Mitochondrial Function is Differentially Altered in the Basal Ganglia of Chronic Schizophrenics

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In the present study, we have applied a novel strategy involving the postmortem measurement of the mitochondrial respiratory chain enzyme cytochrome-c oxidase (COX; complex IV) to identify regional changes in energy metabolism in the basal ganglia of chronic, medicated schizophrenics. COX activity was decreased in the caudate nucleus but increased in the putamen and nucleus accumbens. An increase in succinate dehydrogenase (complex II) was evident in the putamen and nucleus accumbens, but changes were not seen with NADH dehydrogenase (complex I). An analysis of interregional correlations in energy metabolism revealed several anomalies in the connections between the caudate and putamen and the globus pallidus in schizophrenics. Results provide strong evidence that changes in baseline energy metabolism in specific regions of the basal ganglia may exist in the disease. Based upon the high degree of input it receives from associative cortical areas, results suggest that a defect in the caudate may underlie certain aspects of cognitive decline in schizophrenics. In contrast, an increase in COX in the putamen, which receives extensive projections from the sensorimotor cortex, may reflect an effect of chronic neuroleptic treatment on motor function.
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The main contributors to the search for functional brain changes in schizophrenia have employed imaging techniques such as positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI) to identify potential candidate regions which may define disease symptomatology. To date, over 300 published articles exist (Medline database 1966–1997) in which imaging techniques have been utilized in schizophrenics. While the power of PET as a tool for understanding 'state-dependent' brain function and receptor characteristics, as well as offering excellent patient control, is generally undisputed, the sheer bulk of data created by researchers in the field has generated a disconcertingly diffuse base of evidence for which regions may be involved in the disease. Although a general trend implicating reduced metabolism in cortical and sub-cortical regions has formed the foundation for assumptions about a possible defect in schizophrenics (Buchsbaum et al. 1982, 1992; Tamminga et al. 1992; Siegel et al. 1993), opposite findings have been made (Early et al. 1987; Wiesel et al. 1987; Cleghorn et al. 1989).

As a complement to imaging studies, our laboratory has applied a strategy involving the post-mortem measurement of the mitochondrial respiratory chain en-

zyme cytochrome-c oxidase (COX) in an attempt to localize altered brain function in schizophrenia. This approach is based upon a strong body of evidence which indicates that neuronal COX is highly regulated by the energy demands of the cell and as such represents an endogenous marker of cellular energy metabolism over time (Wong-Riley 1989). Interest in COX as a marker of neuronal function rests upon many of the same assumptions implicit in the use of PET in the measurement of regional glucose metabolism and blood flow. Neurons are highly dependent upon oxidative phosphorylation as the primary pathway for the generation of ATP, of which 40–60 % is utilized in the maintenance of ion gradients by ATPases. In support of this, a strong correlation has been demonstrated between the regulation of COX and Na+,K+ ATPase in brain tissue (Hevner et al. 1992). Traditionally however, the strongest evidence that COX is coupled to neuronal energy demands comes from studies in which changes in COX activity can be induced by experimental interventions which alter neuronal activity. Perhaps the most interesting study in this regard was performed utilizing a histochemical technique to demonstrate reduced COX activity in the brains of cats in which chronic neuronal inactivity was induced in visual cortex by monocular suture (Wong-Riley 1979). In addition, studies have shown that monocular retinal impulse inhibition with tetrodotoxin results in a decrease in COX activity in specific regions of the monkey visual cortex and thalamus (Wong-Riley and Carrol 1984). In terms of localization, evidence suggests that as much as 60% of COX activity in a particular region reflects dendritic activity whereas glial cell activity is responsible for less than 5% (Wong-Riley 1989). While contemporary research on mitochondrial function has focused primarily on the role of energy metabolism in neurodegenerative disease (Beal 1992), the concept that neuronal function and the energy demands of the cell govern the activity of its energy producing structures forms the basis of our assumptions in the present work. Thus, while defects in the genes which control cellular energy production can undoubtedly lead to disease, possibly including psychosis, animal studies in our laboratory as well as extensive PET studies suggest that changes in energy metabolism, probably including the long term regulation of COX, are the result, rather than the cause, of altered neuronal signalling.

Preliminary findings from our laboratory indicated that COX activity may be reduced in the striatum and frontal cortex of schizophrenics, consistent with the concept that altered activity in cortico-striatal circuits may underlie the disorder (Cavelier et al., 1995). Subsequent studies on the effects of neuroleptics, PCP, and methamphetamine on animals, have provided additional evidence that a state of dopaminergic overactivity or glutamatergic underactivity produces a hypomet-abolic state similar to that which is evident in the brains of schizophrenics (Prince et al. 1997a,b,c). We sought, in the present study, to expand the search for changes in energy metabolism which may exist in the basal ganglia of chronic, medicated schizophrenics.

MATERIALS AND METHODS

Materials

Ubiquinone-1 was purchased non-commercially through F. Hoffman-La Roche AG (Basel, Switzerland). PD1O columns were obtained from Pharmacia (Uppsala, Sweden). PicoGreen was purchased from Molecular Probes (Leiden, The Netherlands). Cytochrome-c (horse heart) and all other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Subjects and Tissue Preparation. Brain samples were obtained from 11 schizophrenics and 16 controls with no known history of psychiatric or neurodegenerative disorders. Patients were diagnosed according to the Diagnostic and Statistical Manual (DSM) III-R. In the schizophrenic population, seven were diagnosed as paranoid, two disorganized, and two undetermined (non ultra descriptus, NUD). In addition, one individual who suffered from organic psychosis and one schizoaffective patient were analyzed, but excluded from group comparisons. All schizophrenics had suffered from a chronic course of the disease and been treated extensively with various neuroleptics. No evidence of Alzheimer's disease or other neuropathological features of degenerative disease could be found in either schizophrenics or controls. The mean age of the patients (6 male, 5 female) was 78 ± 7 (SD) years, and 81 ± 9 (SD) years for controls (9 male, 7 female). To address the influence of PMI on biochemical parameters, five controls were included with PMIs in excess of 100 hrs. The mean PMI for schizophrenics was 47 ± 20 (SD) hrs and 53 ± 24 (SD) for 11 controls. Brain tissue specimens from the caudate nucleus, putamen, nucleus accumbens, globus pallidus, thalamus, and mesencephalon were obtained at autopsy from all individuals. Dissections were performed according to anatomical landmarks: the nucleus accumbens was taken from the junction between the frontal caudate nucleus and putamen. The mesencephalon was a cross section of brainstem below the superior and inferior colliculi. This rough dissection of the mesencephalon includes portions of the A8, A9, A10, and raphe nucleus. Following dissection, brain samples were frozen in liquid nitrogen and crushed into a course powder prior to storage at −70°C. Brain samples were homogenized in a buffer consisting of 10 mM potassium phosphate (pH 7.6), 1

mM EDTA, 0.25 M sucrose in an Ultra-Turrax set on full speed for 30 sec. Homogenates were then frozen at −70°C until assayed.

Cytochrome-c Oxidase Assay.   Cytochrome-c oxidase was assayed according to a modification of the spectrophotometric method of Yonetani and Ray (1965). Reduced Cytochrome c was prepared by the addition of 30 mg $Na_2S_2O_4$/100 mg cytochrome c in a 10 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA, and separated on a PD1O column (Pharmacia). Incubations were performed in 10 mM potassium phosphate buffer (pH 7.6), 1 mM EDTA, and 25 μM reduced cytochrome c at 25°C. Upon the addition of approximately 100 μg protein, the change in absorbance at 550 nm was determined on a Jasco V-550 spectrophotometer for 5 min. Initial rates were determined differentially where-d[ferrocytochrome c]/dt is derived from polynomial plots at zero time using an extinction coefficient of 19.0 $mM^{-1}cm^{-1}$ (Yonetani and Ray 1965).

NADH:ubiquinone Oxidoreductase Assay.   NADH:ubiquinone oxidoreductase (complex I) was assayed according to a modification of the method described by Whitfield et al. (1981) in the presence and absence of 5 μM rotenone. Approximately 500 μg of brain homogenate was added to a reaction mixture containing 10 mM potassium phosphate buffer (pH 7.6), 1 mM EDTA, 80 μM coenzyme-Q_1, and 2 mM KCN. Reaction mixtures were equilibrated to 25°C for 3 min prior to the addition of 0.1 mM NADH to initiate the reaction. The decrease in absorbance at 340 nm was monitored for 2 min and activity calculated from linear plots using a combined extinction coefficient for Q_1 and NADH of 6.81 $mM^{-1}cm^{-1}$.

Succinate Dehydrogenase Assay.   Succinate dehydrogenase (complex II) was assayed according to a modification of the method of Ackrell (1978). Brain homogenates (50 μg) were added to a solution consisting of 10 mM potassium phosphate buffer (pH 7.6), 1 mM EDTA, 10 mM succinate, and 1 mM KCN. Reactions were initiated with the addition of 1.625 mM phenazine methosulphate and 70 μM 2,6-dichloroindophenol (DPIP) to the incubation mixture. The decrease in absorbance at 600 nm was then monitored for 3 min at 37°C and the activity of the enzyme was calculated using an extinction coefficient of 21 $mM^{-1}cm^{-1}$ for DPIP.

Protein and DNA Measurements.   The total amount of protein in the samples was determined according to the Markwell modification of the Lowry procedure using bovine albumin as a standard (Markwell et al. 1978). Based upon extensive evidence which suggests that DNA is a sensitive indicator of cell number (Downs and Wilfinger 1983; Rago et al. 1990) the total quantity of ds-DNA in samples was determined fluorometrically using PicoGreen (Molecular Probes) based upon its superior selectivity for DNA over RNA (Singer et al.

1997). Samples (10 μl) were added to a 50 μl TE buffer (0.01 M Tris-HCl buffer, pH 8.0, 1 mM EDTA) containing 0.01% SDS, incubated at RT for 10 minutes, and then sonicated at low power for 5 sec on a Branson B15 cell disruptor. A 1 ml solution containing 0.6 μM PicoGreen was then added to the samples and incubated for 5 min at RT. Fluorescence was then determined on a Jasco FP-777 spectrofluorometer using 480 nm and 520 nm excitation and emission wavelengths. Calf thymus DNA was used as a standard and a value of 7.23 pg DNA/cell was used to calculate cell number.

Statistics

All statistical analyses were performed on a Power Macintosh 7300/200 using StatView v4.5.1 (Abacus Concepts) and Prism (GraphPad). Statistically significant differences in the means of biochemical data were established using Student's t-test and significance between subgroups was established using ANOVA and Fisher's PLSD. Coefficients of determination (r^2) were calculated by simple linear regression analysis and p-values were obtained using paired t-tests. Significance differences between regression lines were determined with t-values after testing for parallelism (Prism).

RESULTS

Consistent with previous results (Cavelier et al. 1995) cytochrome-c oxidase activity was found to be reduced in the caudate nucleus in the total population of schizophrenics (Figure 1). However, differences were much less pronounced in the present study. We extended our analysis to include several other brain regions which essentially define the primary structures of the basal ganglia. We also addressed the specificity of the change in COX by assaying two other respiratory chain complexes; NADH dehydrogenase and succinate dehydrogenase.

While the reduction in COX seen in the caudate was shown to be specific among the regions investigated, the most prominent findings in the present study were significant increases in COX and SDH enzyme activity in the putamen (Figure 1). In addressing differences in other mitochondrial enzymes, no significant changes were found in NADH dehydrogenase. Interestingly, a strong correlation between SDH and COX in the total population was observed in the putamen ($r^2 = 0.587$; $p < .0001$), but was essentially absent in the caudate ($r^2 = 0.134$; $p = .1226$). There were no differences in this relationship between controls and schizophrenics.

Because the population was small, attempts to address differences between sub groups among schizophrenics was not feasible. Based upon its size however, comparisons could be made with the paranoid group.
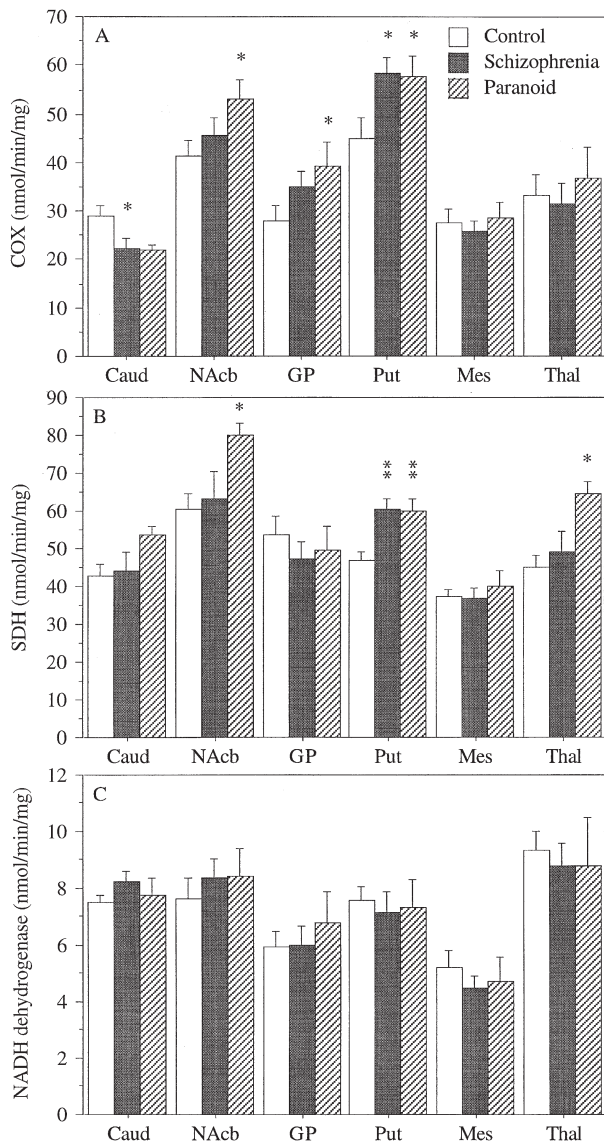
Figure 1. Activity of various mitochondrial respiratory chain enzyme complexes in the basal ganglia of schizophrenics (N = 11; N = 7 for paranoid) and controls (N = 11). Values are expressed as mean ± SEM. Significance was determined using ANOVA and Fisher's PLSD. *p < .05; **p < .005. The legend in A applies to B and C as well. Caud, caudate nucleus; Nacb, nucleus accumbens; GP, globus pallidus; Put, putamen; Mes, mesencephalon; Thal, thalamus.

In paranoid schizophrenics, COX was increased significantly in the nucleus accumbens (p = .0274) and globus pallidus (p = .0476) in addition to the putamen (p = .0075) (Figure 1). Reductions in the caudate were not significant when the paranoid subgroup was compared to controls (p = .0892). Compared with controls, SDH activity was also significantly elevated in the paranoid group in the nucleus accumbens (p = .0019) and putamen (p = .0027).

A second strategy involving metabolic variation in schizophrenics, largely propagated by PET researchers,

has been to address interregional correlations in energy metabolism. We have applied a similar approach under the premise that our observations using COX reflect baseline metabolism in the brain. To address this phenomenon, a simple linear regression matrix was constructed in which each region was compared with every other region in both controls and schizophrenics. To establish the degree of significance between the regression lines of controls and schizophrenics, a simple test of parallelism was applied. Consistent with a prospect that connectivity may be disturbed in the disease, several significant anomalies emerged between schizophrenics and controls. A summary of the results is shown (Table 1). A strong correlation between the putamen and globus pallidus could be seen in the controls, but was lacking in the schizophrenic population. As well, the correlation between the caudate nucleus and globus pallidus was higher in the controls compared to the schizophrenic population, although the difference between the two did not reach significance (Table 1).

The most common practice in reporting enzyme activities is to relate them to total protein levels. In this study, we have included an analysis of total cell number in brain homogenates. The relationship between cell number and total protein was highly linear as expected and significant differences in enzyme activities between schizophrenics and controls remained regardless if they were related to protein or cell number. Total cell number related to protein levels was significantly increased in the mesencephalon, but no other brain region of schizophrenics (Table 2).

On the basis that no information exists in the literature about the effects of age and postmortem time on mitochondrial enzyme function, we utilized linear regression to analyze these factors as potential covariates. No significant correlations between age and activity with any of the three enzymes in any of the brain regions could be established (ages 64–96 yrs). A significant effect of postmortem time could be established for complex I in all of the brain regions studied (p = .0001), but this was not evident for either COX or SDH (PMI 12–168 hrs). No differences between males and females were evident with any of the enzymes studied.

DISCUSSION

In our present study, we present evidence that abnormalities in energy metabolism exist in the basal ganglia of chronic schizophrenics. While several significant findings were made, the most prominent and consistent were the increases in both COX and SDH in the putamen and nucleus accumbens in the paranoid group of schizophrenics. On the basis that the schizophrenic population in this study had been undergoing extensive neuroleptic treatment up to the time of death, it

Table 1. Interregional Coefficients of Determination (r^2) for Cytochrome Oxidase in the Basal Ganglia of Schizophrenics and Controls

Brain Regions	Controls	Schizophrenics	Slope Significance (p)
Caudate/Putamen	0.716 (0.0080)	0.764 (0.1257)	NS
Caudate/Accumbens	0.065 (0.6792)	0.352 (0.1213)	NS
Caudate/Pallidus	0.414 (0.1683)	0.001 (0.9294)	.082
Caudate/Mesenceph	0.801 (0.0159)	0.729 (0.0144)	NS
Caudate/Thalamus	0.937 (0.0003)	0.490 (0.1215)	.076
Putamen/Pallidus	0.649 (0.0288)	0.004 (0.8829)	.033
Putamen/Accumbens	0.304 (0.3357)	0.082 (0.5337)	NS
Putamen/Thalamus	0.905 (0.0035)	0.546 (0.2613)	.092
Putamen/Mesenceph	0.810 (0.0058)	0.084 (0.5285)	NS
Accumbens/Pallidus	0.427 (0.0791)	0.294 (0.0685)	NS
Accumbens/Mesenceph	0.076 (0.5495)	0.637 (0.0032)	NS
Accumbens/Thalamus	0.870 (0.0206)	0.250 (0.2073)	.061
Pallidus/Mesenceph	0.335 (0.1024)	0.318 (0.0560)	NS
Pallidus/Thalamus	0.655 (0.0509)	0.475 (0.0584)	NS
Thalamus/Mesenceph	0.747 (0.0264)	0.536 (0.0390)	.056

Values are coefficients of determination (r^2) for linear regression lines. N = 11 for both controls and schizophrenics. Values in parenthesis are P values for individual regression lines (t-test). p-values represent significance of parallelism between regression lines (t-test).

seems prudent to interpret findings of increased metabolism as representing a primary effect of medication. In support of this, we have previously demonstrated that chronic treatment with neuroleptics increases COX activity in specific regions of the rat brain, primarily the striatum (Prince et al. 1997a,b). Extensive human studies using PET have also implicated increased striatal metabolism in conjunction with a reduction in symptomatology in schizophrenics after chronic neuroleptic treatment (DeLisi et al. 1985; Wolkin et al. 1985; Buchsbaum et al. 1987; Wolkin et al. 1996; Miller et al. 1997).

The decrease in COX in the caudate represents a unique anomaly which may result from disease pathology and not from neuroleptic treatment. In line with this concept, the largest study yet performed using PET involving 70 schizophrenics found a reduction in caudate nucleus glucose metabolism in patients who were neuroleptic-naive (Siegel et al. 1993). Others have also found reduced glucose metabolism, primarily in the caudate and frontal cortex, in never medicated schizophrenics (Buchsbaum et al. 1992). It should be pointed out however that findings also exist which indicate in-

creased metabolic function in the globus pallidus, but not in other regions of the basal ganglia in neuroleptic-naive patients (Early et al. 1987). Nonetheless, together with our previous results (Cavelier et al. 1995), these findings shed light upon the prospect that a pathologically significant reduction in COX may be present in the caudate nucleus of schizophrenics.

Perhaps the most enigmatic finding in this study was that the activities of COX in the caudate and putamen appear to be affected differentially. The discrepancies between the two regions are however, unlikely to be due to opposing effects of neuroleptics, a prospect which is strengthened by extensive evidence that schizophrenics generally exhibit decreased brain energy metabolism which is reversed by neuroleptics (Buchsbaum et al. 1992; Holcomb et al. 1996). The two possibilities which exist are that either the caudate is responding relatively poorly to neuroleptics, or that an underlying deficit is more prominent in the caudate than i.e. the putamen. While literature on the matter is scarce, specific differences between the caudate and putamen in schizophrenia have been noted previously.

Table 2. Total Cell Density in Various Brain Regions in Schizophrenics and Controls

Brain Region	Controls	Schizophrenics	p-value
Caudate Nucleus	10.38 ± 0.37	10.85 ± 0.44	.4370
Putamen	9.06 ± 0.35	9.01 ± 0.61	.9319
Nucleus Accumbens	8.91 ± 0.32	8.60 ± 0.18	.3941
Globus Pallidus	7.02 ± 0.35	7.44 ± 0.42	.4676
Mesencephalon	6.34 ± 0.19	6.90 ± 0.21	.0390
Thalamus	7.61 ± 0.19	7.16 ± 0.32	.2670

Values are expressed as mean cell number $\times 10^6$/mg protein ± SEM. N = 11 for both controls and schizophrenics. p-values were determined using Student's t-test.

In one study, changes in synaptic density were observed in the caudate, but not in the putamen of schizophrenics (Kung et al. 1998). A second finding, which is perhaps more difficult to interpret, suggested that the caudate nucleus in schizophrenics may be diminished in size whereas the putamen is enlarged (Shihabuddin et al. 1998).

Differences in the putamen and caudate may be of significance for understanding the neurophysiology of schizophrenia. Of particular importance in this regard is the fact that projections to the striatum from associative regions of the cortex such as the dorsolateral prefrontal cortex generally innervate the caudate nucleus whereas projections from motor areas generally target the putamen (Parent 1990). The primary influence the frontal cortex has on caudate function suggests that a defect in either could result in deficits in a variety of cognitive functions, including working memory (Levy et al. 1997). Indeed, considerable evidence exists that the disorganization of thought and behavior in schizophrenics may result from deficits in information processing (Goldman-Rakic and Selemon 1997) a phenomenon which may be reflected in altered caudate function.

Previous studies involving postmortem material have tended to concentrate on single regions, a limited approach which has left open the question of whether or not similar changes exist in related areas. We have therefore also attempted to address this concern based upon growing interest in 'circuitry' as opposed to restricted regional effects (Andreasen et al. 1996). In this regard, a major focal point in contemporary schizophrenia research has been in identifying 'circuits' which may be dysfunctional in the disorder. While connectivity in brain regions cannot be conclusively demonstrated by the fact that metabolic correlations exist between them, the hypothesis is that differences in metabolic gradients in regions which have defined anatomical connectivity may be suggestive of dysfunction in a given patient population (Clark et al. 1984; Katz et al. 1996; Andreasen et al. 1996). Traditionally, the search for functional anomalies in schizophrenia has almost exclusively concentrated on various cortical regions and the relationships between them (Andreasen et al. 1997; Katz et al. 1996; Siegel et al. 1993; Clark et al. 1984). However, since the original correlational studies by Clark et al. (1984), findings have been diffuse in terms of establishing clear abnormalities in metabolic correlations in schizophrenics. The data accumulated in the present study also facilitated a statistical approach in establishing correlations between the structures of the basal ganglia. We observed a high degree of positive correlations between brain regions in controls, but these were lacking in specific regions of schizophrenics. This effect was evident in several areas, but was most prominent in the connections between the caudate and putamen and the globus pallidus. While it is tempting

to speculate that this may be intrinsic to the disease, a neuroleptic effect seems more plausible. Thus, it follows logically that a chemical intervention which alters metabolism in a brain region downstream from its affector region will naturally interrupt the normal associations between those two regions. However, as to why both the caudate and putamen elicit this effect with regards to correlations in COX but not in absolute activity is at present unknown. Correlational findings are generally subject to criticism based upon sample size, a problem which applies to our results as well. However, the general theme which has emerged in terms of reduced correlations in schizophrenics merits further investigations using this approach.

Our main emphasis in this work has been on identifying regional metabolic alterations which may offer evidence as to which brain areas are involved in the expression and development of psychosis. An abundance of previous literature, primarily involving the use of PET, alludes to the prospect that hypometabolism contributes to the pathophysiology of schizophrenia (Buchsbaum et al. 1982, 1992; Siegel et al. 1993). In the wake of the increasing use of PET in the study of schizophrenia, we have employed a novel strategy involving the search for altered brain function using endogenous levels of COX as a marker of neuronal function. The results from this study provide evidence that mitochondrial function is differentially altered within the structures of the basal ganglia in schizophrenics. While an increase in energy metabolism in the putamen may reflect an effect of neuroleptics on motor function, a deficiency in the caudate may be indicative of impaired input from associative cortical regions.

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