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COMT Genetic Reduction Produces Sexually Divergent Effects on Cortical Anatomy and Working Memory in Mice and Humans

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Genetic variations in catechol-O-methyltransferase (COMT) that modulate cortical dopamine have been associated with pleiotropic behavioral effects in humans and mice. Recent data suggest that some of these effects may vary among sexes. However, the specific brain substrates underlying COMT sexual dimorphisms remain unknown. Here, we report that genetically driven reduction in COMT enzyme activity increased cortical thickness in the prefrontal cortex (PFC) and postero-parieto-temporal cortex of male, but not female adult mice and humans. Dichotomous changes in PFC cytoarchitecture were also observed: reduced COMT increased a measure of neuronal density in males, while reducing it in female mice. Consistent with the neuroanatomical findings, COMT-dependent sex-specific morphological brain changes were paralleled by divergent effects on PFC-dependent working memory in both mice and humans. These findings emphasize a specific sex–gene interaction that can modulate brain morphological substrates with influence on behavioral outcomes in healthy subjects and, potentially, in neuropsychiatric populations.

Keywords: cognition, cortical thickness, dopamine, postero-parietal cortex, prefrontal cortex

Introduction

Catechol-O-methyltransferase (COMT) is an enzyme that catalyzes the methylation of catechol structures, including dopamine, norepinephrine, epinephrine, caffeine, and catechol estrogens (Axelrod and Tomchick 1958). Throughout evolution, COMT seems to have progressively shifted from a primary role in peripheral detoxification of xenobiotics in non-mammalian species (Guldberg and Marsden 1975; Li et al. 2010; Alazizi et al. 2011), to a fundamental role in higher cognitive processes in mammals via the modulation of central neurotransmission, especially dopamine (Chen et al. 2004; Papaleo et al. 2008; Kaenmaki et al. 2010). In particular, multiple findings demonstrate a critical and specific role for COMT in the catabolism of cortical dopamine as a consequence of the lack of dopamine transporters at cortical synapses (Chen et al. 2004; Yavich et al. 2007; Kaenmaki et al. 2010). This relatively selective effect is thought to be the mechanistic basis by which functional COMT genetic variations modulate multiple spheres of mammalian behavior, with remarkable similarities between humans and mice (Egan et al. 2001; Heinz and Smolka 2006; Papaleo et al. 2008; Mier et al. 2010; O'Tuathaigh et al. 2012; Scheggia et al. 2012; Papaleo, Erickson et al. 2012a). Perhaps not surprisingly, COMT genetic variations have been associated with diverse behavioral effects, in both human and mouse studies, suggesting that a relative reduction in COMT might confer certain cognitive advantages, but less adaptive affective behaviors (Drabant et al. 2006; Papaleo et al. 2008; Mier et al. 2010; Scheggia et al. 2012).

Dopaminergic innervation of rodents' cortex is linked not only with physiology and behavior but also with cortical thickness (Kalsbeek et al. 1989). In agreement, human magnetic resonance imaging (MRI) studies have suggested an association between COMT genetic variations and the morphology of the temporal and prefrontal cortex (PFC), although the exact findings remain inconsistent (Cerasa et al. 2008; Honea et al. 2009; Witte and Floel 2012; Ira et al. 2013). Increasing evidence suggests that the sex of the subject might be a critical factor to consider in interpreting COMT-dependent effects. Indeed, COMT mutant mice showed sexually dimorphic effects on emotional reactivity, aggressiveness, and cognitive responses to environmental manipulations in domains such as impulsivity and attentional control (Gogos et al. 1998; Papaleo, Erickson et al. 2012a). Similarly, functional genetic variations in COMT in humans have been associated with sexually dimorphic effects in aspects of cognitive abilities, personality, and predisposition to psychiatric disorders (Harrison and Tunbridge 2007; Chen et al. 2011; Papaleo, Erickson et al. 2012a). Finally, both COMT expression and prefrontal dopamine levels are regulated by estrogens, and COMT enzymatic activity appears to be different between males and females (Xie et al. 1999; Chen et al. 2004; Harrison and Tunbridge 2007; Jacobs and D'Esposito 2011). It is therefore conceivable that if a COMT–sex interaction exists, the brain substrates influenced by COMT genetic variants should also interfere with or be modulated by the sex of the subject.

To test this hypothesis, we analyzed COMT-dependent changes in brain anatomy using structural MRI in mice and humans, combined with a finer histological evaluation in COMT mutant mice and parallel behavioral assessments in mice and humans. The use of COMT genetically modified mice allows for a precise dissection of the biological role played by COMT, avoiding the complexity of human polymorphisms,

genetic, and clinical heterogeneity and the uncontrolled impact of gene–gene and gene–environment interactions in adult human data. In particular, COMT knockout mice have partial reduction (COMT+/−) or complete absence (COMT−/−) of COMT enzyme activity, and PFC tissue dopamine levels are increased in COMT knockout male but not female mice (Gogos et al. 1998; Yavich et al. 2007). Human studies, guided by findings in genetically modified mice, have provided the essential translational validity. This approach may provide a critical perspective to better understand the enormous amount of work done in humans related to COMT-dependent cerebral and behavioral changes.

Materials and Methods

Mice Subjects

All procedures were approved by the Italian Ministry of Health (permit no. 230/2009-B) and local Animal Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the European Community Council Directives. COMT null mutant mice (COMT−/−), their heterozygous (COMT+/−), and wild-type (COMT+/+) littermates were bred by heterozygous (+/−) mating and were identified by PCR analysis of tail DNA. Mice were group-housed in a climate-controlled animal facility (22 ± 2 °C) and maintained on a 12-h light/dark cycle, with free access to food and water, unless otherwise specified in particular experiments. Testing was conducted on male and female adult mice during the light phase. We limited the age range to mice of 3–7 months of age, as in this species, this time window is considered only as "adulthood," avoiding the developmental and senescence-related phenomena that might interact with the genetic mutations. Different cohorts of naïve mice were used for each single experiment. Experimenters were blind to the genotype during testing.

Structural Magnetic Resonance Imaging in Mice

High-resolution morpho-anatomical T_2-weighted (T_2W) and diffusion-weighted MR imaging was performed in paraformaldehyde (4% PFA) fixed mouse brains. This procedure permits to obtain artifact-free high-resolution images devoid of physiological or motion artifacts (Lerch et al. 2012). Animals were anesthetized with an intraperitoneal Avertin injection (375 mg/kg) and perfused intracardially with phosphate buffered-saline (PBS) followed by 4% PFA. Both perfusion solutions were added with a Gadolinium chelate (Prohance, Bracco, Milan, Italy) at a concentration of 10 and 5 mM, respectively, to reduce longitudinal relaxation times. Brains were imaged inside intact skulls to avoid post-extraction deformations. MR images were acquired within 6 days from perfusion at 7.0 Tesla using a 72-mm birdcage transmit coil, and a custom-built saddle-shaped solenoid coil for signal reception with the following imaging parameters: 3D rapid acquisition with relaxation enhancement (RARE) spin-echo sequence, Repetition Time (TR) = 550 ms, echo time = 33 ms, RARE factor = 8, echo spacing 11 ms, and voxel size of 90 μm^3 (isotropic). Diffusion tensor images (DTI) were acquired with 81 different gradient orientations at a b value of 1262 s/mm^2 ($\partial = 5$ ms $\Delta = 10$ ms), in-plane spatial resolution of 130×130 μm^2, and slice thickness of 350 µm in the coronal plane, using a 4-shot echo-planar images sequence with TR = 5500 ms and effective echo time = 26 ms, 20 averages for a total acquisition time of 10 h52 m. For each DWI dataset, 8 co-centered volumes were acquired with no diffusion weighting ($b = 0$). Co-centered T_2W images were also acquired with the same resolution of the diffusion tensor imaging volumes, using a 2D fast spin-echo sequence.

Voxel-Based Morphometry

Intergroup differences in local gray-matter volume were mapped using voxel-based morphometry (VBM) (Ashburner and Friston 2000). A study-based template was created aligning high-resolution T_2W images of the control population to a common reference space via a 12 degrees-of-freedom (DOF) affine alignment, followed by 5 consecutive symmetric diffeomorphic registrations (Avants et al. 2010; Avants, Tustison, Song et al. 2011a) Individual T_2W images of 2 groups of subjects were then nonlinearly registered to the study-based template using diffeomorphic registration. Gray matter of spatially normalized subjects was then segmented using a Markov random field model using a 6-class segmentation of the study-based template as a prior to initialize the process (Avants, Tustison, Wu et al. 2011b). The Jacobian determinants of the deformation were then used to modulate the GM probability maps calculated during the segmentation step. The modulation compensates for the deformation introduced after the spatial normalization so that there is no variation of the total amount of gray matter, focusing the analysis on the local volumetric variation of the GM instead of the tissue density (Ashburner and Friston 2000). The resulting modulated GM probability maps were then smoothed using a Gaussian kernel with a sigma of 3 voxels for voxel-wise statistical parametric comparison. Voxel-wise cross-subject statistic was performed using a nonparametric permutation test with 5000 permutations (Nichols and Holmes 2002). Data were corrected for multiple comparisons using a cluster-based threshold of 0.01.

Histology and Immunofluorescence in Mice

Mice were anesthetized with 20% urethane in saline (dose 0.01 mL/g) and then transcardially perfused with 4% PFA in PBS (0.1 M, pH7.4). Brains were postfixed with 4% PFA overnight at 4 °C. Serial coronal sections (60 µm thick) of the PFC (Bregma 1.9) and of the temporal cortex (Bregma −2.8) were prepared with a vibratome and then stored at 4 °C in PBS prior to histological staining. Nissl staining was performed with Cresyl Violet acetate (Sigma) using standard protocols (Banny and Clark 1950; Brinks et al. 2004). Slices were always kept floating, to avoid possible morphological artifacts due to tissue dehydration. Immunofluorescence was performed as follows: Sections were incubated with mouse anti-NeuN (MAB377, Millipore) and rabbit anti Cux1 (M222; Santa Cruz, CA, USA) diluted 1:100 in blocking solution (0.3% Triton X-100, 5% Normal Goat Serum, 0.1% BSA) overnight at 4 °C, and subsequently with secondary anti-mouse IgG antibody (Alexa-568-coniugated, Invitrogen) diluted 1:600 in blocking solution, containing Hoechst (Invitrogen) at a final concentration of 50 µg/mL, for 2 h and 30 min at room temperature, and mounted on glass slides with aqueous mounting medium (Vectashield, Invitrogen). For details on microscopy analyses, see Supplementary Materials.

Human Subjects

Right-handed healthy individuals were recruited by local advertisements. To parallel mouse studies and to limit potential interacting effects of COMT mutations with major developmental brain and behavioral changes in preadolescence and elderly (for review see Sambataro et al. 2012; Scheggia et al. 2012), we focused our investigation on adult subjects between 18 and 60 years old. To reduce the possibility of artifactual association caused by ethnic stratification, the final sample included only individuals of Italian–Caucasian ancestry, born and educated in Italy. Exclusion criteria were 1) major medical illnesses and/or known or suspected history of alcoholism or drug dependency and abuse; 2) mental disorders (i.e., schizophrenia, mood, anxiety, personality, and/or any other significant mental disorders) according to the DSM-IV-TR criteria assessed by the Structured Clinical Interviews for DSM-IV-TR (First and Pincus 2002) and/or neurological disorders diagnosed by an accurate clinical neurological examination; 3) presence of vascular brain lesions, brain tumor, and/or marked cortical and/or subcortical atrophy on MRI scan; and 4) presence of dementia. We included only subjects with Mini Mental State Examination score ≥24 or if they did not present dementia diagnoses according to DSM-IV-TR criteria. After a careful evaluation of these exclusion criteria, 131 subjects were eligible for this study. All of these subjects were assessed for working memory abilities in the *n*-back task while MRI scans were available for only 121 subjects. According to the COMT $Val^{158}Met$ polymorphism, we categorized individuals into 3 groups: the homozygous Val/Val group, the Val/Met group, and the homozygous Met/Met group (see Supplementary Tables 1 and 3 for genotypic and demographic data). We focused on this single nucleotide polymorphism, as it is the most established and widely studied functional mutation

in the COMT gene (Chen et al. 2004; Scheggia et al. 2012). Future investigations with larger samples size would be required to assess the potential effects played by other COMT functional polymorphisms/haplotypes (Lachman et al. 1996; Bray et al. 2003; Meyer-Lindenberg et al. 2006; Nackley et al. 2006; Gothelf et al. 2014). Written, informed consent was obtained from all subjects participating in the study, which was approved by the local ethics committee at the Santa Lucia Foundation of Rome. Standard procedures were used for human genotyping (Supplementary Materials for details).

Structural Magnetic Resonance Imaging in Humans

Structural MRI was performed according to our routine protocol (Cerasa et al. 2011) by a 3 Tesla scanner 3-T Allegra MR imager (Siemens, Erlangen, Germany) with a standard quadrature head coil. MRI-based quantification of cortical thickness was performed using "Freesurfer" (v. 4.05) software package (http://surfer.nmr.mgh.harvard.edu). This method has been previously described in detail (Dale et al. 1999; Fischl and Dale 2000). Brain images for cortical thickness analysis were first corrected for intensity of nonuniformity and registered via affine transformation (12 parameters) to MNI space. Then, images underwent a further intensity normalization using a different automated algorithm and were automatically skull stripped (Dale et al. 1999). Next, the entire cortex was visually inspected prior to analysis, and data from 121 subjects were deemed to require manual correction, which included 1) manually realigning each subject's image to the MNI template, 2) setting intensity normalization control points where brain matter was erroneously skull stripped, 3) adjustment watershed parameters of the skull strip, and 4) visual inspection and correction of the automatic subcortical segmentation. All subjects were inspected by a neuroradiologist with a high level of neuroanatomical expertise who was blinded to the MRI results. MRIs of inferior quality, not suitable for reliable tissue segmentation with Freesurfer even after manual editing of cortical surfaces and subcortical regions, were discarded. For each subject, thickness measures across the cortex were computed by finding the point on the gray–white boundary surface that was closest to a given point on the estimated pial surface (and vice versa) and averaging between these 2 values (Dale et al. 1999; Fischl and Dale 2000). The accuracy of the thickness measures derived from this technique have been validated by direct comparisons with manual measures on postmortem brains (Rosas et al. 2002) as well as direct comparisons with manual measures on MRI data (Cerasa et al. 2009). The surface representing the gray–white border was "inflated" (Dale et al. 1999), differences among individuals in the depth of gyri–sulci were normalized, and each subject's reconstructed brain was then morphed and registered to an average spherical surface representation that optimally aligned sulcal and gyral features across subjects. This spherical morphing procedure was used to map the thickness measurements at each vertex on each subject's cortical surface into a common spherical coordinate system. Finally, cortical maps were smoothed with a 10-mm full-width at half-maximum Gaussian kernel. For each hemisphere, differences in cortical thickness were tested by computing a general linear model of "genotype" and "sex" effects on structural neuroanatomy at each vertex. A false discovery rate (FDR) of $P \leq 0.05$ was applied to correct for multiple comparisons at whole brain. Age was included in the models as a covariate of no-interest.

Physical Health Assessments in Mice

Measures of general health and neurological reflexes were assessed as previously described (Papaleo et al. 2008; Papaleo, Yang et al. 2012b).

Discrete Paired-Trial Variable-Delay T-Maze Task for Working Memory and Spatial T-Maze Task for Reference Memory

Two different cohorts of naïve females COMT−/−, +/−, and +/+ littermates were tested in these 2 T-maze paradigms as described previously (Papaleo et al. 2008, 2011; Papaleo, Yang et al. 2012b). Briefly, in the discrete paired-trial variable-delay T-maze task, mice were presented with a sequence of randomly chosen forced runs, each followed by a choice run so that they were required to integrate information held online (the forced run) with the learned rule (nonmatch to sample). In the spatial T-maze task of reference memory, we used the same T-maze apparatus but mice were only required to acquire a simple spatial rule: The same arm of the maze was baited on every trial.

Spatial and Visual Working Memory n-Back Tasks in Humans

Participants were required to continuously monitor a sequence of spatial or visual stimuli (a total of 22 items for each task, visually presented on a screen) and to select items that appeared as *n*-back items in any sequence (Cerasa et al. 2011). The number of correct responses was generally considered as index of working memory performance.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM) throughout. Mice's MRI, histology, and immunofluorescence morphological measurements were examined using two-way analyses of variance (ANOVAs), with genotype (COMT+/+, +/−, −/−) and sex (male, female) as between-subjects factors. Cells counting in mice were analyzed using separate one-way ANOVAs with genotype (COMT+/+, +/−, −/−) as between-subjects factor because slices from male and female mice were stained separately, thus their data are kept separate as direct comparisons might not be performed. One-way ANOVA was used to compare COMT+/+, +/−, and −/− female mice on number of days required to reach criterion on the discrete paired-trial T-maze and reference memory T-maze task. The percentages of mice of each genotype that acquired the 2 T-maze tasks were subjected to separate 2×2 χ^2 analyses. Latency to eat in both tasks was analyzed via two-way ANOVAs, using genotype (COMT+/+, +/−, and −/−) as between-subjects factors and day of habituation (first or second) as a repeated-measures within-subjects factor. Correct responses in humans' *n*-back task were examined using two-way ANOVAs, with genotype (COMT Val/Val, Val/Met, Met/Met) and sex (male, female) as between-subjects factors. Post hoc analyses for individual group comparisons were carried out with Newman–Keuls post hoc test, when statistical significance emerged. The accepted value for significance was $P \leq 0.05$. The software STATISTICA (StatSoft, version10) was used.

Results

Male but Not Female COMT Knockout Mice Show Increased Fronto-Cortical and Postero-Parieto-Temporal Gray Matter Volume

To investigate whether genetically driven COMT reductions differentially alter brain morphology depending on the sex of the subject, we performed an unbiased, hypothesis-independent MRI VBM of gray matter in the whole brain of adult male and female COMT knockout littermates. This allowed us to contrast life-long effects of genetic variations resulting in relatively low COMT activity (i.e., COMT+/− mice), complete absence of COMT (i.e., COMT−/− mice), and normal endogenous expression of COMT (i.e., wild-type COMT+/+ mice). This is important as it offers the possibility to unravel potentially different compensatory effects of a relative reduction in COMT gene function (that might also better mimic human genetic conditions) versus complete loss of gene activity.

VBM highlighted bilateral foci of increased prefrontal cortical (PFC) and postero-parieto-temporal gray-matter volume in males but not females COMT−/− and +/− mice compared with +/+ littermates (Fig. 1). When the same dataset was analyzed using a region-of-interest (ROI) approach, a significant COMT–sex interaction effect was evident in the frontal ($F_{2,32} = 3.28$; $P = 0.05$) and in the postero-parieto-temporal cortices ($F_{2,32} = 3.70$, $P < 0.04$). Male COMT−/− and +/− mice had significantly increased volume of the gray matter in the frontal

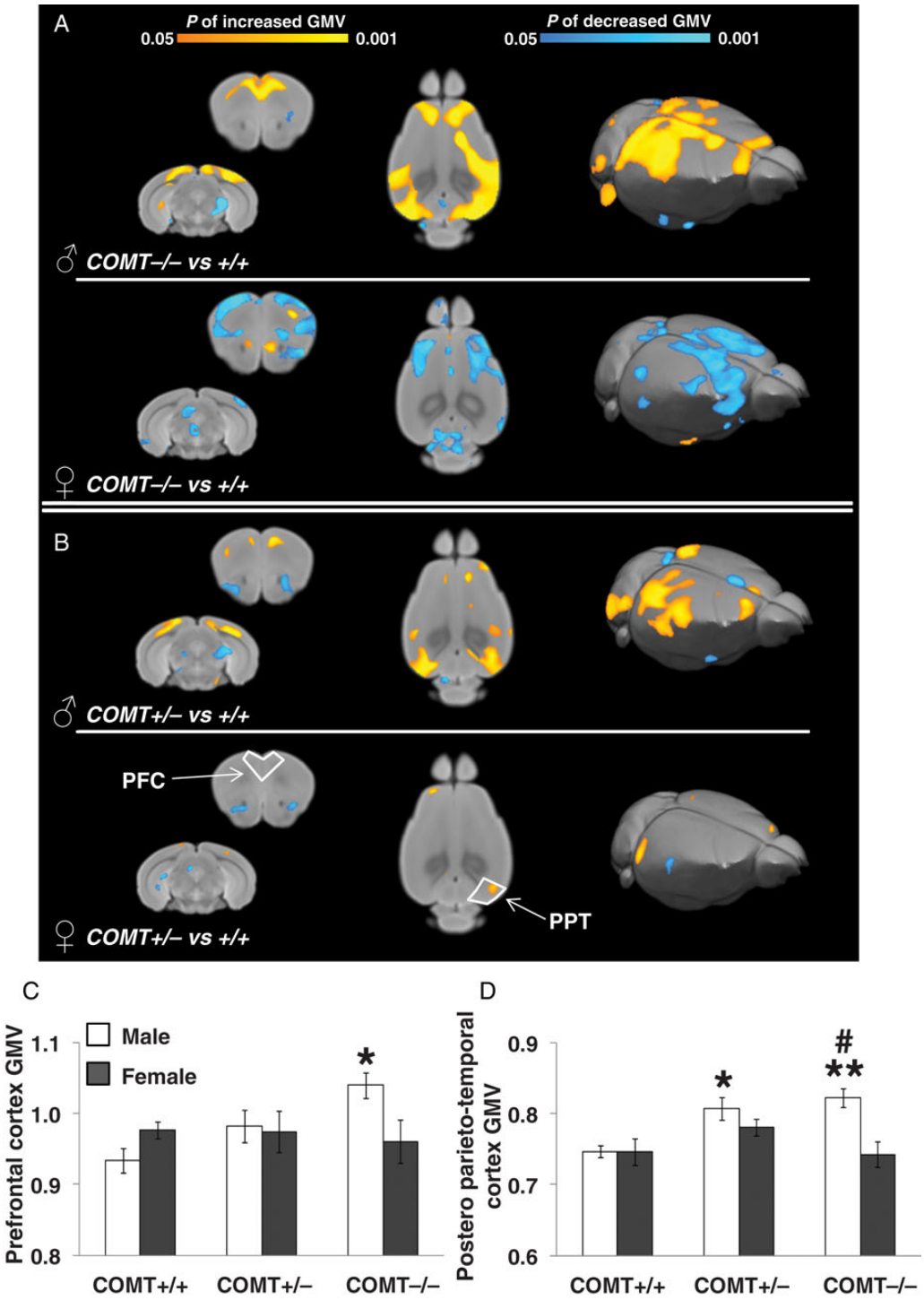

Figure 1. Increased prefrontal and postero-parieto-temporal gray-matter volume in male but not female COMT knockout mice. (*A*, *B*) Representative coronal (left), horizontal (middle), and 3D volumetric reconstruction (right) of the areas showing statistically significant increased or decreased in gray-matter volume (GMV) (*A*) in COMT–/– and (*B*) in COMT+/– compared with wild-type COMT+/+ littermates ($P < 0.05$, TFCE corrected). Areas of increased gray-matter volume were apparent in prefrontal (PFC) and postero-parieto-temporal (PPT) cortical areas of male (♂) but not female (♀) mice. The arrows indicate the placement of region of interest for post hoc analyses in the PFC and PPT areas. Bar graphs illustrate mean GMV within (*C*) the PFC and (*D*) the PPT plotted as a function of genotype and sex. COMT+/+ males $N = 7$, females $N = 6$; COMT+/– males $N = 6$, females $N = 8$; COMT–/– males $N = 7$, females $N = 4$. Values represent mean ± SEM in all figures. $^{*}P < 0.05$, $^{**}P < 0.01$ versus COMT+/+. $^{\#}P < 0.01$ versus COMT–/– females.

($P < 0.05$; Fig. 1*A–C*) and postero-parieto-temporal cortex ($P < 0.005$; Fig. 1*A*,*B*,*D*) compared with +/+ mice. In contrast, no differences were observed between COMT+/+, +/–, and –/– female littermates in the PFC ($P > 0.87$; Fig. 1*A–C*) and postero-parieto-temporal cortex ($P > 0.18$; Fig. 1*A*,*B*,*D*). These findings suggest that life-long genetic variations resulting in reduced COMT enzymatic activity and possibly increased synaptic dopamine levels produce MRI measures of increased gray-matter volume in fronto-cortical and postero-parieto-temporal areas in male but not female mice.

COMT Knockout Male Mice, but Not Females, Exhibit Increased Thickness in Cingulate, mPFC, and Posterior Parietal Cortex

To evaluate in greater depth the cortical substructural effects of the COMT–sex interaction evinced by MRI analyses, we performed histological and immunofluorescence analyses in brain slices from 2 other separate cohorts of COMT genetically modified mice. We first focused on frontal cortical areas, because COMT modulation of dopamine levels and behaviors has been extensively ascribed to the PFC (Tunbridge et al. 2004; Yavich et al. 2007; Papaleo et al. 2008; Kaenmaki et al. 2010; Scheggia et al. 2012).

To rule out the potential effect of tissue shrinkage induced by dehydration (Schuz and Palm 1989), and to better identify cells populations and cortical lamination (Gittins and Harrison 2004), coronal brain slices were prepared by vibratome, and stained using fluorescent markers: NeuN immunostaining selectively revealed neuronal cells while Hoechst counterstained revealed total cell nuclei. NeuN- and Hoechst-stained brain sections revealed a COMT–sex interaction effect on total cortical thickness in the mPFC ($F_{2,24} = 3.4$, $P = 0.05$; Fig. 2*A–E*). In particular, COMT−/− male mice had thicker mPFC than all other groups ($P < 0.03$; Fig. 2*C*). In contrast, COMT knockout females did not show any difference in mPFC total cortical thickness compared with +/+ littermates ($P > 0.7$; Fig. 2*C*). Interestingly, the thickness of morphologically identified superficial layers II/III were not affected by COMT genetic reduction ($F_{2,24} = 0.2$, $P = 0.8$), by sex ($F_{1,24} = 2.6$, $P = 0.1$), nor by their interaction ($F_{2,24} = 0.05$, $P = 0.9$; Fig. 2*D*). In contrast, analysis of the thickness in deeper layers V/VI revealed a COMT–sex interaction effect ($F_{2,24} = 3.7$; $P = 0.05$). Deeper layers V/VI were enlarged in COMT−/− male mice compared with their +/+ counterparts ($P = 0.006$; Fig. 2*E*), while no differences were present in females ($P > 0.8$; Fig. 2*E*). Consistent with these results, Nissl-staining analyses revealed that only COMT −/− male mice had thicker mPFC and cingulate cortices than their +/+ littermates ($P < 0.05$), but no differences in the adjacent motor cortex ($P > 0.9$; Supplementary Results and Fig. 1).

Overall, these results show that reduced COMT enzymatic activity increased the thickness of the medial and cingulate PFC regions in males but not in females. Moreover, these data suggest that the increased PFC thickness found in COMT knockout male mice may depend on an enlargement of deeper but not superficial cortical layers.

Histological analyses of the total cortical thickness in the posterior parietal cortex revealed a significant effect of genotype ($F_{2,28} = 3.6$, $P = 0.04$) and sex ($F_{1,28} = 4.4$, $P = 0.04$), but no COMT–sex interaction effect ($F_{2,28} = 2.1$, $P = 0.1$; Fig. 2*F–J*). Planned comparison in each sex-group revealed that the effect of COMT on the total posterior parietal cortical thickness was driven by the male mice ($F_{2,16} = 5.7$, $P = 0.01$), but not the female mice ($F_{2,12} = 0.1$, $P = 0.9$). In particular, COMT+/− and −/− male mice exhibited increased posterior parietal thickness compared with +/+ male mice ($P < 0.03$; Fig. 2*H*). These results are consistent with the male-specific effect of COMT genotype in the posterior parietal cortex established in the MRI experiments.

We then directly measured the relative thickness of the different cortical layers of the posterior parietal cortex. The analysis of the thickness of the superficial layers II/III revealed a significant COMT–sex interaction effect ($F_{2,28} = 4.2$, $P = 0.02$). In particular, superficial layers II/III were enlarged in COMT +/− male mice compared with their +/+ counterparts ($P = 0.004$; Fig. 2*I*), whereas no difference was present in female mice ($P > 0.3$; Fig. 2*I*). No COMT–sex interaction effect was observed for the thickness of the layer IV ($F_{2,28} = 0.5$, $P = 0.6$; not shown), nor of the layers V/VI ($F_{2,28} = 0.3$, $P = 0.7$; Fig. 2*I*). These results suggest that the sexually dimorphic associations of COMT knockout mice in posterior parietal cortex involve primarily the superficial compared with the deeper cortical layers, the inverse of the laminar patterns found in PFC.

COMT Genetic Reduction Increased Cortical Neuronal Density in Male Mice, but Reduced It in Female Mice in the mPFC

To investigate at the cellular level, the effects of genetically driven COMT reduction in the mPFC and in the posterior parietal cortex, we quantified the relative density of neuronal (NeuN-labeled) and total cells (Hoechst-labeled). Notably, COMT seemed to affect the neuronal density in a sexually dimorphic way.

Cell quantifications in deeper layers V/VI of the mPFC revealed a COMT genotype effect on the density of neurons in both males ($F_{2,57} = 4.08$; $P = 0.02$) and females ($F_{2,45} = 3.3$, $P = 0.04$). In particular, neuronal cells per field were significantly increased in male mice with reduced COMT activity ($P = 0.03$; Fig. 3*B* and Supplementary Fig. 3). Conversely, neuronal cells per field were decreased in females with genetic reduction of COMT ($P = 0.03$; Fig. 3*B* and Supplementary Fig. 4).

In contrast, the density of the total number of cells and the ratio between NeuN and Hoechst positive cells were not significantly affected by COMT genotype in the mPFC in males (COMT genotype on Hoechst counting: $F_{2,57} = 2.573$, $P = 0.09$ and NeuN/Hoechst ratio: $F_{2,57} = 0.904$, $P = 0.4$) or in females (COMT genotype on Hoechst counting: $F_{2,45} = 0.6$, $P = 0.5$ and NeuN/Hoechst ratio: $F_{2,45} = 0.8$, $P = 0.4$; Fig. 3*C,D*). Overall, these results suggest that COMT genetic mutations affect neuronal density in the mPFC in opposite ways in males and females. In particular, in the deeper cortical layers V/VI of the mPFC, genetically driven reduction of COMT resulted in increased neuronal density in males, but decreased neuronal density in females.

Cell counting in superficial layers II/III of the posterior parietal cortex revealed no significant effect of COMT genotype on the density of neurons (i.e., NeuN positive cells) in both males ($F_{2,29} = 1.6$, $P = 0.2$; Supplementary Fig. 2*A,B*) and females ($F_{2,25} = 1.0$, $P = 0.4$; Supplementary Fig. 2*A,B*). Similarly, we found no effect of COMT genotype on the density of all cells (i.e., Hoechst positive cells) in both males ($F_{2,29} = 0.03$, $P = 0.97$; Supplementary Fig. 2*A,C*) and females ($F_{2,25} = 0.1$, $P = 0.9$; Supplementary Fig. 2*A,C*). However, the ratio between the density of neurons and the total cells revealed a significant COMT genotype effect in males ($F_{2,29} = 6.5$, $P = 0.004$; Fig. 5*A,D*) but not in females ($F_{2,25} = 0.4$, $P = 0.7$; Supplementary Fig. 2*A,D*). Specifically, COMT−/− male mice showed an increased percentage of neuronal cells relative to total cells in the superficial layers II/III of the posterior parietal cortex compared with +/+ littermates. These results suggest that COMT genetic mutations differentially affected neuronal density in the posterior parietal cortex in males and females.

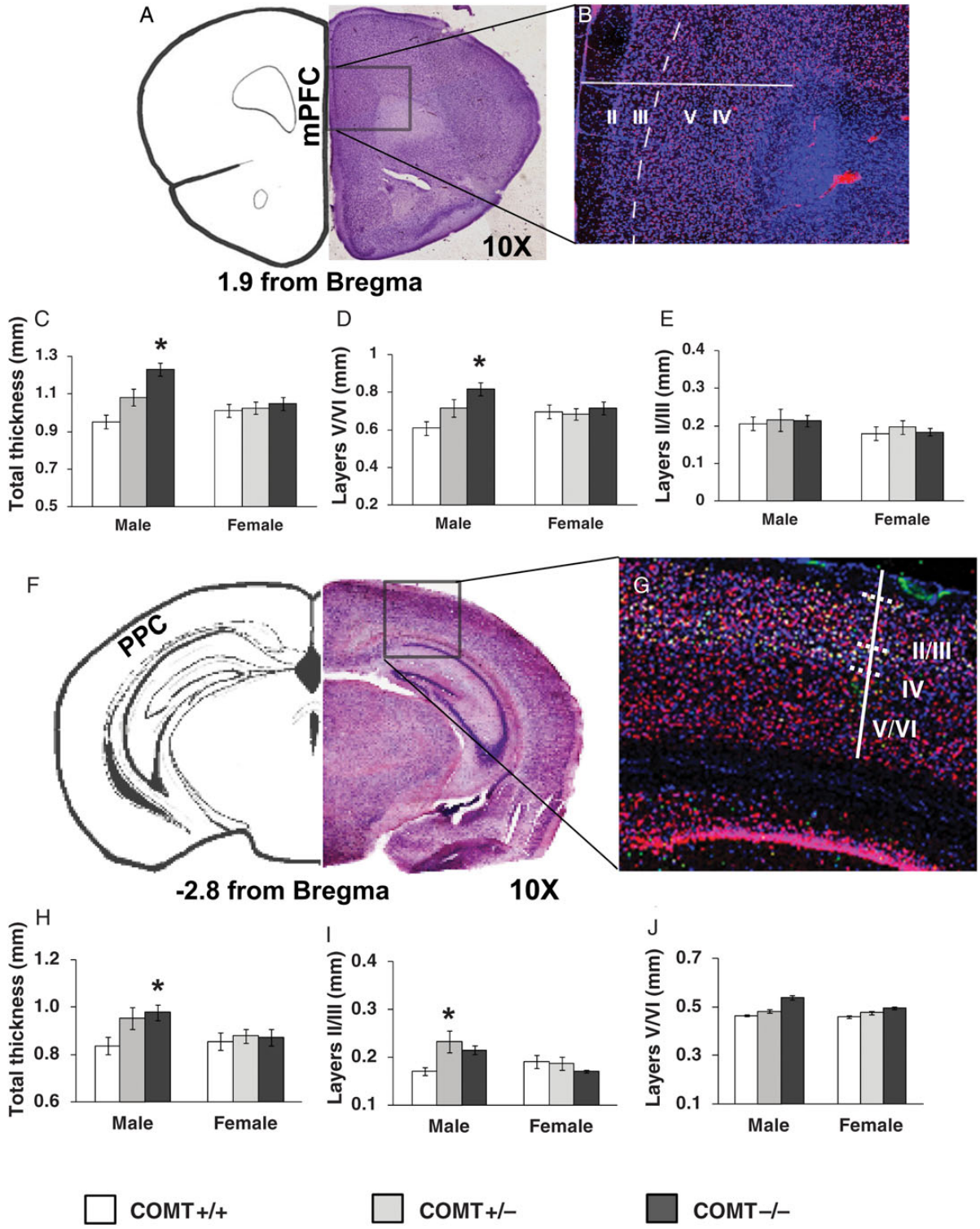

Figure 2. COMT knockout male mice show increased thickness in deeper layers V/VI of the medial PFC and in superficial layers II/III of the posterio-parietal cortex. (*A*) Representative coronal section of mice mPFC. (*B*) Confocal image of the mPFC after staining for 2 cell markers: Hoechst (in blue) and NeuN (in red). The continuous white line represents the cortical thickness of the mPFC. The statistical analysis revealed an increased (*C*) total thickness of the PFC in males (*$P < 0.05$ vs. all other groups) and, in particular, (*D*) of layers V/VI (*$P < 0.05$ vs. +/+ male mice), but (*E*) not of layers II/III. COMT+/+ males $N = 5$, females $N = 5$; COMT+/– males $N = 5$, females $N = 5$; COMT–/– males $N = 5$, females $N = 5$. (*F*) Representative coronal section of mice posterior parietal cortex (PPC). (*G*) Confocal image of the PPC stained for: Hoechst (blue), NeuN (red), and Cux1 (white), note that the pattern of expression of Cux 1 helped to confirm the identification of layers V/VI from layers IV and II/III (see also Supplementary Material). The continuous white line represents the thickness of the PPC. The statistical analysis revealed an increase in (*H*) the total thickness of the PPC and, in particular, (*I*) of layers II/III, and (*J*) layers V/VI. COMT+/+ males $N = 5$, females $N = 5$; COMT+/– males $N = 5$, females $N = 5$; COMT–/– males $N = 9$, females $N = 5$. *$P < 0.05$ versus +/+ mice.

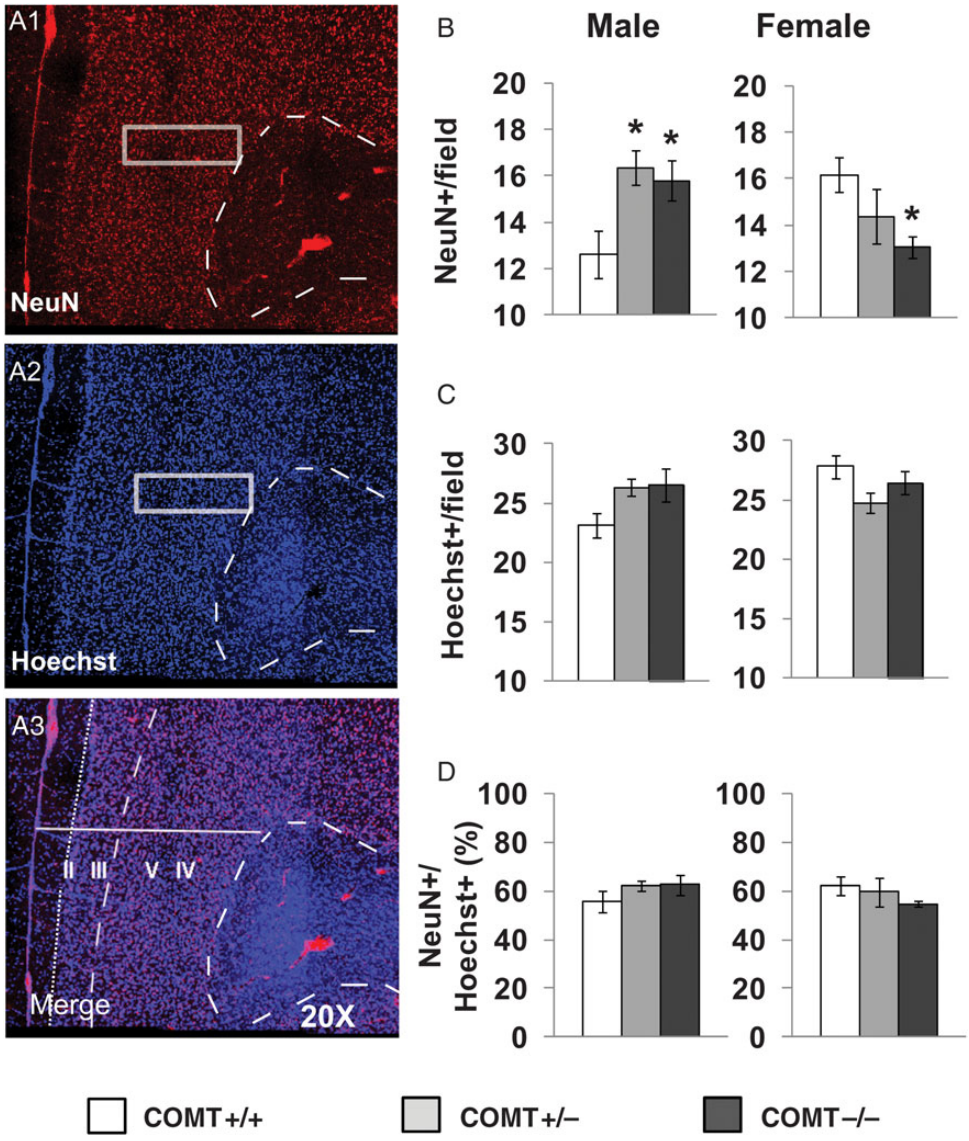

Figure 3. In the medial PFC, COMT genetic reduction increased cortical neuronal density in male mice but reduced density in female mice. (*A*) Confocal images of coronal brain slice immunostained for NeuN (A1), Hoechst (A2), and merge (A3). Fields (white rectangles in A1–2) were predefined in deep layers, and positive cells were counted. (*B*) Number of NeuN positive cells and (*C*) of Hoechst positive cells per field. (*D*) Percentage of NeuN/Hoechst positive cells. COMT+/+ males $N = 20$, females, $N = 16$; COMT+/– males $N = 20$, females $N = 16$; COMT–/– males $N = 20$, females $N = 20$. *$P < 0.05$ versus +/+ mice.

COMT Met/Met Healthy Human Males, but Not Females, Show Increased Thickness of the Inferior Frontal Sulcus and Superior Temporal Sulcus

Evidence from COMT knockout mice indicated that COMT genetic modifications were associated with altered PFC and postero-parieto-temporal thickness in a sex-dependent manner (Figs 1–3). We next asked whether these sexually dimorphic COMT effects were predictive of similar effects in humans using the rs4680 Val/Met polymorphism. This single mutation results in a substantial change in stability and enzymatic activity of COMT: the Met form leads to reduced COMT protein levels and ~40% lower enzymatic activity compared with the Val allelic variant (Chen et al. 2004). Because the 2 alleles are codominant, Val/Met heterozygotes show intermediate COMT activity, leading to a trimodal distribution of COMT activity across the human population (Floderus et al. 1981).

We selected healthy adult participants between 18 and 60 years old (total $N = 121$). The allelic distribution of COMT genotypes was in Hardy–Weinberg equilibrium ($\chi^2 = 0.3$; P level = 0.58). No significant difference was detected for demographic variables among groups (Supplementary Table 1).

Neuroimaging of cortical thickness revealed a significant COMT–sex interaction effect in the right superior temporal sulcus (STS; $P_{FDR} < 0.05$) and in the right inferior frontal sulcus (IFS; $P_{FDR} < 0.05$), which survived the correction for multiple comparisons at a whole-brain level (Fig. 4*A*). Post hoc analyses showed that homozygous males carrying the Met variant showed significantly increased thickness compared with females of all genotypes and to both Val/Met and Val/Val males in the STS ($P < 0.0001$; Fig. 4*B*). Similarly, Met/Met homozygote males exhibited increased thickness of the IFS compared with all other groups ($P < 0.0001$; Fig. 4*C*). These findings concord with previous data on MRI brain morphology

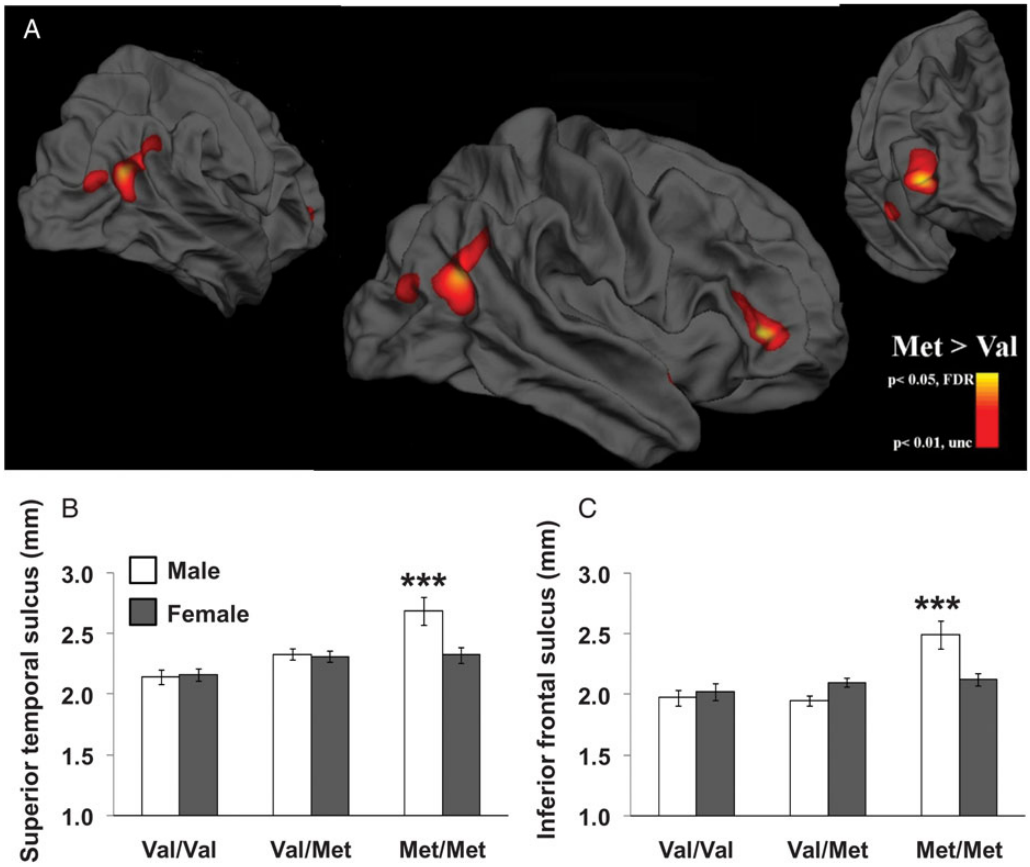

Figure 4. COMT Met/Met male healthy humans, but not female, show increased thickness of the inferior frontal sulcus and superior temporal sulcus. (*A*) Whole-brain vertex-wise analysis of cortical thickness for the COMT Val/Met by sex interaction effect. The regions displayed in red (uncorrected level <0.01) and yellow (correction for multiple comparisons, FDR <0.05) represent areas where males homozygous for the Met allele have significantly thicker cortex than other groups. The effect on the posterior cortex is widespread with cortical thickness alterations encompassing both inferior parietal cortex and middle occipital cortex. The pial view has been used to highlight the 3D gyral and sulcal anatomy. (*B*, *C*) Bar graphs represent mean cortical thickness within the right superior temporal sulcus and the right inferior frontal sulcus plotted as a function of genotype and sex. COMT Val/Val: males $N = 16$, females $N = 15$; COMT Val/Met: males $N = 27$, females $N = 37$; COMT Met/Met: males $N = 9$, females $N = 17$. $^{***}P < 0.0001$ versus all other groups.

in humans, in which participants with the Met allele were characterized by a significant increase in cortical thickness (FDR <0.05) relative to Val-carrier counterparts (Shaw et al. 2009; Cerasa et al. 2010). More importantly, our data suggest sexually dimorphic effects of COMT genetic variations in humans. This is remarkably analogous to the COMT–sex interaction effects found in the COMT knockout mice.

Sexual Dimorphism of COMT Genotype on Visuo-Spatial Working Memory in Mice

PFC–postero-parieto-temporal circuitries have been consistently implicated in working memory (D'Esposito et al. 1998), and our MRI, histological, and immunofluorescence analyses consistently revealed that COMT genetic reductions produced sexual dimorphisms in cortical anatomy of the PFC and postero-parieto-temporal cortex. To explore if COMT genetic mutations differentially modulate working memory in males and females, we tested COMT knockout female mice in the same mPFC-dependent visuo-spatial working memory T-maze task in which we previously tested male COMT knockouts (Papaleo et al. 2008).

Surprisingly, fewer COMT−/− female mice were able to acquire this PFC-dependent discrete paired-trial T-maze task than their +/+ and +/− littermates ($P < 0.05$; Fig. 5*A*). Among the mice that were able to learn this task, no genotype effect was found for the days needed to reach the learning criteria ($F_{2,24} = 0.48$; $P = 0.62$; Fig. 5*B*). During the habituation phase of the task, no genotype effect was present for the latency to eat ($F_{2,32} = 0.20$; $P = 0.82$), and all mice ran faster on the second day to retrieve the reward pellets ($F_{1,32} = 36.49$; $P < 0.0001$; Fig. 5*C*). These have been used as indexes of simple reference memory, as well as index of motivation and motor abilities (Kellendonk et al. 2006; Papaleo et al. 2008).

Female mice that acquired the task were further tested in the discrete paired-trial T-maze paradigm under more demanding conditions, which consisted of 4 different intratrial delays, and a decrease in the intertrial delay to 20 s, instead of 20 min. Analysis of the percentage of correct choices at the different intratrial delays revealed both a genotype effect ($F_{2,96} = 5.22$, $P < 0.008$) and a delay effect ($F_{3,96} = 8.53$, $P < 0.0001$). All groups displayed delay-dependent performance, but the COMT+/− female mice showed consistently worse performance than their COMT+/+ littermates ($P < 0.005$; Fig. 5*D*). The COMT−/− group exhibited a similar profile, showing poorer performance compared with COMT+/+ littermates, although only with the 60-delay interval ($P < 0.05$). This was likely due to the limited statistical power, as only 6 of the 12 females in this group were able to successful acquire the task. These results indicate that genetic modifications resulting in reduced COMT activity lead

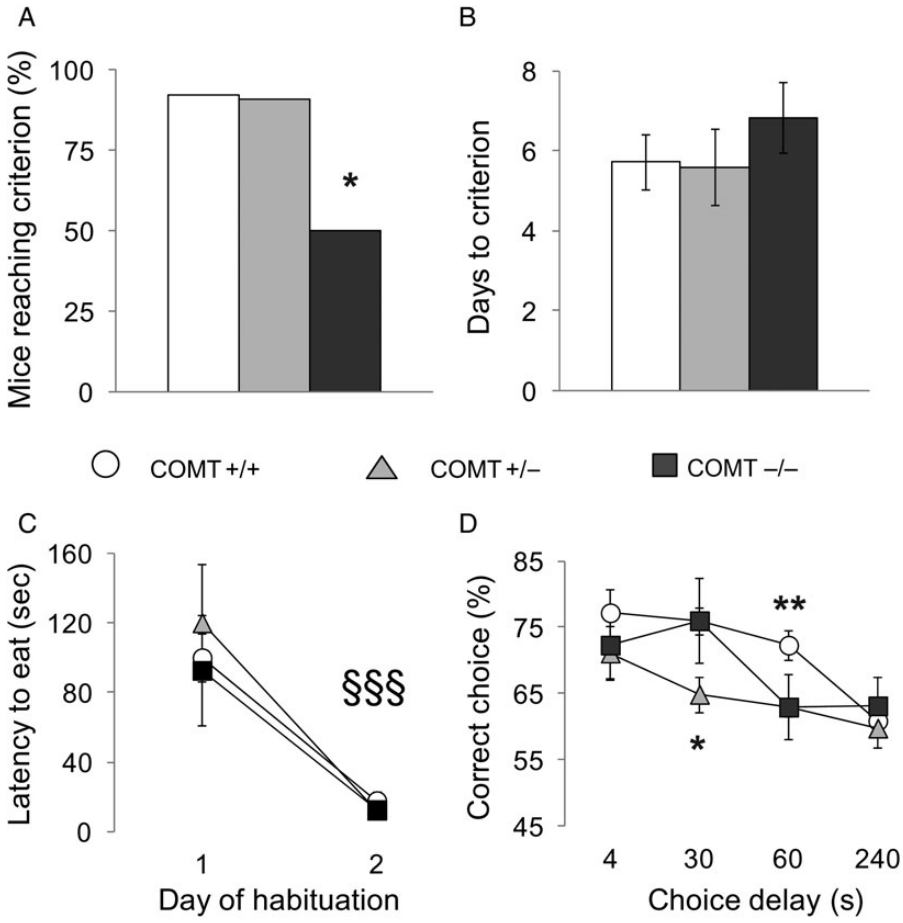

Figure 5. Working memory impairment in COMT knockout female mice. (*A*) Number of mice reaching criteria after 20 days of training, (*B*) days needed to reach the criteria, and (*C*) latency to retrieve the hidden food pellet displayed by COMT+/+, +/–, and –/– female littermates during the discrete paired-trial T-maze task. COMT+/+ females $N = 12$; COMT+/– females $N = 11$; COMT–/– females $N = 12$. *$P < 0.05$ versus +/+ and +/– mice; §§§$P < 0.0005$ versus day 1 of the habituation. (*D*) Percentage of correct choices displayed by COMT+/+, +/–, and –/– during the discrete paired-trial variable-delay T-maze task with different intratrial delays randomly presented (4, 30, 60, 240 s) and an intertrial delay of 20 s. The dotted line corresponds to chance levels (50%) of correct choices. COMT+/+ females $N = 11$; COMT+/– females $N = 10$; COMT–/– females $N = 6$. *$P < 0.05$ versus +/+ and –/– mice; **$P < 0.05$ versus +/– and –/– mice.

to working memory deficits in female mice. This was exactly the opposite of the effect found previously in COMT knockout males, who demonstrated improved working memory performance in this mPFC-dependent task (Papaleo et al. 2008).

COMT Knockout Female Mice do not Have General Cognitive Deficits

To ensure that the working memory deficits found in COMT knockout female mice were specific and not a manifestation of gross cognitive deficits, we tested another cohort of naïve female COMT+/+, +/–, and –/– littermates in the same T-maze apparatus, but this time using a simple spatial reference memory rule: the same arm of the maze was baited on every trial (Kellendonk et al. 2006; Papaleo et al. 2008; Papaleo, Yang et al. 2012b). In contrast to the previous working memory task, every single mouse of all genotypes successfully acquired this reference memory T-maze paradigm ($P = 1.00$), and did so in a similar number of days, irrespective of genotype ($F_{2,18} = 0{,}15$; $P = 0.86$; Supplementary Fig. 5). As with the previous T-maze, no COMT genotype effect was evident for the latency to eat when exposed to the T-maze ($F_{2,18} = 0.03$; $P = 0.97$), and all genotypes ran faster to eat on the second day compared with the first ($F_{1,18} = 22.47$; $P = 0.0005$; Supplementary Fig. 5). Finally, as well as for their male counterparts (Papaleo et al. 2008), genetically driven COMT reduction did not affect the general health or physical abilities of female mice (Supplementary Table 2). Overall, these results indicate that female mice with disruption or partial reduction of COMT manifest specific deficits in working memory functions that are not attributable to alterations in reference memory, spatial learning, motivation, food-reward associations, nonspecific cognitive deficits, motor skills, or abnormal general health.

COMT Met-Carrier Healthy Human Males, but Not Females, Show Improved Visuo-Spatial Working Memory

To investigate if the COMT–sex interaction predicted by our mouse studies in visuo-spatial working memory performance would be observable also in humans, we analyzed the performance of healthy human subjects in the *n*-back paradigm for visual and spatial abilities. This working memory task is thought to be an explicit test of information-processing efficiency within the PFC (Egan et al. 2001; Drabant et al. 2006).

One hundred thirty-one healthy humans (18–60 years old) were tested in this working memory task (Supplementary Table 3 for sociodemographic data). Interestingly, their

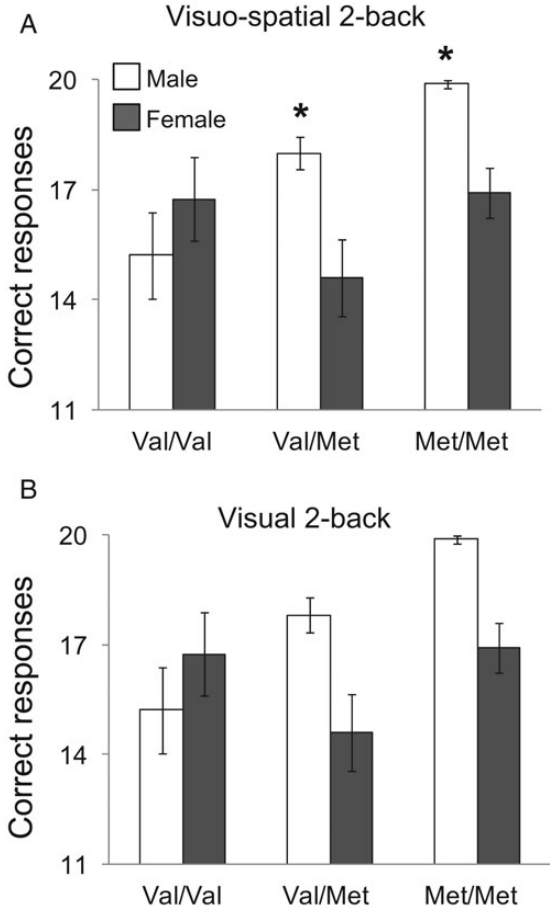

Figure 6. Genetic reduction of COMT leads to improved working memory performance in men but not women. Number of correct responses (*A*) in the visuo-spatial and (*B*) in the visual 2-Back tests displayed by COMT Val/Val, Val/Met and Met/Met male and female healthy adult human subjects. COMT Val/Val males $N = 14$, females, $N = 12$; COMT Val/Met males $N = 32$, females $N = 40$; COMT Met/Met males $N = 8$, females $N = 25$. *$P < 0.02$ versus Val/Met women.

performance in the visuo-spatial working memory 2-back test revealed a significant statistical interaction between COMT genotype and sex ($F_{2,124} = 5.3$; $P = 0.006$). Post hoc analysis revealed that male COMT Met carriers, but not male COMT Val/Val, made more correct responses than Val/Met women ($P < 0.05$; Fig. 6*A*). In the visual version of the test, a similar trend was present; however, the interaction between COMT genotype and sex failed to reach statistical significance ($F_{2,124} = 2.8$; $P = 0.06$; Fig. 6*B*). No significant COMT–sex interactions were present in the easier visuo-spatial and visual 0- and 1-back tests (Supplementary Fig. 5).

Despite the relative small sample sizes in the experimental groups, it is tempting to note that the 2-back visuo-spatial working memory performance was superior in men with relatively decreased COMT, but not in women. This is similar to the findings with the COMT genetically modified mice.

Discussion

We have reported results from adult mice and human healthy subjects that inform on the interaction between COMT genetic modification and sex on cortical anatomy and function. Our main finding was that genetic modifications reducing COMT enzyme activity produced divergent changes in brain morphology depending on the sex of the subject. Genetically driven reduction in COMT enzyme activity was associated with a selective increase in cortical thickening in the PFC and postero-parieto-temporal cortex of male but not female healthy humans and mice. Moreover, COMT genetically driven reduction produced sexually dimorphic effects in neuronal density, especially in the PFC. That is, reduction of COMT resulted in increased neuronal density in males' mPFC, but reduced the neuronal density in female mice. Finally, the potential relevance of these sex-specific morphological changes was observed in pertinent cognitive functions such as working memory which also showed that genotype effects varied with sex.

Previous neuroimaging studies have suggested an effect of the human COMT Val/Met polymorphism on the morphology of human brain (Witte and Floel 2012; Ira et al. 2013). However, the extent to which COMT genetic variations differentially influenced brain anatomy in the 2 sexes was still unclear. Kates et al. reported sex-dependent effects of the COMT Val/Met polymorphism in dorsal versus orbital PFC in 58 children affected by 22q11DS, a genetic syndrome in which COMT is hemi-deleted. Similarly, other studies have investigated potential sexually dimorphic effect of COMT genetic variations on brain morphology, but without conclusive results (Kates et al. 2006; Zinkstok et al. 2006; Barnes et al. 2012). The translational approach adopted here allowed us to unravel the biological causal link between mutations in the COMT gene and brain morphological changes. We demonstrated that genetically driven COMT enzymatic reduction in both mice and humans was associated with increased thickness in the PFC and the postero-parieto-temporal cortex in healthy adult males but not in females, an effect that might show some laminar specificity.

Dimorphic effects of COMT were evident on neuronal density in the mPFC and the posterior parietal cortex. The increased neuronal density found in the deeper layers of the mPFC of male COMT knockout mice might be a consequence of the increased dopaminergic stimulus on these neurons in these male mutants. Indeed, the deeper layers of the mPFC are highly targeted by dopamine projections (van Eden et al. 1987) and express the highest level of dopamine receptors within the cortex (Al-Tikriti et al. 1992). Importantly, PFC dopamine levels are increased in adult COMT knockout male but not female mice (Gogos et al. 1998; Yavich et al. 2007). In contrast, dopaminergic innervations are less abundant in the posterior parietal cortex (Levitt et al. 1984; Haber and Fudge 1997). Thus, the effects we found on the postero-parieto-temporal cortex might be a consequence of COMT-dependent effects in the PFC. Indeed, PFC and postero-parieto-temporal cortex present strong anatomical and functional connections through the inferior fronto-occipital fasciculus (Reep et al. 1994). This interpretation might be also supported by the fact that the principal changes in neuronal density in the parietal cortex were found in layers II/III, which receives primarily intracortical connections (Miller 1996).

The present data are unable to specify the mechanisms by which these sexual dimorphisms occur, but these mechanisms are unlikely to be simple. We might speculate that the observed increased thickness and neuronal density in the mPFC of males following COMT reduction is likely a consequence of the increased dopaminergic stimulus acting as a neurotrophic factor

(Robinson and Kolb 1997; Jones et al. 2000). Notably, our data suggest that differences in neuronal density did not closely parallel the differences found in cortical thickness. In particular, COMT−/− males exhibited the greatest thickening of the PFC, but their neuronal density was comparable with that of +/− heterozygous males. Moreover, in females, reduced COMT enzyme activity did not alter cortical thickening but did reduce cortical neuronal density. It is conceivable that different compensatory changes in cells size and/or dendritic arborizations might be at the base of these discrepancies. The conundrum, however, is why these COMT-dependent effects were different and even opposite between males and females? Perhaps of critical interest is evidence that baseline extracellular mPFC dopamine levels, mesocortical dopaminergic cells, and the release of dopamine after stimulation are all higher in females than in males (Becker 1999; Kritzer and Creutz 2008; Staiti et al. 2011). As dopamine and cortical function show a well-characterized inverted *U*-shaped relationship (Papaleo et al. 2008; Mier et al. 2010), it is conceivable that females have much greater dopamine stimulation than males for the same genotype, leading them to fall off the right hand limb of the inverted U curve if they have COMT-reduction genotypes. The effect of this sex-specific hyperdopaminergic overdrive during development may be counter-trophic and of negative influence on cortical development and function. Thus, specific sex-dependent differences in the brain dopaminergic organization might interact with the prolonged alterations in the PFC dopaminergic system produced by COMT genetic reductions altering the neurodevelopmental trajectories of the PFC. Finally, because different levels of COMT finely modulate dopamine levels in PFC (Gogos et al. 1998; Chen et al. 2004), these same mechanisms might be also at the base of the discrepancies between the different measures of brain anatomy in the context of partial reduction (+/−) versus complete absence (−/−) of COMT.

Our results add to previous work (Bruder et al. 2005; Papaleo et al. 2008; Papaleo, Erickson et al. 2012a) highlighting that COMT is a "hot-spot" gene involved in finely modulating working memory, despite exerting less influence on other cognitive processes such as sustained attention, reference memory, and reversal learning. Caution should be observed when comparing tasks performed in humans and rodents. Nevertheless, we would note that, there is evidence that suggests that higher order cognitive processes such as working memory depend on the reciprocal connection between the PFC and posterior parietal cortical areas in both primates and rodents (Reep et al. 1994; Fox et al. 2003; Katsuki and Constantinidis 2012). Although alterations in cortical thickness per se might not explain specific behaviors or pathological states, our combined morphological/behavioral findings support the presence of this functionally analogous cortical network in mice as well as humans. Moreover, we provided initial evidence suggesting that neuronal density in the mPFC also predicts COMT modulatory effects in working memory. That is, COMT knockout male mice showed increased cortical neuronal density in the mPFC and improved PFC-dependent working memory abilities relative to COMT+/+ mice. Conversely, COMT knockout female mice had decreased neuronal density in the mPFC and impaired PFC-dependent working memory. This suggests that beyond mere thickness, neuronal density may predict cognitive outcomes. In agreement, recent findings with mice have shown that neuronal density in the mPFC correlates with cognitive abilities such as reversal learning performance (Meechan et al. 2013).

In summary, we report that functional genetic mutations in COMT modulate cortical thickening, neuronal density, and working memory performance with different profiles strongly dependent on the sex of the subject. Alterations in brain morphology and related cognitive functions represent important intermediate phenotypes in individuals at risk for psychiatric disorders. Because these disorders are often characterized by sex-dependent differences, our findings may explain some of the discrepancies related to COMT and human behavior and psychiatric disorders. In particular, in consideration of our present findings, imaging and behavioral studies on the effects of COMT genetic modifications that have overlooked the sex of the subjects should be reconsidered. This might be particularly important when comparing the overall COMT-dependent effects in 2 populations that might have a sex bias (e.g., healthy subjects with normal distribution of males and females versus patients with schizophrenia that might have higher numbers of males). Similarly, a distinction between males and females in relationship to COMT associations may be important in pharmacogenetic studies. Our results emphasize the importance of taking into account the combined effect of sex and genetics to improve the outcomes of diagnostic and therapeutic strategies.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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Notes

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