

Catechol O-Methyltransferase (COMT) mRNA Expression in the Dorsolateral Prefrontal Cortex of Patients with Schizophrenia

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Human prefrontal cortical neurons express catechol O-methyltransferase (COMT), an enzyme that inactivates the neurotransmitter dopamine. A functional polymorphism of COMT, Val^{108/158} Met, affects prefrontal function, and the high-activity Val allele has been reported to be a genetic risk factor for schizophrenia. We used *in situ* hybridization histochemistry to measure mRNA levels of COMT in the dorsolateral prefrontal cortex (DLPFC) of patients with schizophrenia ($N = 14$) and of normal controls ($N = 15$). While the groups did not differ in terms of mean level of COMT mRNA, there was a significantly different laminar pattern of COMT mRNA expression in pyramidal neurons ($F = 2.68$, $df = 4, 108$, $P < 0.04$); patients with schizophrenia had relatively lower levels in the superficial (II/III) layers and higher levels in the intermediate/deep (IV/V) layers ($P < 0.01$), while in controls, the expression was homogeneous across layers. Neither the mean level nor the laminar distribution of COMT mRNA was related to the Val^{108/158} Met genotype, suggesting that the feedback regulation of mRNA level is not a compensation for the functional effect of the COMT polymorphism. The disease-related laminar difference of COMT expression may be involved in dysregulation of dopamine signaling circuits in the DLPFC of patients with schizophrenia.

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INTRODUCTION

Catechol O-methyltransferase (COMT) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to dopamine, generating 3-methoxytyramine, which is one of the mechanisms for inactivating dopamine after it is secreted (Lundstrom *et al*, 1995; Mannisto and Kaakkola, 1999). Previous experimental data indicate that COMT is especially relevant in modulating prefrontal cortical dopamine neurotransmission. First, studies of COMT knockout mice have demonstrated that dopamine levels are increased in the prefrontal cortex of these mice under varying conditions, but not in the striatum (Gogos *et al*, 1998; Huotari *et al*, 2002). Pharmacological studies in rats have also shown that the catabolic flux of synaptic dopamine through the COMT pathway is much greater in the

prefrontal cortex as compared to the striatum (Karoum *et al*, 1994). Our group has recently demonstrated that COMT mRNA is expressed primarily in neurons in the prefrontal cortex in both rodents and humans, correcting earlier assumptions that COMT was a non-neuronal enzyme in the brain. We have also found that COMT mRNA levels in the prefrontal cortex are significantly higher than in the striatum in both species (Matsumoto *et al*, 2003). The relatively high levels of COMT and the low levels of dopamine transporters at synapses in the prefrontal cortex probably contribute to the relative functional specificity for COMT to prefrontal dopamine metabolism, in comparison to the striatum, where neuronal uptake by abundant dopamine transporters terminates the synaptic action of dopamine (Garris *et al*, 1993; Giros *et al*, 1996; Lewis *et al*, 2001; Mazei *et al*, 2002; Sesack *et al*, 1998; Wayment *et al*, 2001). Finally, we and other groups have demonstrated that a functional polymorphism of the COMT gene, a valine to methionine substitution at codon 108/158 generating a three- to fourfold less active COMT enzyme (Lachman *et al*, 1996; Lotta *et al*, 1995), affects dopamine-related prefrontal cortical function in humans (Bilder *et al*, 2002; Egan *et al*, 2001; Goldberg *et al*, in press; Jooper *et al*, 2002; Malhotra *et al*, 2002; Mattay *et al*, 2003; Rosa *et al*, 2002).

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Deficits in prefrontal-related executive cognition and working memory are prominent components of cognitive dysfunction in schizophrenia (Park and Holzman, 1992; Weickert *et al*, 2000; Weinberger *et al*, 1986, 2001; Wexler *et al*, 1998), and have been linked to evidence of diminished prefrontal dopamine signaling (Akil *et al*, 1999; Weinberger *et al*, 1988, 2001). Consistent with this notion, several [although not all (Semwal *et al*, 2001)] studies using the family-based transmission disequilibrium test have shown the high-activity Val allele of COMT, which presumably increases dopamine catabolism, to be preferentially transmitted to schizophrenic offspring (Egan *et al*, 2001; Kunugi *et al*, 1997; Li *et al*, 2000, 1996). Interestingly, this Val^{108/158} Met functional polymorphism has been reported to be associated with schizotypal personality traits even in healthy individuals, and in the same direction as observed in schizophrenic patients, that is, increased Val allele frequency (Avramopoulos *et al*, 2002). Given the pivotal role of prefrontal cortex in the pathogenesis of schizophrenia and the apparent relevance of COMT for prefrontal dopamine metabolism, we have investigated the expression of COMT mRNA in the prefrontal cortex in patients with schizophrenia compared to control subjects by *in situ* hybridization histochemistry. The relation of Val/Met genotype upon COMT mRNA expression has also been assessed.

MATERIALS AND METHODS

Brain Tissue Preparation

Post-mortem human brains were collected at the Clinical Brain Disorders Branch (NIMH) as previously described (Kleinman *et al*, 1995). The collection of human brain specimens was approved by the Institutional Review Board of the NIMH Intramural Research Program. Cases were screened by police and/or by telephone interviews of family members for a history of medical and/or psychiatric problems, including alcohol abuse and illicit drug use. Any positive history of a psychiatric problem or excessive alcohol or drug use led to the exclusion of that case from the normal subjects group. In addition, 80% of all cases had toxicological screens of blood. If the medical examiner believed that the index of suspicion for illicit drugs or alcohol abuse was low, a toxicological screen was not performed. All brains (both normal subjects and patients with schizophrenia) were screened for signs of macroscopic pathology at the time of autopsy; brain sections were examined microscopically using Bielschowsky's silver stain on multiple cerebral areas to exclude the presence of neuritic pathology as seen in Alzheimer's disease. Those cases with an unclear psychiatric diagnosis, evidence of cocaine or PCP abuse (history and/or toxicology), cerebrovascular disease, autolysis, subdural hematoma, neuritic pathology, or other pathological features were excluded from the analysis.

Briefly, 1.0–1.5 cm coronal slabs through the hemisected cerebrum of each human brain were rapidly frozen in a prechilled dry ice-isopentane slurry bath and stored at -80°C . Cryostat sections (14 μm thick) were prepared from dorsolateral prefrontal cortex (DLPFC) from 14 patients with a diagnosis of schizophrenia and 15 normal subjects.

Wherever possible, normal subjects were matched with those with schizophrenia with respect to gender, age at death, post-mortem interval (PMI), and pH of tissue (Table 1). The total dose of neuroleptic medication given to the patients was calculated by adding the various daily medication levels as determined from available medical records and converting these levels to chlorpromazine (CPZ) equivalents as previously formulated (Torrey, 1983).

In Situ Hybridization

T7/T3 promoter-tagged COMT riboprobe templates were generated by PCR as described (Matsumoto *et al*, 2003). The COMT riboprobe consisted of 321 bases, from the start codon of soluble COMT to just before the functional Val/Met polymorphic site, which recognizes both the long (1.5 kb) and short (1.3 kb) COMT transcripts. Expression of COMT mRNA isoforms in the DLPFC of subjects in both diagnostic groups was determined by Northern blot analysis. Total RNA (2 μg) extracted from DLPFC of five subjects from each diagnostic group (ID #1, 3, 8, 11, 12 from normal control; ID #1, 4, 7, 9, 14 from patients with schizophrenia) were pooled, electrophoresed, and transferred to a nylon membrane. The COMT riboprobe generation, hybridization, and signal detection for Northern analysis were performed as described (Matsumoto *et al*, 2003).

Fixation, acetylation, delipidation, and dehydration of the slices were performed according to standard protocols (Whitfield *et al*, 1990). ³⁵S-UTP labeled riboprobes for COMT with a specific activity of 1×10^9 cpm/ μg were synthesized using T3 (for antisense) and T7 (for sense) RNA polymerase from the T7/T3 promoter tagged COMT riboprobe templates. Two sections from each subject were used (duplicate). A total volume of 200 μl of hybridization buffer containing the ³⁵S-UTP labeled species-specific COMT riboprobes (5 ng/ml) were added to each section and hybridization was allowed to occur at 55°C overnight in humidified chambers. After hybridization, the slides were treated with RNase A and then washed under high stringency conditions ($0.2 \times \text{SSC}$, 55°C for 1 h and $0.2 \times \text{SSC}$, 60°C for 1 h). After dehydration, hybridized slides, along with ¹⁴C standards (American Radiolabeled Chemicals, Inc., St Louis, MO), were exposed to Kodak BioMax film for 1 week. The slides were dipped in an NT-B2 emulsion (Kodak), stored in light-tight boxes in the dark for 2 months and then developed in a D-19 developer (Kodak), dehydrated, and lightly stained with a Nissl counterstain. All the slides were processed together, so that direct quantitative comparisons could be made.

Image Analysis

Optical density measurements of the X-ray film were performed with the aid of NIH Image (Rasband, NIH). The boundary of Brodmann's area 46 (BA 46) was delineated on Nissl-stained sections, applying the criteria described by Rajkowska and Goldman-Rakic (1995). The cytoarchitectonic criteria used were as follows: (i) the presence of a well-defined granular layer IV; (ii) the columnar arrangement of pyramidal neurons in layer III; (iii) the increase in size of pyramidal neurons in layer III

Table 1 Demographic data of patients with schizophrenia and normal subjects

ID	Diagnosis	COMT genotype	Gender	Race	Age at death (years)	PMI (h)	pH	Cause of death (manner)	Last CPZ equivalent (mg/day)	Daily CPZ equivalent (mg/day)	Lifetime CPZ equivalent (10 ⁶ mg)	
<i>Patients and schizophrenia</i>												
1	CUS	VV	M	AA	75	42.5	6.29	Undetermined	400	400	5.3	
2	CUS	VV	F	AA	67	38.5	6.63	COPD	80	100	1.3	
3	CUS	MM	M	C	31	14	6.46	Undetermined	200	250	0.4	
4	CUS	VM	M	AA	23	43	6.48	Anoxia 2° seizure disorder	400	480	0.033	
5	CPS	VM	F	AA	60	35	6.38	ASCVD	100	100	0.7	
6	CUS	VM	M	AA	30	72.5	6.32	Pneumonia cardiac hypertrophy	1900	500	2.2	
7	CUS	VM	M	AA	35	79.5	6.7	PE 2° DVT (suicide)	N/A	N/A	N/A	
8	CPS	VV	M	C	80	13.5	6.05	GI bleed	30	50	0.7	
9	CUS	VM	F	C	81	15	6.78	ASCVD	100	150	2.1	
10	CUS	VM	F	AA	61	21.5	6.74	Aspiration of food (accident)	N/A	200	2.0	
11	CUS/TD	VM	M	AA	38	61	6.5	Acute peritonitis (accident)	800	60	12	
12	CUS	VV	M	AA	44	34	6.28	Pulmonary abscess and empyema	300	350	2.7	
13	CDS	VM	F	AA	41	51	6.08	ASCVD	2400	1135	10	
14	CUS	VV	M	AA	41	32	6.63	ASCVD	50	400	2.5	
					50.5 ± 19.7	39.5 ± 20.9	6.45 ± 0.23					
Mean ± SD												

ID	Diagnosis	COMT genotype	Gender	Race	Age at death (years)	PMI (h)	pH	Cause of death (manner)	
<i>Control subjects</i>									
1	CON	VV	F	AA	52	12	6.87	Ruptured aorta	
2	CON	VV	M	AA	35	49.5	5.88	ASCVD	
3	CON	VM	M	AA	41	14.5	6.72	Stab wound to chest (homicide)	
4	CON	VM	F	C	66	37.5	6.37	Ruptured aorta	
5	CON	VV	M	AA	32	15.5	6.77	GSW to chest (homicide)	
6	CON	VV	M	AA	24	12.5	6.59	Pericarditis	
7	CON	VV	M	AA	38	32.5	6.14	PE (accident)	
8	CON	VV	M	AA	18	14.5	6.51	GSW to back (homicide)	
9	CON	VV	M	AA	83	66.5	6.01	PE 2°DVT (accident)	
10	CON	VM	M	AA	56	34	6.09	PE 2°DVT	
11	CON	VM	M	C	63	19.5	6.54	ASCVD with myocardial fibrosis	
12	CON	VM	F	AA	52	26	6.38	Pericarditis with tamponade	
13	CON	VM	F	AA	57	19	6.43	ASCVD	
14	CON	VM	F	AA	59	37	6.57	Cirrhosis of the liver	
15	CON	VV	F	AA	67	34	6.69	Dilated cardiomyopathy	
					49.5 ± 17.8	28.3 ± 15.5	6.43 ± 0.29		
Mean ± SD									

Means and standard deviations are displayed below the last individual in each group. Gender, race, the mean age at death ($P > 0.71$), post-mortem interval ($P > 0.28$), and pH ($P > 0.39$) did not differ between the diagnostic groups. Abbreviations are as follows: CON = normal control, CDS = chronic disorganized schizophrenia, CPS = chronic paranoid schizophrenia, CUS = chronic undifferentiated schizophrenia, TD = tardive dyskinesia, MM = Met/Met, VM = Val/Met, VV = Val/Val, F = female, M = male, AA = African American, C = Caucasian, PMI = post-mortem interval, 2° = secondary to, ASCVD = atherosclerotic coronary vascular disease, COPD = chronic obstructive pulmonary disease, DVT = deep vein thrombosis, GSW = gunshot wound, PE = pulmonary embolism, CPZ = chlorpromazine, N/A = not available. Unless otherwise specified, manner of death was natural. Chlorpromazine equivalents are shown as milligram equivalents.

with an increase in cortical depth; and (iv) the presence of a clear transition from layer VI into the white matter. Sampling was carried out in BA 46, where the cortical layers were aligned parallel to the pial surface and conducted blind to diagnosis. A rectangle (approximately 2.1 mm²) was placed along the middle frontal sulcus, and the optical density for COMT mRNA in the gray matter (layers II–VI) was obtained by taking the average of six such boxes from two tissue sections per subject. For the white matter regions, the optical density was obtained by taking the average of six circles (approximately 1.7 mm²) from two tissue sections per subject. Optical densities normalized for sampled area ($\mu\text{Ci/g}$) per region were averaged across slices per subject.

In order to determine if COMT mRNA varied according to neuronal type (large pyramidal neurons *vs* smaller, presumably nonpyramidal neurons) or according to anatomical layer, silver grain analysis of neurons from layers II to VI was conducted blind to diagnosis on a Zeiss Axiophot (Gottingen, Germany) microscope equipped with a video camera and a Bioquant image analysis system (BIOQUANT). We counted grains on large pyramidal neurons (large cell size, light Nissl stain, and triangular shape) and smaller neurons (small cell size, light Nissl, and round shape) separately from layers II, III, IV, V, and VI. Although cells with a small, darkly stained nucleus with little cytoplasmic stain, presumably glial cells, occasionally appeared to have a positive hybridization signal, typically the signal was indistinguishable from the background. Therefore, glial cells were not analyzed. Circles of 30 μm diameter for large pyramidal cells and 15 μm diameter for smaller cells were drawn and centered over every neuron under the bright field. The illumination was then switched to dark field and the silver grains overlying a neuron, within the boundaries of this circle, were counted with the aid of a grain-counting macroprogram written using Bioquant software. Background silver grain level was determined by averaging the number of grains in three 30 μm -diameter circles (for pyramidal neurons) and in three 15 μm -diameter circles (for smaller neurons) in the neuropil of layer I from each sample. A total of 20 large pyramidal and 20 smaller neurons within each layer for each case from one tissue section were sampled from contiguous fields within BA46. The average number of grains per cell (background subtracted) from 20 neurons for each layer for each case was used in the statistical analysis. Images of silver grains from these slides were captured using a video camera mounted on a microscope Axiophot (ZEISS, Gottingen, Germany).

Genotyping of COMT Val^{108/158} Met Allele

The COMT genotype was determined by restriction fragment length polymorphism analysis with subtle modifications to the earlier method (Lachman *et al*, 1996). A 109 bp PCR product was generated from brain genomic DNA from each subject using the primers 5'-CTCATCACCATCGA-GATCAA-3' (sense, position 1881–1900 according to the GenBank accession number Z26491) and 5'-CCAGGTCT-GACAACGGTCA-3' (antisense, 1970–1989). The PCR product was digested with restriction enzyme NlaIII. The digested PCR fragments were separated on an agarose gel

and visualized by ethidium bromide staining. The Val allele was recognized by 86 and 23 bp fragments and the Met allele by 68, 18, and 23 bp fragments.

Statistical Analysis

Diagnostic group differences in demographic variables, age at death, PMI, and pH were tested with unpaired *t*-tests (Statistica, Statsoft). Pearson Product-Moment correlation coefficients were used to assess the relations between COMT mRNA levels and the demographic variables, while unpaired *t*-tests were used to determine if gender differences in COMT mRNA existed. Paired *t*-tests were used to compare COMT mRNA levels between the gray matter and white matter area (optical density) or between large pyramidal and smaller neurons (silver grain). Comparison of COMT mRNA optical densities between the diagnostic groups and between the COMT genotype-groups was accomplished using unpaired *t*-tests. Two-way ANOVAs were run to explore interactions between diagnosis and COMT genotype. In order to analyze for differences in the laminar expression of COMT mRNA per neuron based on either diagnosis or COMT genotype, two-way repeated measures ANOVAs were run with diagnosis and COMT genotype as between group factors and the cortical layer as a within-group factor (the number of silver grains was the dependent variable). To explore interactions between diagnosis and lamina, one-way repeated measures ANOVAs were run testing for differences in COMT mRNA levels across layers in each diagnostic group followed by least significant difference *post hoc* tests. Additionally, unpaired *t*-tests comparing COMT mRNA levels in schizophrenia *vs* control were run for each layer. A three-way repeated measures ANOVA was used to determine interaction across diagnosis, COMT genotype, and laminar expression.

RESULTS

In Situ Hybridization Histochemistry

The specificity of the human COMT riboprobe was determined previously (Matsumoto *et al*, 2003). Consistent with the previous data, the long isoform of COMT mRNA (1.5 kb) but not the short isoform (1.3 kb), was detected in the DLPFC of subjects in both diagnostic groups by Northern blot analysis (data not shown; Hong *et al*, 1998; Matsumoto *et al*, 2003; Tenhunen *et al*, 1994). Hybridization of human DLPFC tissue sections with the ³⁵S-labeled COMT antisense riboprobe generated a fairly robust signal, whereas hybridization with the sense riboprobe showed no obvious background signal (Figure 1a). Representative X-ray film autoradiograms and high-power bright field images of DLPFC from patients with schizophrenia and normal subjects are shown in Figure 1b. The mean optical density of the COMT hybridization signal on X-ray film was significantly higher in the gray matter than in the white matter area in both diagnostic groups (Figures 1b and 2). Silver grains were more numerous overlying large lightly stained neurons than over the small non-neuronal glial profiles, where the signal was typically indistinguishable or was occasionally slightly higher than the background level in both groups (Figure 1b). We have, however, consistently

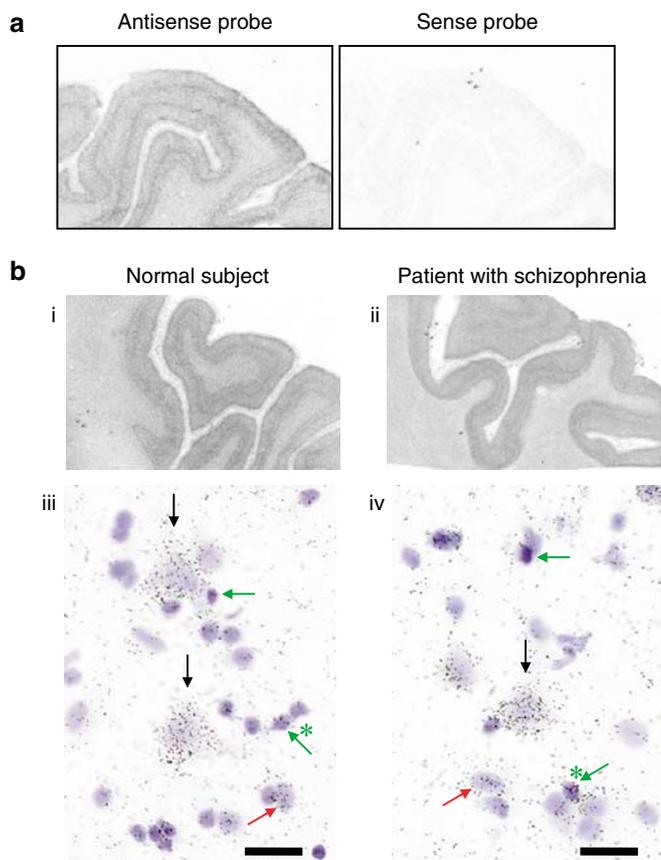


Figure 1 X-ray film autoradiogram images and high-power bright field images of *in situ* analysis of COMT mRNA in the DLPFC. (a) Representative X-ray film autoradiogram images from adjacent sections (normal subject) showing a robust signal with the antisense riboprobe, but no obvious background signal with the sense riboprobe. (b) Representative X-ray film autoradiogram images and photomicrographs (layer V, scale bar = 20 μm) from coronal DLPFC sections of normal subjects (i, iii) and patients with schizophrenia (ii, iv). Note that the large pyramidal neurons contain COMT silver grains at high densities (black arrows), while smaller-sized neurons contain relatively lower densities of silver grains (red arrows). Glial cells, identifiable by small darkly Nissl-stained nuclei, have silver grains at background levels (green arrows), but are occasionally slightly higher than background levels (green arrows with asterisk).

detected low levels of COMT mRNA signal in the white matter area (Figures 1 and 2), which implicates some glial expression of COMT mRNA, albeit at lower levels as compared to neurons. The number of silver grains was significantly greater on large pyramidal neurons as compared to smaller neurons (Figures 1 and 3).

Comparison of COMT mRNA Levels between Diagnostic Groups

Demographic variables, gender, mean age at death, PMI, and pH of tissue did not differ between the two groups of samples (Table 1). Furthermore, no relations were found between COMT mRNA levels in the gray matter of DLPFC and demographic variables; namely age at death ($r = -0.11$, $P = 0.56$), PMI ($r = -0.18$, $P = 0.35$), pH of tissue ($r = 0.24$, $P = 0.21$), and gender ($t = 0.47$, $df = 27$, $P = 0.64$). No correlations were detected between the COMT mRNA levels in patients with schizophrenia and neuroleptic medication

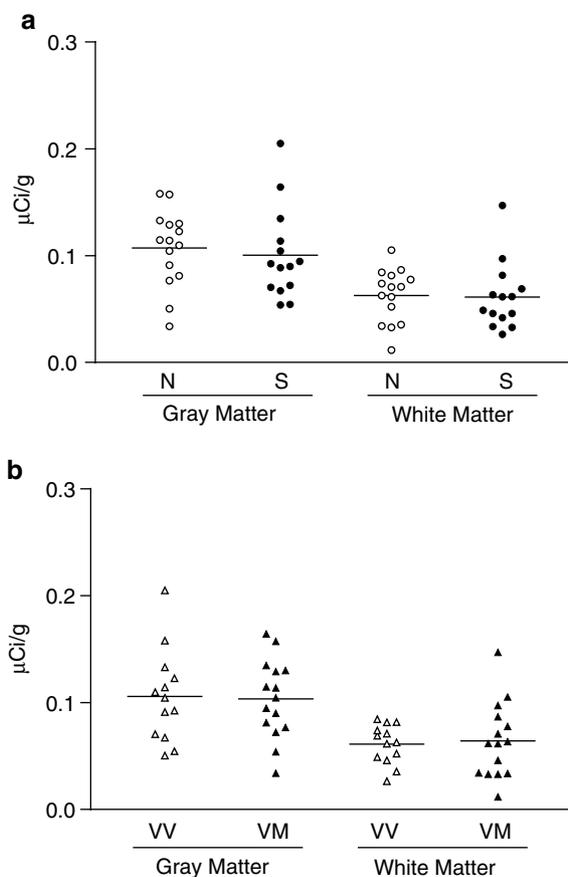


Figure 2 Comparison of COMT mRNA optical densities in the DLPFC on X-ray films (a) between diagnostic groups and (b) between COMT genotype-groups. (a) The mean COMT mRNA level in normal subjects (N) was $0.107 \pm 0.035 \mu\text{Ci/g}$ (mean \pm SD) in the gray matter and $0.063 \pm 0.025 \mu\text{Ci/g}$ in the white matter. The mean COMT mRNA level in patients with schizophrenia (S) was $0.100 \pm 0.043 \mu\text{Ci/g}$ (mean \pm SD) in the gray matter and $0.061 \pm 0.031 \mu\text{Ci/g}$ in the white matter. There was a significant difference in COMT mRNA levels between the gray and white matter in both groups of subjects (paired *t*-test, $P < 0.0001$). (b) The mean COMT mRNA level in subjects who were homozygous for the Val allele (VV) was $0.106 \pm 0.043 \mu\text{Ci/g}$ (mean \pm SD) in the gray matter and $0.061 \pm 0.018 \mu\text{Ci/g}$ in the white matter. The mean COMT mRNA level in subjects who were heterozygous (VM) was $0.103 \pm 0.037 \mu\text{Ci/g}$ (mean \pm SD) in the gray matter and $0.064 \pm 0.035 \mu\text{Ci/g}$ in the white matter. There was a significant difference in COMT mRNA levels between the gray and white matter in both groups of subjects (paired *t*-test, $P < 0.0001$).

histories converted to CPZ equivalents (average daily doses $r = -0.26$, $P = 0.40$, estimated lifetime doses $r = -0.24$, $P = 0.43$, daily doses at death $r = -0.31$, $P = 0.33$). When we analyzed COMT mRNA levels in the white matter area, again, no significant relation with demographic variables or neuroleptic medication was found (data not shown). Therefore, we did not utilize these demographic variables as covariates in further statistical analysis. Analysis of optical density on the X-ray films revealed no significant differences in mean levels of COMT mRNA between the groups of schizophrenic and normal subjects (gray matter, $t = 0.46$, $df = 27$, $P = 0.65$; white matter, $t = 0.15$, $df = 27$, $P = 0.88$; Figure 2a).

Silver grain analysis of COMT mRNA in neurons (large pyramidal and smaller, presumably nonpyramidal neurons

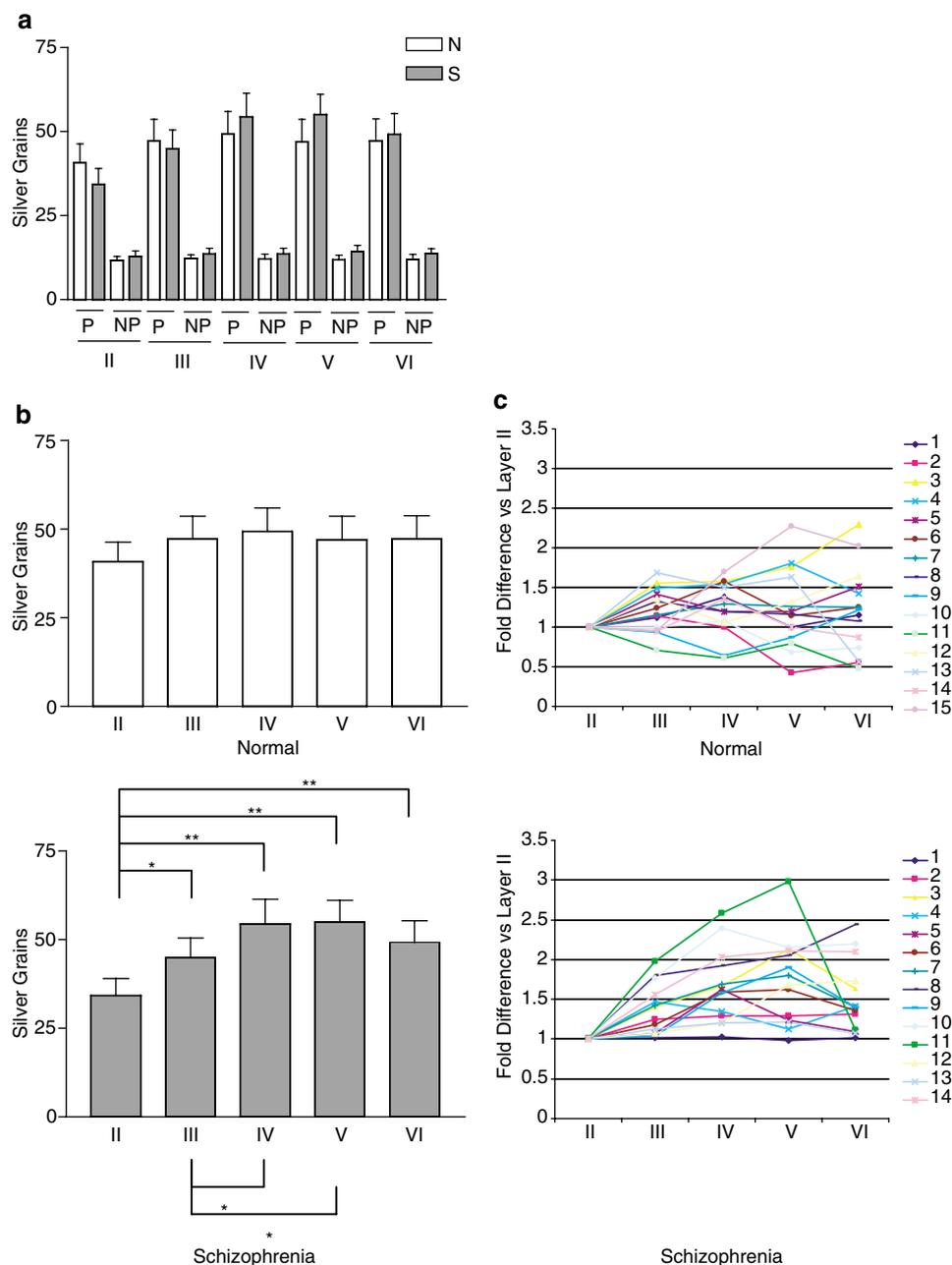


Figure 3 Comparison of silver grains on neurons in the DLPFC between diagnostic groups. (a) The mean number of silver grains in layers II–VI is shown on large pyramidal neurons (P) and on smaller, presumably nonpyramidal neurons (NP) in normal subjects (N) and in patients with schizophrenia (S). Error bars represent the standard error. There is a significant difference in the number of silver grains between the large and small neurons in both subject groups (paired *t*-test, $P < 0.0001$). (b) The mean number of silver grains in layers II–VI on large pyramidal neurons in normal subjects and in patients with schizophrenia. Error bars represent the standard error. Significant laminar differences are indicated by asterisks (* $P < 0.01$; ** $P < 0.0001$). (c) The mean number of silver grains in layers II–VI on large pyramidal neurons in each individual was normalized by that of layer II (fold difference vs layer II).

from layers II to VI) using two-way repeated measures ANOVAs for each class of neuron confirmed the film analysis, revealing no main effect of diagnosis (large neurons, $F = 0.02$, $df = 1,27$, $P = 0.88$; small neurons, $F = 0.76$, $df = 1,27$, $P = 0.39$; Figure 3a). However, the analysis showed a significant effect of cortical layer ($F = 10.5$, $df = 4,108$, $P < 0.0001$) and a significant layer by diagnosis group interaction ($F = 2.68$, $df = 4,108$, $P < 0.04$) in large pyramidal neurons, but not in smaller neurons ($P = 0.83$ and 0.90 , respectively). The laminar difference in COMT mRNA levels was still robust when the analysis was

restricted to patients with schizophrenia ($P < 0.0001$), but no significant laminar effect was observed in normal subjects ($P = 0.26$). *Post hoc* comparison indicated that schizophrenic patients had lower levels of COMT mRNA in pyramidal neurons in the superficial layers (II/III) and higher levels in the intermediate/deep layers (IV/V) (Figure 3b). The laminar difference in the patient group was represented fairly consistently in each individual (Figure 3c). Levels of COMT mRNA in the individual layers did not differ between diagnostic groups when directly compared (all $P > 0.38$). No relation was detected between

the patients' medication histories and the COMT mRNA levels in any layer (large neurons, all $P > 0.16$; small neurons, all $P > 0.11$).

Comparison of COMT mRNA Levels between the COMT Genotype-Groups

In order to determine whether COMT genotype affects its own mRNA level, all subjects were genotyped at Val^{108/158} Met and divided into two groups: homozygous for the Val allele (Val/Val) and heterozygous (Val/Met). Since there was only one subject who was homozygous for the Met allele (Met/Met) in the present cohort, we did not include this subject in the statistical analysis. There was no difference between the two genotype-groups in the mean levels of optical density of COMT mRNA on X-ray film (gray matter, $t = 0.15$, $df = 26$, $P = 0.88$; white matter, $t = -0.28$, $df = 26$, $P = 0.78$; Figure 2b). When the analysis was restricted to patients with schizophrenia or to normal subjects, again there were no differences between the two genotype-groups ($P > 0.48$). There were no interactions between diagnosis and COMT genotype ($P > 0.40$). Likewise, silver grain analysis of COMT mRNA in neurons showed no main effect of COMT genotype (large neurons, $F = 0.47$, $df = 1,26$, $P = 0.50$; small neurons, $F = 0.15$, $df = 1,26$, $P = 0.70$). There was also no interaction between the cortical lamina and COMT genotype (large neurons, $P = 0.35$; small neurons, $P = 0.11$), or across cortical lamina and diagnosis and COMT genotype (large neurons, $P = 0.79$; small neurons, $P = 0.95$).

DISCUSSION

We have examined the expression of COMT mRNA in brain tissue from patients with schizophrenia and from normal comparison subjects. COMT mRNA was detected at higher levels in large pyramidal neurons compared to smaller, presumably nonpyramidal neurons and was distributed from layers II to VI in the DLPFC. Although silver grains were occasionally detected in glial cells with dark Nissl-stained nuclei (Figure 1), overall, the expression in these cells was typically indistinguishable from the background. The neuronal expression profile of COMT was also confirmed by the comparison between gray and white matter, which showed significantly higher levels of COMT mRNA in the gray matter. These results are similar to those reported previously (Matsumoto *et al*, 2003). Although the COMT riboprobe used in the present study recognizes both the long (1.5 kb) and short (1.3 kb) transcripts (Matsumoto *et al*, 2003), we and other groups have shown that the long COMT mRNA is the only isoform detectable in the human cortex (Hong *et al*, 1998; Matsumoto *et al*, 2003; Tenhunen *et al*, 1994). Consistent with this, Northern blot analysis detected only long COMT mRNA expression in the DLPFC of subjects in both diagnostic groups. Therefore, the mRNA signal detected in the present *in situ* study likely represents the long isoform of COMT transcript, which presumably leads to the predominant expression of the membrane bound type of COMT having a high affinity for catechol (Matsumoto *et al*, 2003; Tenhunen *et al*, 1994).

We have also assessed the effect of COMT Val^{108/158} Met genotype on its own mRNA level. The present comparison of individuals who are homozygous for the Val allele (Val/Val) and heterozygous (Val/Met) subjects has demonstrated no significant difference in the levels or laminar distribution of COMT mRNA, making it appear that a transcriptional mechanism aimed at compensating for the functional Val^{108/158} Met polymorphism does not occur. This is consistent with a recent finding by Yan *et al* (2002), showing no relation between COMT mRNA levels and the COMT genotype of 388C/T SNP that is presumably in strong linkage disequilibrium with Val/Met SNP due to their very small distance (< 500 bp). Our data are also consistent with experiments performed in Dr Paul Harisson's laboratory at Oxford University (personal communication). Previous studies have demonstrated that the Met allele COMT protein is thermolabile at body temperature and has less enzymatic activity *in vivo* (Lachman *et al*, 1996; Lotta *et al*, 1995). Together, these data suggest that the Val/Met substitution affects the net enzyme activity of COMT without any change in its mRNA levels in the DLPFC. The presumed direct effect of COMT Val^{108/158} Met on prefrontal dopamine metabolism is supported by previous data associating this functional polymorphism with observable dopamine-related prefrontal phenotypes, that is, working memory performance and prefrontal physiology (Bilder *et al*, 2002; Egan *et al*, 2001; Goldberg *et al*, in press; Joobar *et al*, 2002; Malhotra *et al*, 2002; Rosa *et al*, 2002).

Although we did not find a difference in the mean levels of COMT mRNA in the DLPFC between patients with schizophrenia and normal subjects, we did detect a difference in laminar distribution. Pyramidal neurons of the patients expressed COMT mRNA at significantly lower levels in the superficial layers (II/III) and higher levels in the intermediate/deep layers (IV/V), while COMT mRNA expression in pyramidal neurons of normal subjects did not show such laminar variation. Importantly, we detected these laminar differences only in pyramidal neurons, and not in small, presumably nonpyramidal neurons. It is important to note that these findings reflected a difference in the laminar pattern of expression, as the mean expression in individual lamina did not differ between groups. In other words, while the absolute difference in COMT mRNA expression per pyramidal neuron was relatively small, the synaptic architecture of catecholamine synapses on pyramidal neurons may have undergone reorganization across cortical layers in schizophrenia. This is clearly a novel observation that will require independent replication.

We did not detect any correlation between the COMT mRNA levels and the medication histories of patients. Additionally, an earlier animal study has indicated that chronic neuroleptic treatment (ie haloperidol) does not affect brain COMT activity (Hannan, 1986). These data support the possibility that the disease-related laminar differences we have observed in the schizophrenic subjects reflect aspects of disease biology and are not epiphenomena related to treatment.

The mechanism of this potential rearrangement of the molecular profile of prefrontal pyramidal neurons is a matter of speculation, but likely involves the synaptic organization of these neurons. Since COMT proteins have been detected exclusively in the neuropil of the cerebral

cortex (Kaplan *et al*, 1979; Karhunen *et al*, 1994; Lundstrom *et al*, 1995) and neurons are the primary cell population producing COMT mRNA, it is reasonable to assume that COMT is located at dendrites and/or spines in the prefrontal cortex (Matsumoto *et al*, 2003). Interestingly, the prefrontal cortex of patients with schizophrenia has been reported to show reduced cortical neuropil, particularly in layer II (Selemon *et al*, 1998), and decreased somal size of pyramidal neurons in layer III (Pierri *et al*, 2001; Rajkowska *et al*, 1998), both of which could be related to reduced dendritic arborization. Thus, lower levels of pyramidal COMT mRNA in these external cortical layers may reflect this putative reduction of dendrites and/or spines, where COMT protein is presumably located, and may implicate catecholaminergic synapses as part of this process. In fact, studies in animals indicate that the activity of dopamine inputs to the prefrontal cortex directly covary with the density of spines (Robinson and Kolb, 1999).

Previous anatomical studies in monkey (Goldman-Rakic *et al*, 1989; Krimer *et al*, 1997; Lewis, 1992; Smiley and Goldman-Rakic, 1993) and human (Akil *et al*, 1999; Lewis, 1992) brain have demonstrated that dopaminergic projections to DLPFC are highly organized and distributed in a bilaminar manner at high densities in the superficial (I/II) and deep layers (V/VI), whereas the distribution is more uniform in the dorsomedial region of this cortical area. The majority of dopaminergic terminals synapse on pyramidal neurons, while dopaminergic contacts on nonpyramidal neurons are much less dense and are irregularly distributed (Goldman-Rakic *et al*, 1989; Krimer *et al*, 1997; Smiley and Goldman-Rakic, 1993). Consistent with this, our present data show higher levels of COMT mRNA in pyramidal than in small, presumably nonpyramidal neurons. Electrochemical studies in the monkey brain have demonstrated that dopamine inputs modulate cortical neuronal excitability in a complex manner, with the potentiation of pyramidal postsynaptic currents (Henze *et al*, 2000) and also with inhibition via a presynaptic mechanism (Gao *et al*, 2001). Moreover, these pyramidal neurons are connected horizontally and vertically by intrinsic collaterals in the prefrontal cortex (Levitt *et al*, 1993). The laminar imbalance of COMT expression, which presumably leads to imbalance of dopamine availability at the synaptic level, may contribute to dysregulation of pyramidal neuronal circuitry in the prefrontal cortex, albeit to a relatively small degree. In an earlier immunohistochemical study of dopamine terminals in the dorsomedial prefrontal cortex, patients with schizophrenia displayed significantly reduced dopaminergic innervation in the deeper layers (Akil *et al*, 1999). The reduction of dopaminergic input in deep layers has also been observed in the dorsolateral region (Akil M, personal communication). Importantly, this alternation in patients with schizophrenia is opposite to that of COMT mRNA expression in pyramidal cells, especially in layer V, where decreased dopamine innervation and relatively higher levels of COMT mRNA have been detected. Given that prefrontal pyramidal neurons in layer V project to the striatum and brainstem and appear to tonically inhibit striatal dopamine synthesis/release (Carlson *et al*, 1991; Carr and Sesack, 2000; Ferry *et al*, 2000; Kolachana *et al*, 1995; Pycocock *et al*, 1980; Saunders *et al*, 1998), it is tempting to speculate that these various disease-related changes in layer V, that is,

diminished dopamine innervation and increased COMT expression, synergistically contribute to a relative hypodopaminergic state at the level of prefrontal neuronal signaling and a hyperdopaminergic state at the level of striatal function, which has been implicated in the pathophysiology of schizophrenia (Bertolino *et al*, 2000; Grace, 2000; Laruelle *et al*, 1996; Meyer-Lindenberg *et al*, 2002; Weinberger, 1987; Weinberger *et al*, 1988). Finally, the combined effects of this laminar imbalance of dopamine innervation and COMT expression might explain, at least partly, recent evidence that the COMT Val^{108/158} Met polymorphism has a greater impact upon working memory performance in patients with schizophrenia than in normal subjects (Joober *et al*, 2002).

In conclusion, our present data show that the functional impact of the COMT Val^{108/158} Met polymorphism on DLPFC is not compensated by a genotype-associated change in its own mRNA expression. The disease-dependent laminar differences in COMT mRNA expression provide additional evidence of an abnormality of dopamine neurotransmission in the DLPFC of patients with schizophrenia.

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