 G/C allele, located in the 3'UTR of *GCHI*. *GCHI* is the rate limiting enzyme for the synthesis of tetrahydrobiopterin (BH4), which is a cofactor necessary for the synthesis of catecholamines and nitric oxide- molecules critical for pain [14,22,52,54]. Among individuals with the *COMT met* allele, those with two copies of the *GCHI* minor C allele exhibited normalized COMT activity and increased mechanical pain thresholds. The pain-protective effects of the C allele are in line with previous findings that a haplotype containing rs10483639 C is associated with reduced pain following back surgery and reduced analgesic requirements following oral surgery [38,40,61]. The *GCHI* haplotype produces functional biologic effects, coding for reduced *GCHI* gene expression and reduced BH4 bioavailability [2]. Reduced BH4, in turn, reduces levels of catecholamines and NO to alleviate pain.

In agreement with previous studies [45], we identified a statistical 2-way interaction between *MTHFR* SNPs and *val*<sup>158</sup>*met* on COMT activity. However, there were too few subjects carrying the rs2274976 or rs2066470 minor alleles to characterize their effects stratified by rs4680 genotype. Although the present cohort was underpowered to investigate the interaction on TMD risk and other intermediate phenotypes, it would be valuable to explore this finding in a larger study.

Finally, we developed a three gene model that takes into account the sum of *COMT val*<sup>158</sup>*met*, *GCHI* rs10483639 G/C, and *ESR1* rs3020377 A/G allelic effects on COMT activity and TMD risk. This model illustrates 1) the protective role of *GCHI* rs10483639 C on COMT activity and TMD risk among *met* carriers, 2) the deleterious role of *ESR1* rs3020377 G on COMT activity and TMD risk among *val* carriers with only 0 or 1 copy of the *GCHI* rs10483639 C allele, and 3) the correlation between COMT activity patterns and TMD risk, such that individuals with lower COMT activity are at greater risk for TMD.

It is important to note that the influence of *COMT*, *GCHI*, and *ESR1* polymorphisms on COMT enzymatic activity is likely modified by environmental events that produce physical and emotional stress, inflammation, and/or injury [27,30,51]. An example of gene-

environment interaction was illustrated by Slade and colleagues, who found that among individuals with ATCA or ACCG *COMT* haplotypes, TMD incidence was 23% for those with a history of orthodontic treatment and 0% for those with no history of orthodontic treatment [58]. In addition, the influence of genetic polymorphism on *COMT* activity is likely modified by sex. It is well-established that males and females differ with respect to *COMT* activity levels, with males exhibiting higher levels in brain [13] and peripheral tissues [7], as well as *COMT*-dependent nociception [5] and behavioral traits [31]. Further, a growing number of studies demonstrate sex-dependent differences in the role of *ESR1* SNPs [6,25,37] and *COMT val/met* [4,20,36,48] in disease susceptibility. As the present study enrolled only females, we were unable to explore interactions between sex, genotype, and *COMT* activity. Thus, future studies should also account for the effects of sex and nongenetic factors on *COMT* activity and related phenotypes.

## Conclusion

In conclusion, polymorphisms located in distinct gene loci can act alone or in concert to influence the expression and activity of corresponding pain-relevant molecules such as *COMT*. The *COMT val*<sup>158</sup>*met* allele accounts for the greatest amount of individual variation in *COMT* activity, yet its effects are modified by SNPs in functionally-related *ESR1* and *GCHI* genes. Among individuals with the *COMT val* allele, those with two copies of the *ESR1* rs3020377 minor G allele exhibit reduced *COMT* activity, increased bodily pain, and poorer self-reported health. Among individuals with the *COMT met* allele, those with two copies of the *GCHI* rs10483639 minor C allele exhibit normalized *COMT* activity and increased mechanical pain thresholds. Thus, the *ESR1* minor G allele is deleterious among *val* carriers, while the *GCHI* minor C allele confers a protective advantage among *met* carriers. This finding is further confirmed in the three gene model, which also illustrates the parallels between low *COMT* activity and increased TMD risk. Ultimately, measuring functional levels of *COMT* will provide information representing the sum of molecular regulatory events affected by genetic polymorphisms as well as environmental events.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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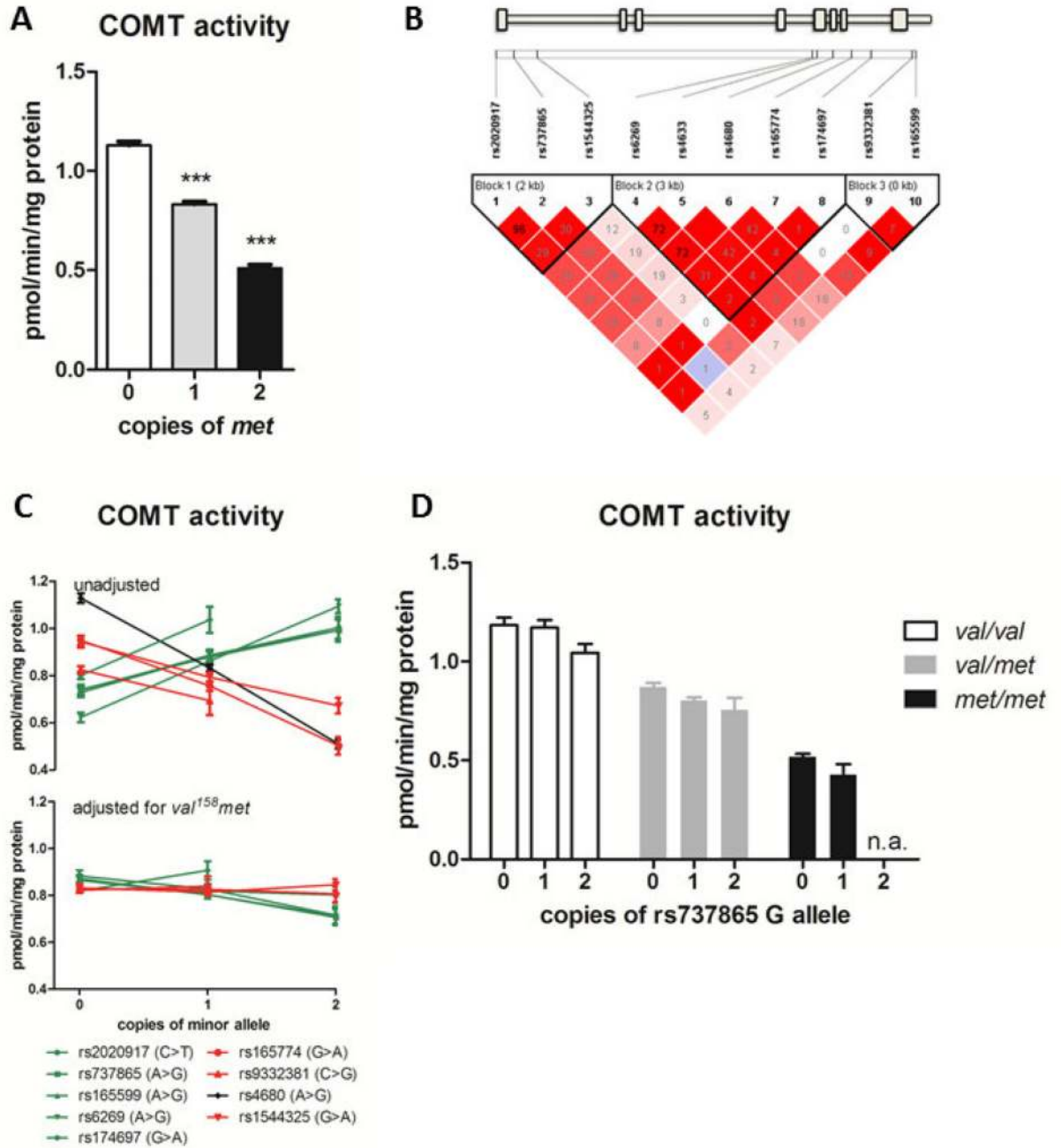
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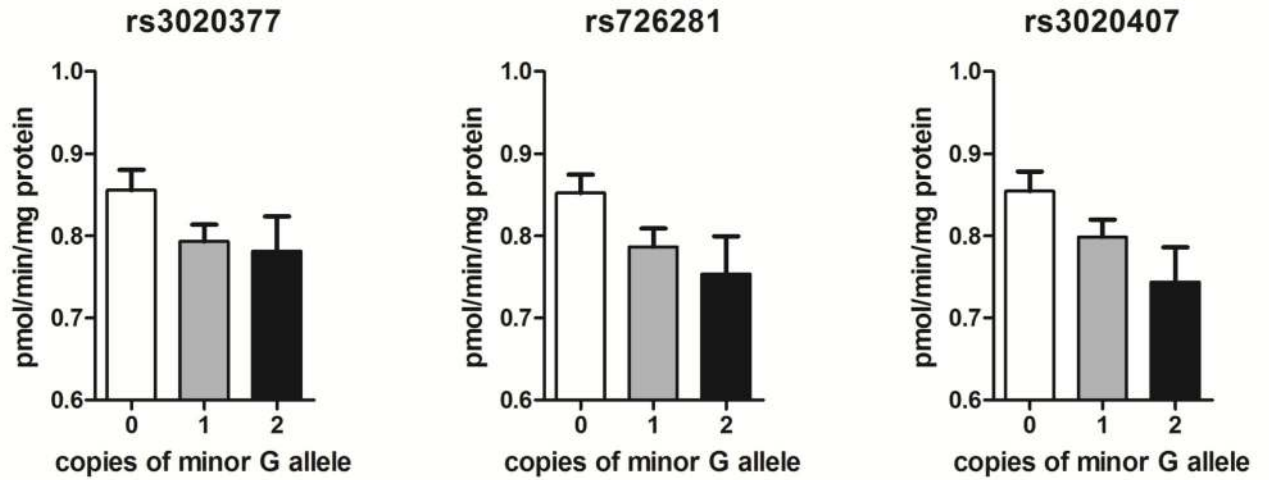
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**Fig. 1. *COMT* genotype effects COMT activity**

**A.** *COMT val*<sup>158</sup>*met* genotype effects COMT activity, such that individuals with 1 or 2 copies of the *met* allele exhibit reduced activity. Data represent mean  $\pm$  s.e.m. \*\*\* $p < 0.0001$ . **B.** Linkage disequilibrium plot of the genotyped SNPs in the *COMT* gene locus. The gene diagram indicates the location of each SNP with respect to exons (boxes) and introns of the gene. The color of each box in the LD plot depicts the strength of LD between aligned SNPs according to  $D'$  (darker red indicates stronger LD), and the number in the box is the corresponding  $r^2$  value. **C.** COMT activity levels of each genotype are shown for all *COMT*

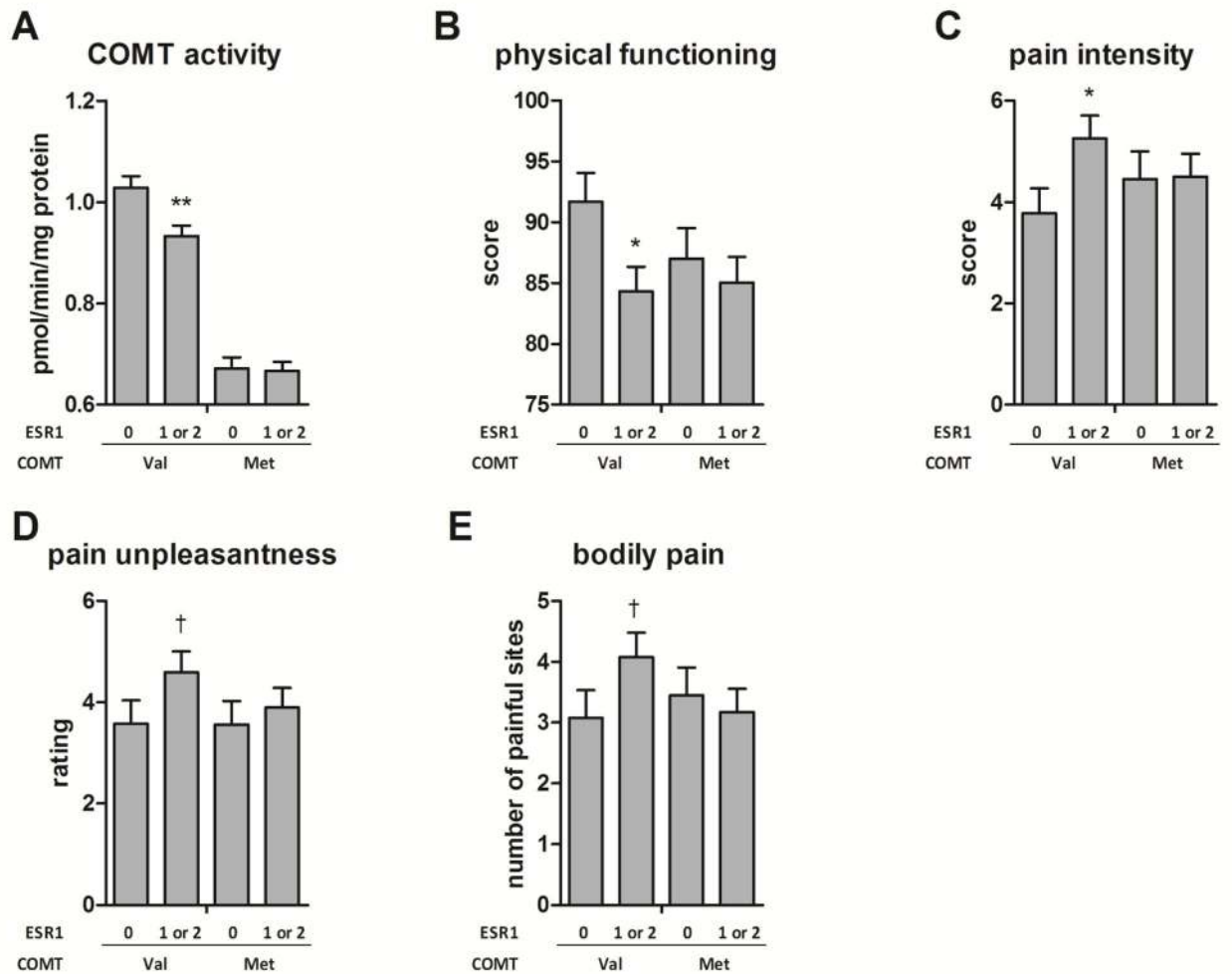
SNPs tested. Symbols and lines are colored green if the respective minor allele is associated with increased COMT activity, and red with decreased COMT activity; the rs4680 *val<sup>158</sup>met* is shown in black. In the top panel genotype effects of each SNP are unadjusted; in the bottom panel each SNP effect is adjusted for *val<sup>158</sup>met* genotype. **D.** COMT activity level of each genotype of rs737865, stratified by *val<sup>158</sup>met* genotype, shows that the minor G allele of this SNP is associated with decreased COMT activity after the confounding effect of *val<sup>158</sup>met* is removed.



**Fig. 2. *ESRI* SNP effects on COMT activity**

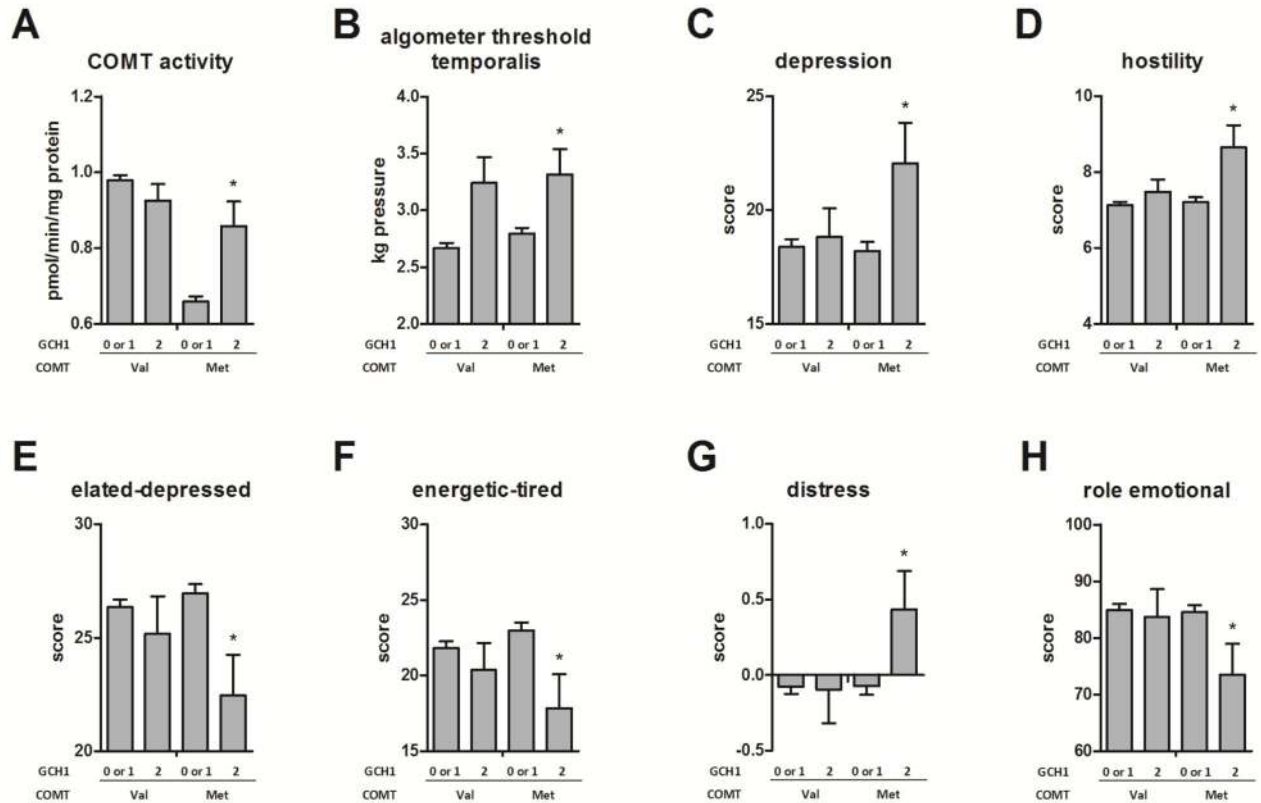
All three *ESRI* SNPs showed significant ( $p < 0.05$ ) association with COMT activity using a codominant model, although the dominant model provides a better fit for the data for rs3020377. Data represent mean  $\pm$  s.e.m.





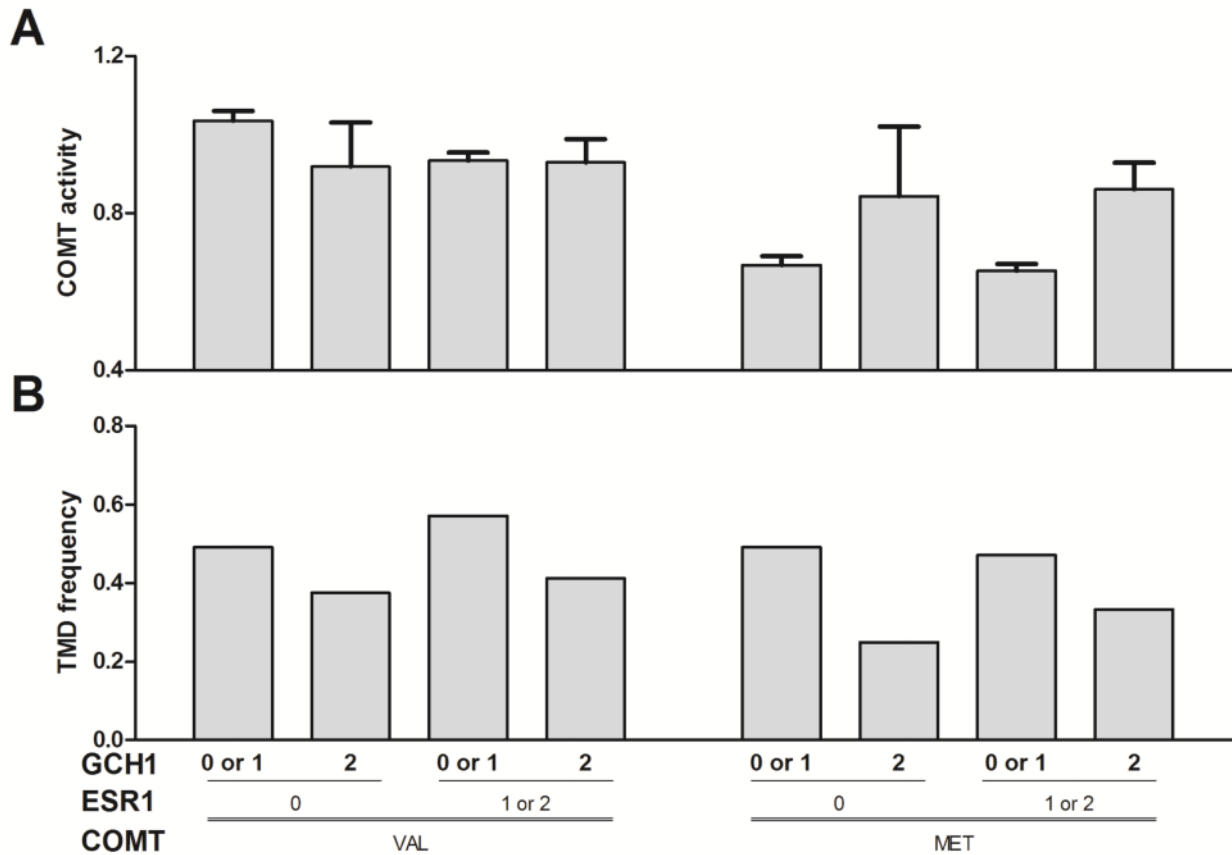
**Fig. 3. *COMT* × *ESRI* effects on *COMT* activity and pain phenotypes**

For genotypes of the *ESRI* SNP rs3020377, all carriers of the minor G allele were combined (dominant test) and stratified by carriers of *COMT* alleles *val* and *met*. Significance level shown is that of the test of the G allele carriers against the reference of the major allele homozygote group, within *val* carriers only. Among *val* carriers, the *ESRI* rs3020377 SNP produced effects on **A**. *COMT* enzymatic activity, **B**. physical functioning subscale of the SF12 questionnaire, **C**. pain intensity rating using the Gracely scale of the SPSR, **D**. pain unpleasantness rating using the Gracely Pain Scale of the SPSR, and **E**. count of the number of bodily sites painful upon palpation by clinical examiner. For each phenotype, rs3020377 modulates the effect of the *val* allele, but not the *met* allele, of *COMT*. The G allele of rs3020377 decreased *COMT* activity and physical functioning, and increased nociceptive phenotypes of pain intensity, unpleasantness, and distribution across body sites. Data are quantitative trait means ± s.e.m. †  $p < 0.1$  \*  $p < 0.05$  \*\*  $p < 0.01$ .



**Fig. 4. *COMT* × *GCH1* effects on *COMT* activity and pain phenotypes**

The *GCH1* SNP rs10483639 showed a recessive mode of inheritance, and was stratified by carriers of the *COMT* alleles *val* and *met*. Significance level shown is that of the test of the C allele homozygotes against the reference of the major allele carriers, within *met* carriers only. Among *met* carriers, the *GCH1* rs10483639 SNP produced effects on **A.** *COMT* enzymatic activity, **B.** algometer pressure at which pain is detected, measured at the temporalis muscle, **C.** depression subscale of the SCL90 questionnaire, **D.** hostility subscale of the SCL90, **E.** elated-depressed axis of the POMS, **F.** energetic-tired axis of the POMS, **G.** distress subscale of the PSS, and **H.** dysfunction in daily activities due to emotional factors, measured by the SF12 questionnaire. Carrying two copies of the rs10483639 C allele raised *COMT* activity in *met* carriers to the level of *val* carriers, but had no effect in the high-activity *val* carriers. The C allele was associated with higher algometer threshold in both *val* and *met* carriers, but was associated with more negative psychological characteristics in *met* carriers only. Data are quantitative trait means ± s.e.m. \*  $p < 0.05$ .



**Fig. 5. Three-way interaction between *COMT*, *ESR1*, and *GCHI* SNPs on COMT activity and TMD risk**

**A.** COMT enzyme activity (means  $\pm$  s.e.m.) and **B.** TMD frequency is plotted for each genotype. The *GCHI* rs10483639 C allele increases COMT activity and reduces TMD risk among *met* carriers, while the *ESR1* rs3020377 G allele decreases COMT activity and increases TMD risk among *val* carriers with only 0 or 1 copy of the *GCHI* rs10483639 C allele.

**Table 1**

COMT pathway SNP effects on S-COMT enzymatic activity

	Val		Met	
	Beta (S.E.M.)	P	Beta (S.E.M.)	P
<b>rs2066470</b> ( <i>MTHFR</i> )	-0.020 (0.03)	0.56	0.080 (0.03)	0.010
<b>rs3020377</b> <b>dom</b> ( <i>ESRI</i> )	0.048 (0.02)	0.0021	0.002 (0.01)	0.87
<b>rs10483639</b> <b>rec</b> ( <i>GCHI</i> )	0.026 (0.03)	0.39	-0.10 (0.03)	0.0030

Selected SNPs showed differential effects on S-COMT activity, based on *val*<sup>158</sup>*met* carrier status. The *MTHFR* (rs2066470) and *GCHI* (rs10483639) SNPs were associated with S-COMT activity only in the low activity *met* carriers; the *ESRI* (rs3020377) SNP was associated only in the high activity *val* carriers. Data represent the beta coefficient and s.e.m. of the SNP genotype term of a univariate regression model of S-COMT activity.