Molecular Psychiatry (2006), 1–11
© 2006 Nature Publishing Group All rights reserved 1359-4184/06 $30.00
www.nature.com/mp

ORIGINAL ARTICLE

Impact of complex genetic variation in *COMT* on human brain function

A Meyer-Lindenberg[1,2,3], T Nichols[4], JH Callicott[2,3], J Ding[4], B Kolachana[3], J Buckholtz[2,3], VS Mattay[2,3], M Egan[3] and DR Weinberger[3]

[1]*Unit for Systems Neuroscience in Psychiatry, National Institute of Mental Health, NIH, DHHS, Bethesda, MD, USA;* [2]*Neuroimaging Core Facility, National Institute of Mental Health, NIH, DHHS, Bethesda, MD, USA;* [3]*Clinical Brain Disorders Branch, Genes, Cognition and Psychosis Program, National Institute of Mental Health, NIH, DHHS, Bethesda, MD, USA and* [4]*Department of Biostatistics, University of Michigan, Ann Arbor, MI, USA*

Catechol-*O*-methyltransferase (COMT) has been shown to be critical for prefrontal dopamine flux, prefrontal cortex-dependent cognition and activation. Several potentially functional variants in the gene have been identified, but considerable controversy exists regarding the contribution of individual alleles and haplotypes to risk for schizophrenia, partly because clinical phenotypes are ill-defined and preclinical studies are limited by lack of adequate models. Here, we propose a neuroimaging approach to overcome these limitations by characterizing the functional impact of ambiguous haplotypes on a neural system-level intermediate phenotype in humans. Studying 126 healthy control subjects during a working-memory paradigm, we find that a previously described risk variant in a functional Val158Met (rs4680) polymorphism interacts with a P2 promoter region SNP (rs2097603) and an SNP in the 3′ region (rs165599) in predicting inefficient prefrontal working memory response. We report evidence that the nonlinear response of prefrontal neurons to dopaminergic stimulation is a neural mechanism underlying these nonadditive genetic effects. This work provides an *in vivo* approach to functional validation in brain of the biological impact of complex genetic variations within a gene that may be critical for its clinical association.
Molecular Psychiatry advance online publication, 20 June 2006; doi:10.1038/sj.mp.4001860

Keywords: COMT; n-back; haplotypes; prefrontal cortex; schizophrenia; fMRI

Introduction

Most diseases of interest for molecular psychiatry are genetically complex. The dissection of genetic susceptibility to these common disorders depends on understanding interactions between multiple genetic variants of usually weak isolated impact, environment and phenotype.[1] In this setting, the study of haplotypes, which characterize the linkage disequilibrium (LD) structure of several markers, is considered highly advantageous, since haplotypes may show stronger association with illness than individual markers because they contain more information about the underlying causative genetic variants in the population.[2] If, as is commonly the case, causative variant(s) are unknown, haplotype analysis is desirable because it likely reflects the genetic background on which these variants arose in a given population.[2] Knowledge of LD across the genome also greatly increases the efficiency of large-scale or genome-wide association studies, a major impetus underlying the Human Hapmap project.[3] In addition to these general considerations, haplotype analyses are not only advantageous but essential if multiple functional sites are implicated within a single gene or haplotype block, for example, through a combination of *cis*-acting variants affecting transcription and coding variants affecting protein function[4,5] because analysis of these interacting effects then critically depends on a characterization of which variants appear together on the chromosome.

These issues become especially pertinent for uniquely human disorders such as psychiatric illnesses, in which clinical phenotypes are often ill-defined and model systems are of restricted value, limiting the options to evaluate the functional implications of interacting loci within a gene *in vitro*. A gene of central interest for neuropsychiatry where multiple potential interacting loci have been described is *COMT*, encoding a major enzyme degrading cortical dopamine. Due to the paucity of dopamine transporters in prefrontal cortex (PFC),[6] COMT is a critical determinant of prefrontal dopamine flux.[7] The gene is located at 22q11.2, a region implicated in schizophrenia by linkage[8] and by the 22q11.2 syndrome (MIM#192430), a hemideletion associated with

Correspondence: Dr A Meyer-Lindenberg, Neuroimaging Core Facility, National Institute for Mental Health, NIH, DHHS, 10-3C103, 9000 Rockville Pike, Bethesda, MD 20892-1257, USA.
E-mail: andreasml@nih.gov
Received 16 March 2006; revised 12 May 2006; accepted 26 May 2006

Figure 1 Diagram of the *COMT* gene, showing locations of genotyped SNPs. Redrawn after Palmatier *et al.*[20]

strongly increased risk of schizophrenia-like illness.[9] A common val$^{108/158}$met substitution affects the stability of the protein and leads to a significant decrease in the activity of the enzyme in brain and lymphocytes.[10] Several studies have demonstrated that this coding variant impacts on indices of PFC function, including PFC activation during working memory,[11] PFC-dependent neuropsychological performance,[12] response to amphetamine in healthy humans,[13] and prefrontal regulation of midbrain dopamine synthesis[14,15]

However, despite the consistent findings implicating the *COMT* val$^{108/158}$met polymorphism in prefrontal function, the evidence supporting association of this coding variant with schizophrenia is inconclusive[16,17] and the isolated association of this polymorphism with the schizophrenia phenotype is likely to be small. One reason contributing to this apparent discrepancy is that additonal genetic variability in *COMT* may be important. In support additional genetic of this hypothesis, a haplotype combining rs4680 with two common SNPs, one upstream in intron 1 (rs737865) and the other in the 3′ untranslated region (rs165599), was highly associated with schizophrenia in a large sample of Israelis of Ashkenazi descent,[18] and differentially affected expression of rs4680 alleles in human brain tissue,[19] suggesting the presence of a *cis* acting functional locus within *COMT* interacting with val/met. Another population study found that this three marker haplotype is markedly heterogeneous in populations worldwide[20] despite the relatively constant prevalence of schizophrenia and suggested the relevance of another possible *cis* functional variant (rs2097603) linked upstream in the P2 promoter driving transcription of the predominant form of *COMT* in the brain (MB-*COMT*) (Figure 1). This variant also affects COMT activity in lymphocytes and post-mortem brain tissue.[10] Thus, *COMT* may contain at least three functional polymorphisms that impact on its biologic actions and confound its clinical associations. The potential complex interactions of functional variations in *COMT* imply that the overall functional state of the gene in an individual, presumably critical for phenotypic association, may not be easily deduced from genotype information alone.

To jointly characterize multiple loci in *COMT*, haplotype information is essential. However, since currently used high-throughput genotyping technology does not provide certain phase information, haplotypes cannot be directly observed in unrelated individuals if more than one marker locus is heterozygous and must be estimated, commonly through an expectation-maximization algorithm[21,22] or a Bayesian approach.[23] Simply using the most likely haplotype for each subject is unadvisable as it has been shown to introduce bias[24] and increased error[25] into the estimated effects, and will reduce power because some individuals cannot be reliably scored. Similarly, restricting analysis to only those individuals who happen to have uniquely determined haplotypes produces a biased subsample of lower power. Therefore, a more powerful approach is desirable that can incorporate haplotype probabilities into the analysis. For single quantitative traits, both score-based[26] and regression-based[4,27] approaches have been proposed, but the application of these methods to high-dimensional phenotype data sets such as that derived from neuroimaging is not straightforward. Here, we describe a novel approach to this problem, which operates by a two-step method: first, we adapt a regression method[27] to estimate the effects of ambiguous haplotypes on imaging data. In a second step, we apply Gaussian Random fields (GRF) theory[28] to effect stringent multiple comparison control over the multiple datapoints studied in neuroimaging.

As a proof of principle for this new approach, we studied the 3-SNP haplotype rs737865–rs4680–rs165599, previously implicated in risk for schizophrenia in population studies.[18,19] However, the focus of the present analysis are the three functional SNPs implicated in the literature: the coding val^{158}met polymorphism (rs4680),[10] a P2 promoter region SNP (rs2097603)[10] and a SNP in the 3′ region implicated in differential expression (rs165599).[19] Proceeding hierarchically, we first studied single SNP effects, then a 2-SNP haplotype composed of rs4680 and rs2097603, and finally the 3-SNP haplotype composed of these possibly functional variants. We tested whether complex genetic variation impacts on prefrontal function in humans in a pattern consistent with interacting functional genetic variation, and hypothesized that inefficient prefrontal response would be related to genetic risk for schizophrenia. These hypotheses were largely confirmed; in particular, our analysis provided evidence in humans for a likely neural mechanism underlying a nonlinear genetic effect.

Results

Genetics

All alleles were in Hardy–Weinberg equilibrium in the entire clinical population from which our imaging sample is derived, but rs165599 deviated from HWE ($P<0.02$, exact test) in the imaging subsample of 126 subjects (Table 1), rs165599 deviated from HWE ($P<0.02$, exact test, Table 2). Estimated allele frequencies were similar to previously reported results in Caucasians (Table 2).[11,16,17,20,29] Age, gender and task performance were balanced between genotype groups (Supplementary Table). With one exception, pairwise LD was significant and extensive, but not complete, underscoring the uncertainty of haplotype information (Table 3).

Main effects of task on brain activation

Confirming previous studies, the working memory task strongly activated dorsolateral, ventrolateral and anterior cingulate PFC as well as the superior parietal lobule (Supplementary Figure 1). Based on the *a priori* evidence on the relevance of COMT for prefrontal function, inference was restricted to activated prefrontal regions to increase sensitivity to genetic variation.[7,11–15]

Single SNP effects

Imaging of the effects of rs4680 genotype showed higher prefrontal activation during the working memory task for val-allele carriers and -homozygotes compared to met homozygotes (Figure 2, Table 4). The relation between the number of val alleles and prefrontal inefficiency was linear. Testing for interactions between genotype and gender did not result in significant effects. Variation at the other individual SNPs (rs2097603, rs737865, rs165599) had no effect on brain activation at the chosen thresholds, both in the whole group and for subjects homozygous for the rs4680.

Haplotype analysis of combined functional variants

We performed an analysis of two haplotypes consisting of combinations of the putative functional SNPs (see also Supplementary Figure 2 for a summary diagram). First, we combined the P2 promoter region

Table 1 Demographic data ($n=126$)

Age	32.2±9.8
Gender (Male:female)	68:58
Performance on task (percent correct during 2-back)	80±17%
Reaction time during 2-back task (s)	0.26±0.10

Mean±s.d. shown.

Table 2 Genotype(coding strand)/estimated haplotype frequencies (%)

Genotype/haplotype	*Genotype/estimated haplotype frequency*
rs2097603 (1:A 2:G)	31 (11) 55 (12) 14 (22)
rs737865 (1:T(A) 2:C(G))[a]	50 (11) 41 (12) 9 (22)
rs4680 (1:G-Val, 2:A-Met)	24 (11) 54 (12) 22 (22)
rs165599 (1:A 2:G)	41 (11) 53 (12) 6 (22)
2-SNP haplotype (rs4680–rs2097603)	
11	42±0.5
12	18±0.5
21	10±0.5
22	30±0.5
3-SNP haplotype (rs737865–rs4680–rs165599)	
111	11±0.5
112	14±0.6
121	37±0.7
122	6±0.5
211	13±0.6
221	4±0.5
212	14±0.7
3-SNP haplotype (rs2097603–rs4680–rs165599)	
111	18±0.7
112	24±0.7
121	16±0.6
222	5±0.5
211	5±0.5
212	5±0.5
221	25±0.7

[a]Base pairs in parentheses denote forward strand as used by Shifman *et al*.[18]

Table 3 Linkage disequilibrium between typed markers

	rs2097603	*rs737865*	*rs4680*
rs2097603			
rs737865	1 (0.327)**		
	χ^2 38.3, $P<0.0001$		
rs4680	0.518 (0.205)**	0.700 (0.203)**	
	χ^2 23.6, $P<0.0001$	χ^2 16.6, $P<0.0001$	
rs165599	0.606 (0.144)**	0.148 (0.020)	0.653 (0.190)**
	χ^2 10.9, $P<0.001$	χ^2 1.3, $P=0.26$	χ^2 13.5, $P<0.0003$

Lewontin's disequilibrium coefficient D' is shown (in parentheses: delta squared). **Significant at $P<0.01$.

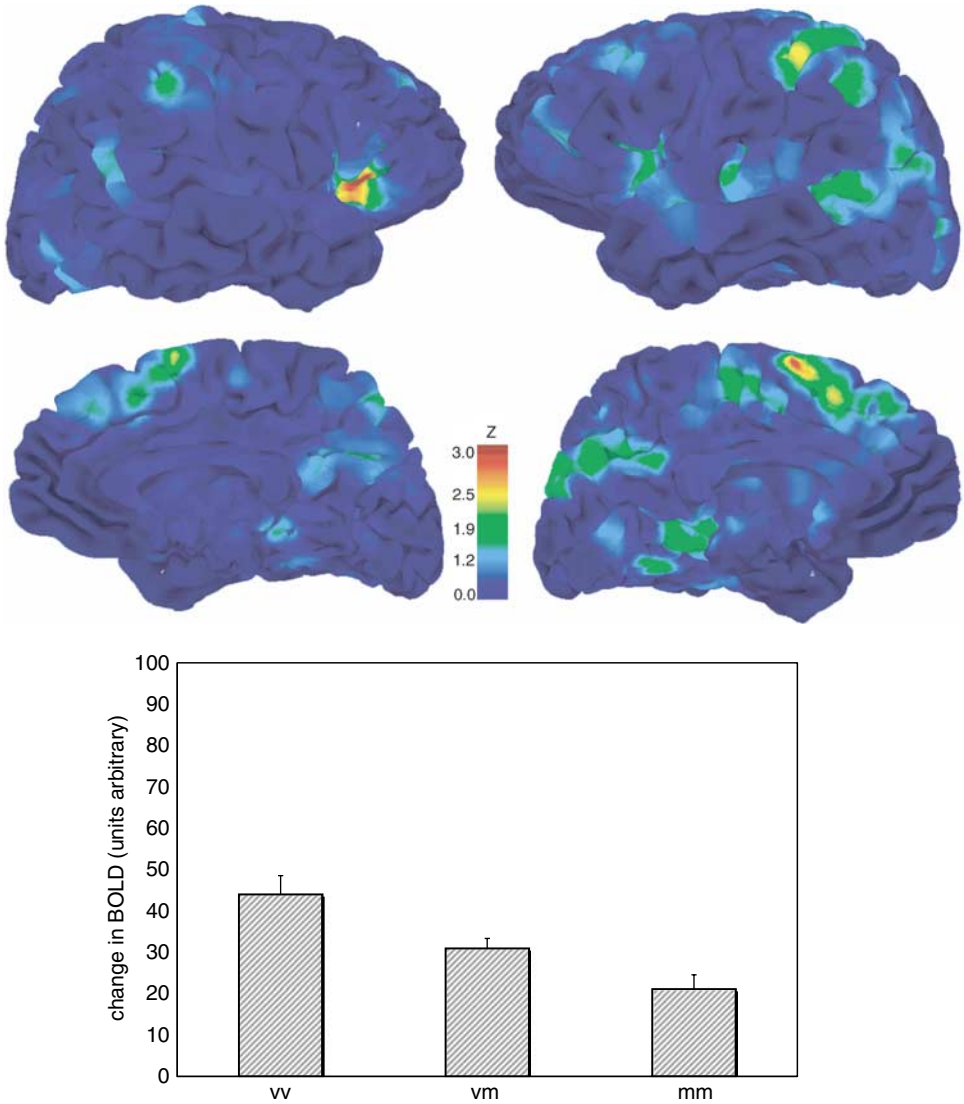

Figure 2 Rendering of regions with significant effect of rs4680 genotype on activation during the 2-back working memory task compared to the 0-back control task. Unthresholded image, see Table 4 for coordinates and corrected significance levels. Inset: activation (change in BOLD, units arbitrary) by genotype for voxel in right ventrolateral PFC (bars are standard errors).

Table 4 Coordinates and statistical information for genotype/haplotype effects on working memory activation

Talairach coordinate (x, y, z)	*Brodmann area*	*Z-score*	*P-value (uncorrected)*	*Cluster size (in voxels)*
rs4680 val-met				
45 18 −1	47/45	4.14	0.000	23
2-SNP haplotype rs2097603–rs4680				
45 18 −1	47/45	3.8	0.000*	17
3-SNP haplotype rs2097603–rs4680–rs165599				
41 22 4	45	4.27	0.000*	20
45 6 33	9	2.62	0.004	1
−49 15 5	44	2.53	0.006	3
3-SNP haplotype rs737865–rs4680–rs165599				
41 22 4	45	3.37	0.000	7
−51 15 5	45	2.70	0.003	2

*Significant, corrected for multiple comparisons ($P<0.05$, corrected).

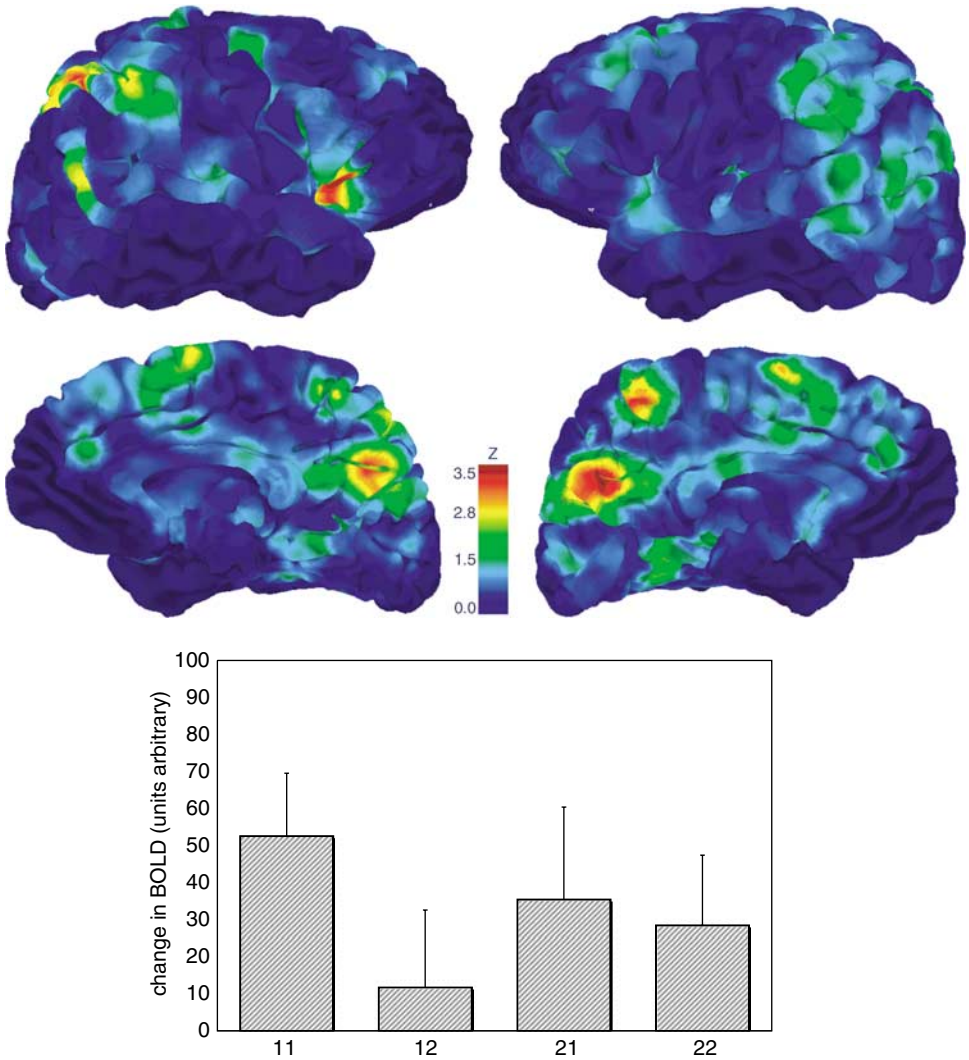

Figure 3 Rendering of regions with significant effect of 2-SNP rs2097603-rs4680 haplotype on activation during the 2-back working memory task compared to the 0-back control task. Unthresholded image, see Table 4 for coordinates and corrected significance levels. Inset: estimated effect sizes (change in BOLD, units arbitrary) by haplotype for voxels in right ventrolateral PFC (bars are standard errors). See Table 2 for haplotype coding.

SNP (rs2097603) with rs4680 to form the 2-SNP haplotype (rs2097603–rs4680). Neuroimaging data showed a strong impact of genetic variation on ventrolateral PFC on the right (Figure 3, Table 4). Since we assume codominance for haplotype effects on the intermediate phenotype studied in neuroimaging, data for all subsequent analyses are plotted as effect sizes for each haplotype rather than as all possible diplotype combinations for individuals. The most efficient prefrontal response was observed for the haplotype promoter-1(A allele)-rs4680-Met (12), the most inefficient response for promoter-1-rs4680 val (11). Responses for haplotypes with the 2(G) promoter allele were intermediate. This was confirmed by *post hoc* testing at this locale using the appropriate contrast ($Z = 4.08$, $P < 0.001$). Secondly, we added rs165599 and analyzed the 3-SNP haplotype rs2097603–rs4680–rs165599, which consisted of only those markers previously associated independently with either variation in COMT enzyme activity or mRNA expression. This haplotype had the statistically strongest impact on prefrontal function (Table 4). Examination of effect sizes at the maximum locale in right ventrolateral PFC (Figure 4, top) showed that haplotypes 111 and 112 differed strongly in efficiency, as confirmed by *post hoc* testing at the ventrolateral prefrontal location ($Z = 3.29$, $P < 0.001$). Otherwise, addition of the 3′ SNP did not reveal additional relevant variation in our neurobiological target measure; in particular, haplotypes 221 and 222 did not differ. No significant interactions with gender were observed in any of the studied markers.

Previously described risk haplotype

In contrast to the previous results, the 3-SNP haplotype (rs737865–rs4680–rs165599) previously

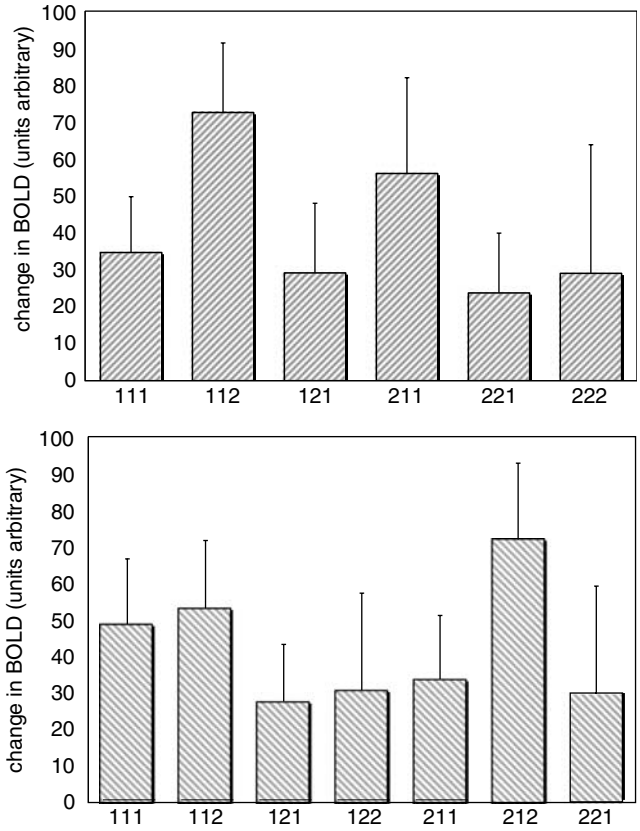

Figure 4 Top: Estimated effect of 3-SNP haplotype rs2097603–rs4680–rs165599. Bottom: Estimated effect of 3-SNP haplotype rs737865–rs4680–rs165599 on working memory activation. Units are arbitrary voxel in right ventrolateral PFC (bars are standard errors). See Table 2 for haplotype coding.

studied in an Ashkenazi population showed only a weak effect on activation during working memory, again with maximum in ventrolateral PFC (Table 4 and Figure 4, bottom). However, while effects did not survive correction for multiple comparisons, the haplotype G-G-G (212), corresponding to the previously identified risk haplotype,[18] showed the most inefficient prefrontal response, as confirmed by *post hoc* testing ($Z = 3.24$, $P < 0.002$).

Discussion

The present study had two goals: demonstrating the feasibility of relating probabilistic haplotype information to brain function using neuroimaging, and using this approach to investigate the potential impact of multiple functional variants in COMT on brain function. We found a significant impact of genetic variation in COMT on cortical, in particular prefrontal function.

When studying single SNPS, the coding val[158]met polymorphism (rs4680) impacted on brain function, with higher prefrontal activation during the working memory task for val-allele carriers and -homozygotes compared to met homozygotes. This replicates previous reports of prefrontal inefficiency associated with the val allele during working memory.[11,13] Our finding that the relation between the number of val alleles and prefrontal inefficiency was linear is in close correspondence with the data on the effect of this genotype on *COMT* activity and abundance in brain and lymphocytes.[10]

Considerably stronger effects than those observed for single SNPs were uncovered when the 2-SNP haplotype derived by adding the P2 promoter region SNP was studied (rs2097603–rs4680). Particularly pronounced effects of haplotype variation were observed in right ventrolateral PFC. While it is possible that this haplotype is superior to single SNPs in monitoring an occult genetic variant that is in LD with these two SNPs defining the haplotype, we propose that our data rather support the alternative hypothesis that genetic variation in the promoter region modulates the val/met effect. This is biologically plausible since a weak effect of rs2097603 on *COMT* activity in lymphocytes was found previously,[10] with higher activity linked to the frequent (A) promoter allele. This effect was found in val/met homozygotes, indicating that it either represents an independent functional variant or is in LD with one. Since this effect was markedly less than that of the rs4680,[10] these previously derived biological data predict that COMT activity as a function of the combination of genetic loci studied here should be ordered as follows: $11 > 21 > 12 > 22$. It is well known that the relationship of extracellular dopamine to prefrontal activity observes an inverted-U shaped curve,[30] and recent neuroimaging studies have shown that rs4680 met-homozygotes are positioned near the peak of that curve[13,15] indicated by efficient prefrontal working memory response.[13] Both further increases of extracellular dopamine by an additional effect of the promoter-2 allele (haplotype 22) on enzyme activity or reduction of dopamine by more efficient enzyme activity (haplotypes 11 and 21) are therefore predicted to lead to less efficient prefrontal response. This is what was observed here (Supplementary Figure 3). Our data thus indicate interacting effects of these SNPs on prefrontal function by both coding and *cis*-acting mechanisms,[20] whose combined effects are not linear. These results are also consistent with a recent study on schizophrenia risk indicating significant separate but interacting effects at two sites in strong LD with these loci.[31] This appears plausible since the tagged variation presumably affects the P2 promoter, which regulates expression of the *COMT*-MB isoform predominantly expressed in brain. Most importantly, these data demonstrate a neural mechanism of a nonadditive genetic interaction on prefrontal efficiency, suggesting that the 2-met haplotype may have a comparable impact on risk for schizophrenia as 1-val containing haplotypes.

Finally, we studied the haplotype rs2097603–rs4680–rs165599 composed of all three SNPs indivi-

dually implicated in functional variation of COMT in previous work.[10,19] We reasoned that if interacting functional variants are the main factor driving the observed haplotype effects, this haplotype should be superior in predicting prefrontal function. Indeed, we found that this haplotype had the statistically strongest impact on prefrontal function. Addition of rs165599 revealed a pronounced difference in prefrontal response between haplotypes 111 and 112, while haplotypes 221 and 222 did not differ significantly. Since it has been suggested that variation at the rs165599 locus affects allele-specific expression, again independent of the val/met effect or effect of the 5″ SNPs,[19] we hypothesize that this differential effect relates to background level of COMT activity: since catalytic activity is high for rs2097603–rs4680 11 genotypes and low in 22,[10] a difference in expression is predicted to elicit greater changes in 11. However, because of the relative low frequency of most of the other haplotypes, additional *post hoc* contrasts are underpowered.

As a supplementary analysis, we examined the previously studied 3-SNP haplotype rs737865–rs4680–rs165599.[18] This showed a weak effect on activation during working memory, again with maximum in ventrolateral PFC. Remarkably, this analysis in a group of healthy controls highlighted the previously identified risk haplotype for schizophrenia[18] as having the most inefficient prefrontal response of other haplotypes containing these SNPs, serving as a useful proof of principle for applying haplotype imaging *vis a vis* population-based studies of clinical phenotypes. Again, this result could be because the haplotype more reliably tags another occult causative variant. However, we suggest that this association also accommodates a functional interpretation, since it has been previously noted[10,20] that variation in this haplotype may be linked to the P2 promoter by asymmetric association of alleles: chromosomes with the intron one G allele almost always show the A allele at the promoter site, whereas the A allele at rs737865 was associated with both the A and G allele. Although there is incomplete LD between the intron 1 and P2 promoter SNP in our data and previously,[20] to the degree that this association holds in the population, the overtransmitted haplotype will be consistently linked to the same promoter allele. This is in agreement with the assumption that genetic variation at the P2 promoter contributes to the risk associated with this haplotype.[10]

It is noteworthy that all analyses localized the maximal effect in the same region of PFC, where the 3-SNP haplotype composed of functional variants showed the strongest effect. Since the degrees of freedom were highest for the single marker analysis, and lowest for the 3-SNP, these results are unlikely to be a simple consequence of the number of regressors employed in the analysis. However, the current literature on efficiency of estimating effects of haplotypes compared to an SNP on a quantitative trait in the context of association studies is inconsistent, with both improved[32] and reduced[33] power reported. Recently developed multilocus tests may offer advantages in power[34] that could be relevant for neuroimaging.[4] In any case, the sample size studied here, while very large for a neuroimaging study, is still small compared with those necessary for the study of clinical phenotypes, illustrating the potential advantage conferred by increased penetrance when studying biologically based quantitative phenotypes,[35] especially when potentially interacting variants are involved. Here, we have used haplotype imaging as a tool to study the functional impact of combinations of multiple loci within a gene. However, the approach presented is generally applicable to all intended uses of ambiguous haplotypes, such as the study of potential functional implications of occult genetic variants on brain function, or in the context of large-scale association studies, extending its usefulness for further study of neuroimaging phenotypes in psychiatric genetics.

For all marker combinations studied, the allele or haplotype showing the strongest prior association with schizophrenia consistently showed the most inefficient prefrontal response to working memory demands, supporting the validity of prefrontal inefficiency as a neural mechanism contributing to genetic risk for schizophrenia.[11,36] In addition, we propose that our results provide a mechanistic explanation for inconsistencies in the clinical genetics literature of association with the val/met polymorphism. Owing to apparent balancing effects of functional variations in putative *cis* elements in *COMT*, which, like val/met, show considerable population variation in allele frequencies,[18,20] all val individuals do not show the same pattern of brain functional associations. Our data demonstrate that to the extent that the association of *COMT* with schizophrenia (or other psychoses) is related to prefrontal functional effects, only a subpopulation of val containing chromosomes will have inefficient prefrontal activity, and some met containing chromosomes will also have a similar functional profile.

Convergent animal[37,38] studies, electrophysiological[30] and modelling work[39] have shown that dopamine critically determines the ratio of task-related to task-unrelated neural firing, or 'tuning' of PFC neurons, with an optimum level of dopaminergic stimulation necessary for highest signal-to-noise ratios.[40] Prefrontal tuning in humans *in vivo* has been shown to be related to dopamine synthesis in the midbrain and rs4680 genetic variation in *COMT*, presumably by modulating the amount of extracellular dopamine in PFC,[15] suggesting that schizophrenia susceptibility linked to prefrontal inefficiency may reflect increased unfocused (detuned) population activity in the setting of decreased physiological signal to noise.[36] Within PFC, it was the right ventrolateral region that was consistently highlighted as the maximum locale for the effect of genetic variation of *COMT*, in good regional agreement with some previous studies.[15,41] This localization is un-

likely to be due to regional variation in dopaminergic tone, since no data suggest prominent asymmetries in dopaminergic projections to human PFC. Previous work has implicated ventrolateral PFC in inhibitory control[42,43] and set shifting,[44] and a right predominance in inhibitory control has been noted.[45] This raises the possibility that optimal dopaminergic tone may be especially relevant for inhibitory control and set shifting, prefrontally dependent cognitive functions that are well known to be impaired in schizophrenia[46,47] and dependent on COMT in rats.[7]

Ultimately, a critical experimental test of the predictions derived here from imaging genetics is afforded by applying the results to association with clinical phenotypes. If the impact of genetic variation on inefficient prefrontal response is validly accessed by the method presented here, and if this biological intermediate phenotype mediates schizophrenia risk, diplotypes composed of combinations of inefficient haplotypes identified in this study should be significantly associated with risk for schizophrenia and should be superior to single SNPs in this regard. This is indeed what was found in a recent case–control association data set from our group, in which none of the individual SNPs studied here showed significant association, but patients were enriched for the inefficient three marker haplotypes compared with controls.[48]

In summary, we present a method to extend the reach of imaging genetics to evaluate the functional impact in brain of ambiguous haplotypes that should be generally applicable both to study interacting functional variants and for using haplotypes to tag occult variation within a gene. Applying this method to COMT and risk for schizophrenia, we show that complex genetic variation can be validated functionally in humans *in vivo* and linked to prefrontal inefficiency, suggesting a common mechanism underlying susceptibility for schizophrenia associated with complex genetic variation in *COMT.*

Materials and methods

Studied sample

Subjects were culled from a larger population after careful screening[11] to ensure they were free of any lifetime history of psychiatric or neurological illness, psychiatric treatment, or drug or alcohol abuse (Table 1). Only Caucasians of European ancestry were studied to avoid stratification artifacts. All available scans of subjects meeting these criteria were used. Subjects gave written informed consent and participated in the study according to the guidelines of the National Institute of Mental Health Institutional Review Board. Functional MRIs of 126 subjects were studied.

SNP genotyping

Genotyping was performed as described previously.[10] See Figure 1 for location of SNPs. All genotypes were determined using a 5′ exonuclease TaqMan assay. The sequences of primers and TaqMan probes for the SNP genotyping were as follows:

(1) P2 promoter SNP (rs2097603), GCCGTGTCTG GACTGTGAGT (forward)/GGGTTCAGAATCACG GATGTG (reverse) and 6FAM-AACAGACAGAAA AGTTTCCCCTTCCCA/VIC-CAGACAGAAAAGCT TCCCCTTCCCATA;
(2) intron 1 SNP (rs737865), GCTTGGAGGGTCACTT TAAACAATA (forward)/TGCTAACAGACCTGCT TTTTGG (reverse) and 6FAM-CAGGACACAAAA AcCCCTGGCTG/VIC-CAGGACACAAAAAtCCCTG GCTGG;
(3) Val/Met SNP (rs4680), TCGAGATCAACCCCGAC TGT (forward)/AACGGGTCAGGCATGCA (reverse) and 6FAM-CCTTGTCCTTCACGCCAGCGA/VIC-A CCTTGTCCTTCATGCCAGCGAAAT;
(4) 3′ flanking SNP (rs165599), CAGCCACAGTGGTG CAGAG (forward)/AAGGTGTGAATGCTGGCTGA (reverse) and 6FAM-TTTCCCAGGCtGGCAGTCGT C/VIC-CGTTTCCCAGGCcGGCAGT.

In addition, our sample was genotyped with a panel of 100 unlinked SNP loci (minor allele frequencies greater 0.4 in all cases) to survey for occult genetic stratification between *COMT* genotype groups and showed no significant variation in frequency at any of these SNPs (available upon request).

LD and haplotype estimation

LD between markers was determined by GOLD.[49] We constructed likely 2- and 3-SNP haplotypes in our samples on the basis of our four polymorphisms by use of PHASE 2.1.[23] Since we had 126 subjects, only haplotypes with estimated frequency $>3\%$ were used in subsequent analysis to ensure we had sufficient numbers of scans per haplotype, resulting in four (out of four) haplotypes composed of two SNPs and seven (out of eight) haplotypes composed of three SNPs. To exclude a density bias in haplotype estimation, we also used the same method to estimate 4-SNP haplotypes, encompassing the SNPs studied here, in a sample of 148 normal controls genotyped for 11 SNPs covering COMT (list available on request). There was no significant difference of the frequencies obtained from this sample to the ones reported here ($\chi^2(15)=0.077$, $P=1$).

fMRI working memory paradigm

During fMRI scanning, a version of the ‘n-back’ working memory task was given as previously described.[11] For this, the numerals 1, 2, 3 or 4 were presented one every 1.8 s in a diamond-shaped array via computer projected on an overhead mirror in the MRI scanner. Subjects held a button-box with four buttons arranged similarly to the visual display. During scanning, task epochs lasted 20 s and were performed in runs of 4 min each. Epochs alternated between the 0-back (control) condition, where the button corresponding to the current number was pressed, and the 2-back (working-memory) condition, in which the button to press corresponded to the

number seen 2 presentations before. Percentage of correct responses was used as the performance measure, and reaction times were also recorded.

fMRI data acquisition and preprocessing

BOLD fMRI was performed on a GE Signa 3 T (Milwaukee, WI, USA) using gradient echo EPI (24 axial slices, 6 mm thickness, TR/TE = 2000/30 ms, FOV = 24 cm, matrix = 64 × 64). Images were processed as described previously using SPM99 (http://www.fil.ion.ucl.ac.uk/spm). Briefly, images were realigned, spatially normalized into a standard stereotactic space (MNI template), smoothed with a 10-mm FWHM Gaussian filter and ratio normalized to the whole-brain global mean. First level intrasubject models were estimated using SPM99, where the two experimental conditions (0-back and 2-back) were represented by boxcars convolved with a canonical hemodynamic response. A contrast image for the 2-back–0-back contrast was estimated for each subject and used for random-effects statistical modelling using haplotype regression as described in the following.

Statistical techniques

We used the haplotype trend regression (HTR) approach[27] to test for association between BOLD signal response and estimated haplotype frequencies. Since we examined the association with an intermediate biological phenotype, we assumed codominance, allowing us to model and analyze the effect of each haplotype separately. We note that the approach would be equally applicable to other genetic models, which would then require explicitly modelling the probabilities of diplotypes instead of haplotypes. The group level model used one contrast image per subject, and regressed the estimated BOLD change on each subject's inferred haplotype frequencies. Justifying this carefully, first consider the case where true haplotype counts are known; we would use these to fit a group model for p subjects and n haplotypes at each voxel,

$$Y = X\beta + \varepsilon$$

where Y is a p-vector of BOLD response magnitudes, X is the (unobservable) p by n matrix of true haplotype counts, and ε is a p-vector of random BOLD errors; x_{ij} of X contains the count of haplotype j for subject i, each row summing to 2. (We implicitly model the grand mean instead of dropping one haplotype.) This would be a standard multiple regression and t- and F-tests could be used to make inference on the effect of haplotype. Either normal-theory parametric P-values or nonparametric P-values (by permutation of subject data) could be inferred.

However, due to phase ambiguity, haplotype frequencies are statistically inferred[23] and we instead have X^*, x^*_{ij} being the estimated frequency of haplotype j for subject i. Yet a key assumption of the GLM is that the predictors are fixed and known without error. Since X^* is subject to estimation error, we have what is known as an error-in-variables problem.[50] Precisely, we have

$$X = X^* + \eta$$

where η is an n by p matrix of random errors attributable to phase ambiguity. Multiple regression with X* will result in biased estimates of haplotype effects (β), though the tests for any effect (H_0: $\beta = 0$) are valid as long as the haplotype estimation errors η are independent of the GLM errors ε.[50] This independence assumption appears reasonable since an influence of (unobservable) haplotyping errors on group-model BOLD residuals is implausible. Hence, we can disregard the error-in-variables problem and proceed with standard inference methods in the framework of the general linear model.[28] By an argument based on the same independence assumption, the null distribution of the t statistic is exchangeable with respect to a permutation of subjects' haplotype data. This means that permutation inference methods are likewise valid, and amount to testing for association between two random variables.

For all models, we used age, gender and task performance as nuisance covariates since an effect of these parameters on brain function during working memory has been described by some and an interaction of susceptibility for schizophrenia with gender has been described for both rs4680 and a 3-SNP risk haplotype considered here.[18] We also considered interactions between inferred genotype and gender by way of moderated multiple regression.

Inference

For statistical inference on the second level, we used the established methods of GRF theory[28] to threshold statistic images, controlling the familywise error rate, the chance of one or more false positives, at a suitable level. This is essential since neuroimages contain many hundreds of voxels and error controls across these multiple tests are necessary. GRF methods are based on theory of continuous random fields and depend on the assumption that the underlying statistical process is spatially smooth relative to the voxel sampling, the validity of which was ensured by smoothing the imaging data as described above. The estimated parameters of the haplotype regression model described above were contrasted to produce a test statistic at each voxel, the resulting statistical parametric map was then assessed for significance (at the threshold of $P < 0.05$, voxel-level corrected). Additional adjustment for the number of genetic markers tested was not performed. Since the work cited above provided a clear *a priori* hypothesis about the relevance of *COMT* for prefrontal information processing, we restricted our analysis to voxels activated by the task in PFC, as defined by an contrast of all ones, corresponding to a one-sample t-test on Y, in Brodmann areas 44,45,46,47 and nine bilaterally, resulting in a search volume of 420 voxels. Since the tests for differential BOLD responses are orthogonal to the contrast of ones, it is valid to use the same data set to create a mask and constrain the search for

significant voxels. For comparability of statistical effects across analyses with differing degrees of freedom, *Z*-scores are reported.

Acknowledgments

We thank John Meyers for analysis of our genomic control panel, David Goldman and Richard Straub for helpful discussion. This work was supported by the NIMH/IRP and a bench-to-bedside award by NIMH, NIAAA and ORD to AML.

References

1 Collins FS, Green ED, Guttmacher AE, Guyer MS. A vision for the future of genomics research. *Nature* 2003; **422**: 835–847.
2 Hinds DA, Stuve LL, Nilsen GB, Halperin E, Eskin E, Ballinger DG *et al.* Whole-genome patterns of common DNA variation in three human populations. *Science* 2005; **307**: 1072–1079.
3 Altshuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, Donnelly P. A haplotype map of the human genome. *Nature* 2005; **437**: 1299–1320.
4 Schaid DJ. Evaluating associations of haplotypes with traits. *Genet Epidemiol* 2004; **27**: 348–364.
5 Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B *et al.* The structure of haplotype blocks in the human genome. *Science* 2002; **296**: 2225–2229.
6 Lewis DA, Melchitzky DS, Sesack SR, Whitehead RE, Auh S, Sampson A. Dopamine transporter immunoreactivity in monkey cerebral cortex: regional, laminar, and ultrastructural localization. *J Comp Neurol* 2001; **432**: 119–136.
7 Tunbridge EM, Bannerman DM, Sharp T, Harrison PJ. Catechol-*o*-methyltransferase inhibition improves set-shifting performance and elevates stimulated dopamine release in the rat prefrontal cortex. *J Neurosci* 2004; **24**: 5331–5335.
8 Harrison PJ, Weinberger DR. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol Psychiatry* 2005; **10**: 40–68; image 45.
9 Murphy KC. Schizophrenia and velo-cardio-facial syndrome. *Lancet* 2002; **359**: 426–430.
10 Chen J, Lipska BK, Halim N, Ma QD, Matsumoto M, Melhem S *et al.* Functional analysis of genetic variation in catechol-*O*-methyltransferase (COMT): effects on mRNA, protein, and enzyme activity in postmortem human brain. *Am J Hum Genet* 2004; **75**: 807–821.
11 Egan MF, Goldberg TE, Kolachana BS, Callicott JH, Mazzanti CM, Straub RE *et al.* Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc Natl Acad Sci USA* 2001; **98**: 6917–6922.
12 Goldberg TE, Egan MF, Gscheidle T, Coppola R, Weickert T, Kolachana BS *et al.* Executive subprocesses in working memory: relationship to catechol-*O*-methyltransferase Val158Met genotype and schizophrenia. *Arch Gen Psychiatry* 2003; **60**: 889–896.
13 Mattay VS, Goldberg TE, Fera F, Hariri AR, Tessitore A, Egan MF *et al.* Catechol *O*-methyltransferase val158-met genotype and individual variation in the brain response to amphetamine. *Proc Natl Acad Sci USA* 2003; **100**: 6186–6191.
14 Akil M, Kolachana BS, Rothmond DA, Hyde TM, Weinberger DR, Kleinman JE. Catechol-*O*-methyltransferase genotype and dopamine regulation in the human brain. *J Neurosci* 2003; **23**: 2008–2013.
15 Meyer-Lindenberg A, Kohn PD, Kolachana B, Kippenhan S, McInerney-Leo A, Nussbaum R *et al.* Midbrain dopamine and prefrontal function in humans: interaction and modulation by COMT genotype. *Nat Neurosci* 2005; **8**: 594–596.
16 Glatt SJ, Faraone SV, Tsuang MT. Association between a functional catechol *O*-methyltransferase gene polymorphism and schizophrenia: meta-analysis of case–control and family-based studies. *Am J Psychiatry* 2003; **160**: 469–476.
17 Fan JB, Zhang CS, Gu NF, Li XW, Sun WW, Wang HY *et al.* Catechol-*O*-methyltransferase gene Val/Met functional polymorphism and risk of schizophrenia: a large-scale association study plus meta-analysis. *Biol Psychiatry* 2005; **57**: 139–144.
18 Shifman S, Bronstein M, Sternfeld M, Pisante-Shalom A, Lev-Lehman E, Weizman A *et al.* A highly significant association between a COMT haplotype and schizophrenia. *Am J Hum Genet* 2002; **71**: 1296–1302.
19 Bray NJ, Buckland PR, Williams NM, Williams HJ, Norton N, Owen MJ *et al.* A haplotype implicated in schizophrenia susceptibility is associated with reduced COMT expression in human brain. *Am J Hum Genet* 2003; **73**: 152–161.
20 Palmatier MA, Pakstis AJ, Speed W, Paschou P, Goldman D, Odunsi A *et al.* COMT haplotypes suggest P2 promoter region relevance for schizophrenia. *Mol Psychiatry* 2004; **9**: 859–870.
21 Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* 1995; **12**: 921–927.
22 Terwilliger JD, Ott J. *Handbook of human genetic linkage*. Johns Hopkins University Press: Baltimore, 1994.
23 Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 2003; **73**: 1162–1169.
24 Zhao LP, Li SS, Khalid N. A method for the assessment of disease associations with single-nucleotide polymorphism haplotypes and environmental variables in case–control studies. *Am J Hum Genet* 2003; **72**: 1231–1250.
25 Tanck MW, Klerkx AH, Jukema JW, De Knijff P, Kastelein JJ, Zwinderman AH. Estimation of multilocus haplotype effects using weighted penalised log-likelihood: analysis of five sequence variations at the cholesteryl ester transfer protein gene locus. *Ann Hum Genet* 2003; **67**(Part 2): 175–184.
26 Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet* 2002; **70**: 425–434.
27 Zaykin DV, Westfall PH, Young SS, Karnoub MA, Wagner MJ, Ehm MG. Testing association of statistically inferred haplotypes with discrete and continuous traits in samples of unrelated individuals. *Hum Hered* 2002; **53**: 79–91.
28 Worsley KJ, Marrett S, Neelin P, Vandal AC, Friston KJ, Evans AC. A unified statistical approach for determining significant signals in images of cerebral activation. *Hum Brain Mapping* 1996; **4**: 58–73.
29 DeMille MM, Kidd JR, Ruggeri V, Palmatier MA, Goldman D, Odunsi A *et al.* Population variation in linkage disequilibrium across the COMT gene considering promoter region and coding region variation. *Hum Genet* 2002; **111**: 521–537.
30 Williams GV, Goldman-Rakic PS. Modulation of memory fields by dopamine D1 receptors in prefrontal cortex. *Nature* 1995; **376**: 572–575.
31 Handoko HY, Nyholt DR, Hayward NK, Nertney DA, Hannah DE, Windus LC *et al.* Separate and interacting effects within the catechol-*O*-methyltransferase (COMT) are associated with schizophrenia. *Mol Psychiatry* 2005; **10**: 589–597.
32 Akey J, Jin L, Xiong M. Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur J Hum Genet* 2001; **9**: 291–300.
33 Long AD, Langley CH. The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. *Genome Res* 1999; **9**: 720–731.
34 Clayton D, Chapman J, Cooper J. Use of unphased multilocus genotype data in indirect association studies. *Genet Epidemiol* 2004; **27**: 415–428.
35 Gottesman II, Gould TD. The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* 2003; **160**: 636–645.
36 Weinberger DR, Egan MF, Bertolino A, Callicott JH, Mattay VS, Lipska BK *et al.* Prefrontal neurons and the genetics of schizophrenia. *Biol Psychiatry* 2001; **50**: 825–844.
37 Sawaguchi T, Goldman-Rakic PS. D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science* 1991; **251**: 947–950.

38 Seamans JK, Floresco SB, Phillips AG. D1 receptor modulation of hippocampal-prefrontal cortical circuits integrating spatial memory with executive functions in the rat. *J Neurosci* 1998; **18**: 1613–1621.
39 Durstewitz D, Kelc M, Gunturkun O. A neurocomputational theory of the dopaminergic modulation of working memory functions. *J Neurosci* 1999; **19**: 2807–2822.
40 Seamans JK, Yang CR. The principal features and mechanisms of dopamine modulation in the prefrontal cortex. *Prog Neurobiol* 2004; **74**: 1–58.
41 Smolka MN, Schumann G, Wrase J, Grusser SM, Flor H, Mann K *et al*. Catechol-*O*-methyltransferase val158met genotype affects processing of emotional stimuli in the amygdala and prefrontal cortex. *J Neurosci* 2005; **25**: 836–842.
42 Liddle PF, Kiehl KA, Smith AM. Event-related fMRI study of response inhibition. *Hum Brain Mapp* 2001; **12**: 100–109.
43 Morita M, Nakahara K, Hayashi T. A rapid presentation event-related functional magnetic resonance imaging study of response inhibition in macaque monkeys. *Neurosci Lett* 2004; **356**: 203–206.
44 Shafritz KM, Kartheiser P, Belger A. Dissociation of neural systems mediating shifts in behavioral response and cognitive set. *Neuroimage* 2005; **25**: 600–606.
45 Garavan H, Ross TJ, Stein EA. Right hemispheric dominance of inhibitory control: an event-related functional MRI study. *Proc Natl Acad Sci USA* 1999; **96**: 8301–8306.
46 Gold JM, Carpenter C, Randolph C, Goldberg TE, Weinberger DR. Auditory working memory and Wisconsin Card Sorting Test performance in schizophrenia. *Arch Gen Psychiatry* 1997; **54**: 159–165.
47 Ford JM, Gray M, Whitfield SL, Turken AU, Glover G, Faustman WO *et al*. Acquiring and inhibiting prepotent responses in schizophrenia: event-related brain potentials and functional magnetic resonance imaging. *Arch Gen Psychiatry* 2004; **61**: 119–129.
48 Nikodemus KK, Kolachana BS, Vakkalanta R, Straub RE, Giegling I, Egan MF *et al*. Evidence for statistical epistasis between catechol-*O*-methyltransferase (COMT) and polymorphisms in RGS4, G72 (DAOA), GRM3 and DISC1: influence on risk of schizophrenia. *Hum Genet*, Submitted.
49 Abecasis GR, Cookson WO. GOLD – graphical overview of linkage disequilibrium. *Bioinformatics* 2000; **16**: 182–183.
50 Fuller WA. *Measurement Error Models*. Wiley: New York, 1987.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)