

Additive Effects of Genetic Variation in Dopamine Regulating Genes on Working Memory Cortical Activity in Human Brain

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Functional polymorphisms in the catechol-*O*-methyltransferase (COMT) and the dopamine transporter (DAT) genes modulate dopamine inactivation, which is crucial for determining neuronal signal-to-noise ratios in prefrontal cortex during working memory. We show that the COMT Met¹⁵⁸ allele and the DAT 3' variable number of tandem repeat 10-repeat allele are independently associated in healthy humans with more focused neuronal activity (as measured with blood oxygen level-dependent functional magnetic resonance imaging) in the working memory cortical network, including the prefrontal cortex. Moreover, subjects homozygous for the COMT Met allele and the DAT 10-repeat allele have the most focused response, whereas the COMT Val and the DAT 9-repeat alleles have the least. These results demonstrate additive genetic effects of genes regulating dopamine signaling on specific neuronal networks subserving working memory.

Key words: dorsolateral prefrontal cortex; anterior cingulate; working memory; dopamine; catechol-*O*-methyltransferase; dopamine transporter

Introduction

Dopaminergic modulation of neuronal signal-to-noise is crucial for working memory processes in prefrontal cortex. More specifically, dopamine directly regulates firing of pyramidal neurons and of their GABA inhibitory surround within prefrontal cortex by differentially acting on D₁ and D₂ dopamine receptors in these neurons to focus prefrontal cortical resources to the task at hand (Seamans and Yang, 2004). Moreover, dopamine indirectly modulates prefrontal signal-to-noise via effects in the striatum, which regulates activity within the cortico-striato-thalamo-cortical circuit (Newman and Grace, 1999). A critical step in determining dopamine signaling is its removal from the extracellular space. Different mechanisms account for inactivation of dopamine at the synaptic and extrasynaptic levels, including methylation by catechol-*O*-methyltransferase (COMT) and reuptake via the dopamine transporter (DAT). COMT is expressed in neurons in the prefrontal cortex (Karoum et al., 1994), especially at neuronal dendritic processes in large pyramidal neurons (Matsumoto et

al., 2003), with a distribution similar to that of D₁ receptors (Diop et al., 1988). Moreover, both genetic and pharmacologic variations in COMT activity modify dopamine levels in the prefrontal cortex (Huotari et al., 2002; Tunbridge et al., 2004). The COMT gene contains a G to A missense variant (Lachman et al., 1996) that translates into a substitution of methionine for valine at codon 158 (Val¹⁵⁸Met), such that the enzyme containing Met¹⁵⁸ has significantly less activity and presumably greater synaptic dopamine than the Val¹⁵⁸ enzyme (Lotta et al., 1995; Chen et al., 2004). Consistently, human subjects carrying the high-activity Val allele show greater engagement of cortical resources during working memory [as assessed with blood oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI)] along with lower behavioral performance (Egan et al., 2001; Mattay et al., 2003; Bertolino et al., 2004b; Blasi et al., 2005).

The DAT critically regulates duration of the cellular actions of dopamine and the extent to which dopamine diffuses in the extracellular space, especially in the striatum. Expression of the DAT is abundant in the striatum where it is mostly found in synapses (Sesack et al., 1998; Lewis et al., 2001). In contrast, in the primary sensory motor cortex, and in prefrontal and cingulate cortices, the DAT is found in low abundance and primarily at a distance from synaptic sites of dopamine release (Sesack et al., 1998; Lewis et al., 2001). Given its ultrastructural localization, the DAT in the striatum is critical in regulating point-to-point dopa-

Received Nov. 22, 2005; revised Jan. 30, 2006; accepted Feb. 27, 2006.

We acknowledge Riccarda Lomuscio, Dr. Mariapia De Candia, Dr. Savino Dimalta, and Dr. Leonardo Fazio for help with data acquisition and all subjects who participated to the study.

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DOI:10.1523/JNEUROSCI.4975-05.2006

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mine neurotransmission, whereas in the cortex, the DAT seems to be better situated to regulate dopamine volume transmission (the three-dimensional spillover of dopamine from a release site reaching the extrasynaptic space) (Cragg and Rice, 2004). Volume transmission is thought to mediate interneuronal communication along with synaptic point-to-point transmission (Zoli et al., 1998). Considering its anatomical distribution and the importance in dopamine neurotransmission, the DAT may play a critical role in regulating cortical signal-to-noise ratio during working memory both directly and indirectly. DATs can directly affect prefrontal pyramidal neurons by regulating dopamine volume transmission on surrounding GABA inhibitory neurons. In contrast, the effect of the DAT can also be indirect via the cortico-striato-thalamo-cortical pathway in which the net effect of dopamine in the striatum is of increasing activity of thalamo-cortical pathways with facilitation of cortically initiated action (Newman and Grace, 1999; Mattay et al., 2002). A functional variable number of tandem repeat (VNTR) polymorphism in the 3'-untranslated region of the DAT gene has been described previously (Vandenberg et al., 1992). Alleles of this polymorphism range from 3 to 11 repeats, with the 9- and 10-repeat alleles by far the most common (Vandenberg et al., 1992). Compared with the 9-repeat allele, the 10-repeat allele has been associated with increased gene expression both *in vitro* (Mill et al., 2002) and *in vivo* (Heinz et al., 2000), although reports of the opposite allelic associations have also appeared (van Dyck et al., 2005). A more recent study under carefully controlled experimental conditions using the DAT protein itself as the reporter signal reported data consistent with the 10-repeat allele being associated with greater expression (VanNess et al., 2005). This study used a targeted stable integration protocol that eliminates confounds to construct comparison attributable to variable transfection efficiency or clonal variance, both common limitations of transient transfection or conventional nontargeted stable integration approaches. These limitations have been generally present in previous studies that have also used heterologous DAT.

Although the potential influence of additive genetic variation on complex behavioral phenotypes has long been predicted, there is no *in vivo* demonstration in humans of the effect of two genes acting together on the same neurobiology. We used fMRI in healthy subjects to explore the relationship of these two functional polymorphisms in dopamine-related genes and neuronal activity measured during working memory, known to involve prefrontal and cingulate activity. Based on the physiological role of dopamine in regulating signal-to-noise ratios in prefrontal neurons (both directly and indirectly via cortico-striato-thalamo-cortical pathways), we hypothesized that individuals homozygous for the COMT Met allele and the DAT 10-repeat allele would show the most focused cortical engagement during working memory compared with COMT Val and DAT 9-repeat.

Materials and Methods

Subjects. We studied 62 healthy subjects (33 males; mean age, 31.8 ± 8.1). After genotype determination, the groups were divided based on COMT, DAT, and COMT-DAT genotypes. The *n* values were as follows: COMT (Val/Val, 14; Val/Met, 34; Met/Met, 14), DAT (9/9-repeat, 7; 9/10-repeat, 30; 10/10-repeat, 25), COMT-DAT (Val/Val 9/10-repeat, 6; Val/Val 10/10-repeat, 8; Val/Met 9/9-repeat, 4; Val/Met 9/10-repeat, 17; Val/Met 10/10-repeat, 13; Met/Met 9/9-repeat, 3; Met/Met 9/10-repeat, 7; Met/Met 10/10-repeat, 4). There were no subjects homozygous for COMT val and DAT 9-repeat alleles in this sample. The allelic distribution of both genes was in Hardy Weinberg equilibrium (COMT χ^2 df 2, $\chi^2 = 0.58$, $p > 0.2$; DAT χ^2 df 2, $\chi^2 = 0.2$, $p > 0.1$). For additional demo-

graphics and genotype determination, see supplemental material (available at www.jneurosci.org).

Working memory paradigm. During fMRI, all subjects completed a blocked paradigm of the N-Back task (Bertolino et al., 2004b), which includes a nonmemory guided control condition (0-Back) and a working memory condition (2-Back). Behavioral performance was recorded as accuracy and reaction time (see supplemental material, available at www.jneurosci.org).

Acquisition and analysis of imaging data. See supplemental material (available at www.jneurosci.org). BOLD fMRI data were acquired with a General Electric (Milwaukee, WI) 3T scanner, as described previously (Bertolino et al., 2004a,b). Data processing with SPM99 included reconstruction, registration, linear detrending, global normalization, and smoothing (10 mm Gaussian kernel). Individual contrast maps were created with *t* statistics (2-Back > 0-Back) and then used in second-level random effects models. All statistical analyses were thresholded at $p < 0.005$, minimum cluster size (k) = 3, with additional Family Wise Error (FWE) small volume correction for multiple comparisons ($p < 0.05$). To identify the independent contribution of both genotypes to activation of the working memory cortical network, we performed multiple regression analyses within SPM99. For this analysis, we entered the single-subject contrasts (2-Back > 0-Back) with the number of Met alleles of COMT genotype (Val/Val, 0; Val/Met, 1; Met/Met, 2) and with the numbers of 10-repeat alleles of DAT genotype (9/9-repeat, 0; 9/10-repeat, 1; 10/10-repeat, 2) as predictors. Furthermore, to evaluate the effect of both genotypes, we used linear regression analyses within SPM99 entering the single-subject contrasts with Met- and 10-repeat alleles as predictors (Val/Val 9/10-repeat, 0; Val/Val 10/10-repeat, 1; Val/Met 9/9-repeat, 2; Val/Met 9/10-repeat, 3; Val/Met 10/10-repeat, 4; Met/Met 9/9-repeat, 5; Met/Met 9/10-repeat, 6; Met/Met 10/10-repeat, 7). We used this analysis to evaluate the parametric effect of both genes with COMT genotype as the major grouping factor for a series of reasons. First, the effect of the COMT polymorphism was already known. Second, the presumed neurobiology of cortical and subcortical dopamine inactivation inside and outside of synapses (see above) makes it reasonable to hypothesize a greater effect of COMT versus that of DAT on cortical signal-to-noise. Third, we calculated the effect size of both genes and found that it was greater for COMT than for DAT. For anatomical localization, statistical maxima of activation were converted to conform to the standard space of Talairach and Tournoux. Brodmann areas (BAs) were determined using the Talairach Daemon software (<http://ric.uthscsa.edu/projects/talairachdaemon.html>). To determine the proportion of variance in BOLD signal accounted for by both genotypes, we used ω^2 (see supplemental material, available at www.jneurosci.org).

Results

Demographics and working memory performance

All genotype groups were matched for age, gender, full scale IQ, and handedness (all, $F < 1.1$; all, $p > 0.3$). They also did not differ in behavioral performance of the working memory tasks (accuracy and reaction time; all, $F < 1.5$; all, $p > 0.15$), thus allowing us

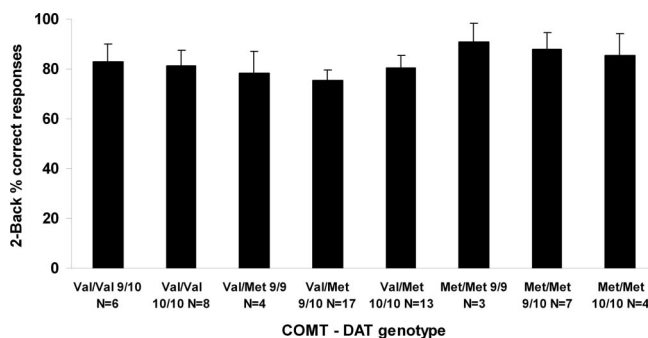


Figure 1. Working memory behavioral results. Performance accuracy results for the 2-Back task across genotype groups. Performance is represented as mean percentage of correct responses (\pm SEM).

to examine the effect of complex genotypes on brain activity independent of behavioral variation in this sample (Fig. 1). All of these analyses were performed separately for each genotype as well as for both genotypes together.

Imaging data

Analysis of the working memory imaging data in the whole sample revealed significant BOLD responses in the working memory cortical and subcortical network, including dorsolateral prefrontal cortex (BA 9), anterior cingulate (BA 24 and BA 32), premotor area (BA 6), parietal cortex (BA 39/40), caudate, and putamen, consistent with previous reports (Callicott et al., 1999, 2000; Egan et al., 2001; Bertolino et al., 2004b). Moreover, multiple regression analyses revealed that both genotypes independently predicted responses in areas of the working memory network. More specifically, the number of Met alleles in the COMT gene was negatively associated with response in the left precentral gyrus/left middle frontal gyrus (Brodmann areas 6/9; Talairach coordinates $x = -40, y = 1, z = 27, k = 27, t = 3.37; p = 0.02$ after FWE correction for multiple comparisons), in the right middle frontal gyrus (BA 9; $x = 33, y = 5, z = 22, k = 8, t = 3.28; p = 0.02$), and in the anterior cingulate (BA 24; $x = 11, y = 12, z = 27, k = 26, t = 3.37; p = 0.02$) (Fig. 2, first row), again consistent with previous reports. Similarly, the number of 10-repeat alleles in the DAT gene was negatively associated with response in the left middle frontal gyrus (Brodmann area 9, $x = -19, y = 26, z = 26, k = 3, t = 3.5, p = 0.01$; BA 8, $x = -51, y = 9, z = 38, k = 3, t = 3, p = 0.04$), in the right middle frontal gyrus (BA 9/8; $x = 33, y = 16, z = 32, k = 25, t = 3.61; p = 0.01$), and in the anterior cingulate (BA 24; $x = 8, y = 12, z = 27, k = 3, t = 2.89; p = 0.05$) (Fig. 2, second row). This approach allows for the unbiased determination of the contribution of independent variables to response of the working memory cortical network, suggesting that both genotypes are independently associated with it. We then used simple regression analyses with genotypes as regressors to address the effect of both genotypes together. Each genotype was attributed a different weight with COMT Val/Val–DAT 9/10-repeat (there was no COMT Val/Val DAT 9/9 individual in this sample) the lowest weight and COMT Met/Met–DAT 10/10-repeat the highest. The results of this analysis indicated that subjects carrying Met and 10-repeat alleles have more focused engagement of areas BA 6/9 ($x = -40, y = 1, z = 27, k = 53, t = 3.44, p = 0.01; x = 33, y = 5, z = 22, k = 10, t = 3.36, p = 0.02$) and BA 24 ($x = 11, y = 12, z = 27, k = 29, t = 3.44; p = 0.01$) than subjects carrying Val COMT and 9/10-repeat DAT (Fig. 2, third row), despite no differences in behavioral performance of the groups. This analysis indicates an overall effect on all groups. An additional *t* test performed outside of image space indicated that the difference in percentage BOLD signal change between Val/Val 9/10-repeat and Met/Met 10/10-repeat groups is statistically significant in all three clusters identified in the linear regression

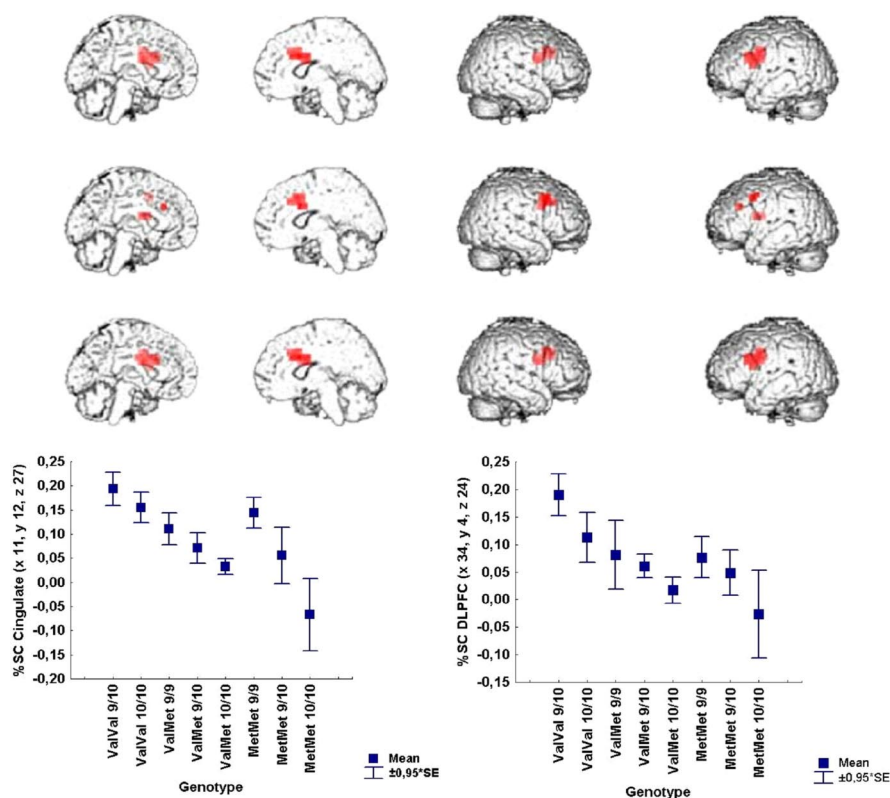


Figure 2. Genotype-based analyses of the working memory BOLD fMRI response. First row, The effect of COMT with Met homozygous subjects having the most focused engagement of areas in BA 6, 9, and 24. Second row, The effect of DAT with 10-repeat homozygous subjects having the most focused engagement of areas in BA 9, 8, and 24. Third row, The combined effect of COMT and DAT with COMT Met homozygous and DAT 10-repeat homozygous subjects having the most focused engagement of areas in BA 6, 9, and 24. Means \pm 0.95 SE plots (4th row) showing percentage of signal change from BA 24 (left) and BA 9 (right) from the latter analyses in all eight genotype groups. These graphs suggest the additive effect of the two genotypes on percentage signal change.

($df = 8$; all, $t > 2.6$; all, $p < 0.03$), suggesting that these two groups have the least and the most focused engagement.

The proportion of variance in the BOLD signal from left BA 6/9, right BA 9, and BA 24 accounted for by both genotypes combined was 22, 11, and 16%, respectively, as calculated with ω^2 . To further demonstrate the additive nature of the action of these two polymorphisms, we reasoned that by inverting the sign of the DAT polymorphism, we would dilute the COMT effect. Therefore, we performed another linear regression with COMT Val/Val–DAT 10/10-repeat having the lowest weight and COMT Met/Met–DAT 9/9-repeat having the highest. This analysis dramatically changed the results, because there was no negative relationship between genotypes and BOLD activation even dropping the statistical threshold at $p < 0.01$, uncorrected. These results, further suggest that the effect of the two polymorphisms is additive. This is also suggested by the effect size (Cohen's *d*) of the difference of signal change in BA 9. The effect size of the difference between COMT Val/Val and Met/Met individuals in BA 9 is $d = 1.42$ (Cohen's *d*); the effect size between 9/9-repeat and 10/10-repeat is $d = 0.64$; the effect size between COMT Val/Val DAT 9/10-repeat and COMT Met/Met DAT 10/10-repeat is $d = 2.7$.

Discussion

As predicted, our results indicate that both COMT and DAT genotypes independently predict BOLD signals in the working memory cortical network with COMT Met/Met and DAT 10/

10-repeat individuals having a more focused response (lesser activation for similar performance). Moreover, these two genes have an additive effect on this phenotype, because subjects homozygous for COMT Met and DAT 10-repeat alleles have the most focused engagement of the working memory network, whereas subjects homozygous for the COMT Val allele and with DAT 9/10-repeats have the least. These differential responses in the working memory cortical network may result from the differential anatomical and ultrastructural expression of COMT and DAT genes. The COMT Met allele is associated with lower activity of the enzyme, presumably leading to relatively increased synaptic levels of dopamine, which would directly increase the signal-to-noise ratio of pyramidal glutamatergic neurons via stimulation of D₁ receptors (Seamans and Yang, 2004). The DAT genotype may exert its effect directly in the prefrontal cortex or indirectly via the striatum. In the cortex, DATs tend to be found on nonvaricose axon segments of small diameter, which make symmetric synapses (Sesack et al., 1998; Lewis et al., 2001), with a distribution similar to that of some D₂ receptors, a proportion of which are extrasynaptic (Pickel et al., 2002; Negyessy and Goldman-Rakic, 2005). Moreover, the functional activity of DAT is regulated by D₂ receptors (Meiergerd et al., 1993; Dickinson et al., 1999; Mortensen and Amara, 2003). Thus, the DAT 10-repeat allele associated with increased expression of the gene may presumably lead to relatively decreased extrasynaptic cortical dopamine levels, which would be associated with reduced D₂ signaling and with increased GABA release, thus secondarily increasing signal-to-noise ratios of pyramidal neurons (Seamans and Yang, 2004). However, it is also important to consider that the DAT is expressed in low abundance in the cortex, but it is very abundant in the striatum, a key component of the cortico-striato-thalamo-cortical system. It has been argued that it is via this complex, re-entrant system that integrated perceptual, mnemonic, and affective information is synthesized into a coherent stream in working memory (Newman and Grace, 1999). Within this complex circuit, the striatum may serve as a gating system filtering out extraneous inputs and binding together of relevant ones to focus the working memory set. In other words, a primary purpose of striatal circuitry may be to increase the signal-to-noise ratio in the cortex, facilitating binding and suppressing cortical activity not directly contributing to the focus of working memory. Because the net effect of dopamine in the striatum is of increasing activity of thalamo-cortical pathways (Tisch et al., 2004), greater expression of the DAT in the striatum would presumably lead to greater dopamine inactivation with a more focused response of the thalamo-prefrontal pathway. Therefore, it is theoretically possible that our data result from a genetic interaction across the cortico-striatal circuitry, where the effect of COMT would take place in the prefrontal cortex and the effect of DAT either in the cortex or in the striatum, or both. In this case, the interaction in our data would be manifest primarily in the prefrontal cortex, because this is the anatomical region more robustly engaged by our working memory task. Our data do not permit a direct test of these different explanations for the neural mechanism of this additive genetic effect. However, these interpretations are consistent with the known role of dopamine in point-to-point and volume neurotransmission, with the cellular localization of COMT and DAT in the cortex and in the striatum, with the cellular localization of D₁ and D₂ receptors in the cortex, and with previous functional imaging studies in humans suggesting a critical role

for cortical regulation of dopamine via both COMT and DAT (Egan et al., 2001; Mattay et al., 2003).

A limitation of the present study is that we have not measured dopamine directly. Therefore, the effects demonstrated with BOLD fMRI are not necessarily related to dopamine concentrations or release. Instead, they might be related to brain plasticity associated with different molecular mechanisms present at the same time but not necessarily related to these two genotypes. It is also important to note that these genotypes may have an effect on other brain functions. Although the presumed neurobiology of dopamine seems to support a more specific role for modulation of higher cognitive functions, it is also possible that genetic variants of these two genes may exert a more general effect on brain activity, including the default mode network (Raichle et al., 2001; Greicius et al., 2003).

It is also important to consider that we used a simple regression model to account for interacting gene effects. This model assumes that the two genes have a meaningful biological relationship that is linear and continuous. However, there is no evidence supporting a “parametric” relationship between the two genes. Therefore, we performed another regression analysis using a “nonparametric” model for the effect of the two genes. This regression was based on the number of hypothetical “beneficial” alleles (Met and 10-repeat) in each individual, resulting in four groups with 1, 2, 3, and 4 of these alleles. Using the same statistical thresholds as in all other statistics, this analysis indicated that the results are very similar (data not shown) to the parametric analysis, suggesting that the effects are not a function of the statistical model used.

To our knowledge, this is the first study in humans of an additive genetic effect of two dopamine regulating genes on the working memory cortical activity phenotype. Elucidation of the genetic factors contributing to individual variation in working memory cortical activity has profound implications for several medical conditions in which brain phenotypes may better elucidate new ways for their assessment and treatment.

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