Published in final edited form as:

J Comp Neurol. 2010 February 1; 518(3): 277–291. doi:10.1002/cne.22206.

Decreased Number of Parvalbumin and Cholinergic Interneurons in the Striatum of Individuals with Tourette Syndrome

Yuko Kataoka¹, Paul S. A. Kalanithi¹, Heidi Grantz¹, Michael L. Schwartz², Clifford Saper³, James F. Leckman¹, and Flora M. Vaccarino^{1,2}

¹ Child Study Center, Yale University, New Haven, CT 06520

² Department of Neurobiology, Yale University, New Haven, CT 06520

³ Department of Neurology & Neuroscience, Harvard Medical School, Boston, MA

Abstract

Cortico-basal ganglia neuronal ensembles bring automatic motor skills into voluntary control and integrate them into ongoing motor behavior. A 5% decrease in caudate (Cd) nucleus volume is the most consistent structural finding in the brain of patients with Tourette syndrome (TS), but the cellular abnormalities that underlie this decrease in volume are unclear. In this paper, the density of different types of interneurons and medium spiny neurons (MSNs) in the striatum was assessed in the postmortem brains of 5 TS subjects as compared with normal controls (NC) by unbiased stereological analyses. TS patients demonstrated a 50–60% decrease of both parvalbumin (PV)+ and choline acetyltransferase (ChAT)+ cholinergic interneurons in the Cd and the putamen (Pt). Cholinergic interneurons were decreased in TS patients in the associative and sensorimotor regions but not in the limbic regions of the striatum, such that the normal gradient in density of cholinergic cells (highest in associative regions, intermediate in sensorimotor and lowest in limbic regions) was abolished. No significant difference was present in the densities of medium-sized calretinin (CR)+ interneurons, MSNs and total neurons. The selective deficit of PV+ and cholinergic striatal interneurons in TS subjects may result in an impaired cortico/thalamic control of striatal neuron firing in TS.

Keywords

Tourette syndrome; acetylcholine; parvalbumin; striatum; postmortem

Introduction

Tourette syndrome (TS) is a childhood-onset neuropsychiatric illness characterized by motor and vocal tics and a high incidence of co-morbid obsessive-compulsive disorder (OCD) and attention-deficit hyperactivity disorder (ADHD) (Graybiel and Canales, 2001; Leckman, 2002). Patients with TS also show deficits in procedural learning (Marsh et al., 2004). Insights from human lesions and degenerative disorders as well as animal models suggest that the basal ganglia are key for the initiation and coordination of sequential motor actions and may play a larger role in procedural learning (Albin et al., 1995; Graybiel et al., 1994). The cerebral cortex projects to the striatum, which comprises the caudate (Cd) and the putamen (Pt). Striatal medium spiny neurons (MSNs) send inhibitory connections, directly or indirectly through the external segment of the globus pallidus (GPe), to the output nuclei of the basal ganglia, which

are the internal segment of the globus pallidus (GPi) and the substantia nigra reticulata (SNr). In turn, the GPi and SNr project inhibitory fibers to motor and intralaminar nuclei of the thalamus, which send fibers back to the striatum and to the cortex. This ensemble forms the cortico-striatal-thalamo-cortical (CSTC) circuitry. This basal ganglia network has been suggested to be crucial for the integration of automatic sequences into goal-directed behavior (Graybiel and Canales, 2001).

A large-scale structural imaging study (Peterson et al., 2003) found a small (5%) but significant decrease in Cd volume in both children and adults with TS, suggesting that this phenotype may represent a useful biomarker for this syndrome. Striatal MSNs projecting to the GP and SNr represent the vast majority of neuronal elements in this region. The MSNs receive cortical inputs; dopaminergic (DA) inputs from the midbrain, and modulatory inputs from four distinct classes of local circuit interneurons, namely, parvalbumin (PV), calretinin (CR), somatostatin/nitric oxide synthase/neuropeptide Y (SOM/NOS/NPY) and cholinergic interneurons. The PV, CR, SOM neurons represent inhibitory GABAergic cells that are involved in various forms of feed-forward inhibition within the striatum (Gurney et al., 2004). The fast-spiking PV+ interneurons are interconnected by gap junctions, forming a widespread, non-habituating inhibitory network (Kawaguchi, 1993; Kita et al., 1990). The CR+ medium-sized aspiny interneurons (10–20 μ m) are the most abundant interneurons in the primate striatum. CR is also expressed by cholinergic interneurons (Cicchetti et al., 1998), which can be distinguished by their large size (24–42 μ m) and by their expression of the acetylcholine synthetic enzyme choline acetyltransferase (ChAT) (DiFiglia, 1987).

A possible biological reason for the reduction in Cd volume in TS is a deficit in DA innervation of the striatum, as abnormal striatal DA function has been reported in TS patients (Albin et al., 2003; Singer et al., 2002). Another possibility is a deficiency in striatal interneurons or in a subset of MSNs. In a recent unbiased stereological study using postmortem basal ganglia tissue from individuals with TS and normal controls (NC), we reported a deficiency in PV+ interneurons in the Cd of three patients with severe TS (Kalanithi et al., 2005). To investigate possible alterations in other striatal cell populations, we undertook a follow-up study in which we assessed the regional density of PV, CR, ChAT-containing interneurons as well as MSNs in five TS patients and five NC. The results suggest that cholinergic, as well as PV+ interneurons, are markedly reduced in the striatum of severely affected TS individuals in a region-specific manner.

Methods

Subjects

The control subjects were collected after routine autopsy at Yale University, Harvard University and Massachusetts General Hospital (Table 1). For TS brains, informed consent was obtained from the next of kin, and donated brain tissue was collected under the sponsorship of the Tourette Syndrome Association (TSA). All diagnoses were made by use of the best-estimate approach according to our standard protocol (Leckman et al., 1982). The TS Diagnostic Confidence Index (DCI) was also estimated (Robertson et al., 1999). All TS subjects had a definitive Diagnostic and Statistical Manual-IV diagnosis of Tourette disorder with a DCI score above 45 and a history of severe tic symptom rated at 36–50 out of 50 points on the Yale Global Tic Severity Scale at the “worst ever” point in their lives (Leckman et al., 1989) (Table 1). These TS subjects were selected from a larger group of donated specimens. Exclusionary criteria included the presence of a neurological condition, e.g., Alzheimer's disease, brain tumors, gross pathological changes indicative of traumatic or ischemic events, problematic agonal events (such as a prolonged interval on a respirator before death), an excessive post mortem interval; an inability to locate the next-of-kin; or the presence of a severe comorbid psychiatric disorder, e.g., schizophrenia, bipolar disorder; or insufficient or

improperly processed tissue. A total of five male subjects with TS (mean age $\pm$ SEM, 43.0 ± 3.6) and five sex-matched NC subjects (61.8 ± 4.3) were used in this study (Table 1). Although there was a statistical difference in the ages between the TS and NC ($p = 0.032$, Mann Whitney U test) age was not statistically significant when introduced as a covariate (see Results). The postmortem interval (PMI) did not differ significantly between the groups (NC = 14.1 ± 2.7 ; TS = 22.3 ± 4.2 ; $p = 0.111$, Mann Whitney U test). One of the five TS subjects (case 4454) was not taking antipsychotic medications at the time of death. The other four subjects were on a variety of medications (Table 1).

Immunocytochemistry

Antibody characterization—See Table 2 for a detailed description of the antibodies used in this paper. For each antibody, we omitted the primary antibody in our immunostaining procedures as a control, which resulted in no staining.

The monoclonal anti-PV (mouse IgG1 isotype) antibody was derived from the PARV-19 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized mouse. It recognizes a single band of 12-kDa apparent M.W. on Western blot analysis of extracts from rabbit leg skeletal muscle (data provided by Sigma-Aldrich Co.). It stains a pattern of neurons that is identical with previous studies in the human brain (Bernácer et al., 2008). The specificity of this antibody for the antigen has been determined by preadsorption with the appropriate purified protein as described by others (Hackney et al., 2005), indicating that after preadsorption there was no staining.

The rabbit polyclonal anti-CR detects a single protein band of the appropriate molecular weight on Western blots of monkey, rat, chicken, and fish brain extracts (Schwaller et al., 1993). It stains a pattern of neurons that is identical with previous studies in human brain (Holt et al., 1999).

The goat polyclonal anti-ChAT antibody stains a single band of 68-70 kDa apparent M.W. on Western blot analysis of mouse brain lysate (manufacturer's technical information). The specificity of this antibody for the antigen has been determined by preadsorption with the appropriate purified protein as described by others (Rico and Cavada, 1998), indicating that after preadsorption there was no staining. The staining pattern was identical with previous studies in the human brain (Bernácer et al., 2007).

The polyclonal anti- *dopamine- and cAMP-regulated phosphoprotein* with an apparent Mr of 32,000 (DARPP-32) detects a single band of 32 kDa apparent M.W. on Western blot analysis of extracts from rat brain cortex (manufacturer's technical information). The specificity of this antibody for the antigen has been determined by preadsorption with the synthetic peptide used to generate the antibody (CVEMIRRRRPTPAML), as described by others (Partida et al., 2004), indicating that after preadsorption there was no staining. The staining that we obtained with this antibody was identical to that described previously in the rat striatum (Yang et al., 2008).

Staining procedures

Intact half brains were stored in 10% formalin. The telencephalon and brainstem were cut coronally into 2.5-cm blocks. Blocks were rinsed in phosphate-buffered saline/0.1% NaN₃ (PBS/azide, pH 7.3) and cryoprotected in 15% sucrose. Frozen tissue was serially sectioned at 50 μ m.

Typically one series of sections per block was reacted free-floating with each antibody. The distance between sections was 1.2 mm for cresyl violet, PV and CR, 2.4 mm for ChAT and 3.6 mm for DARPP-32. Sections were rinsed 3 times in PBST (0.1% Tween-20 in PBS), and

endogenous peroxidase was quenched by incubation in 3% H₂O₂ for 30 min at room temperature. Sections were blocked in PBS containing 5% normal goat serum (normal horse serum for ChAT staining) and 0.1% Triton-X-100 (PBS-serum) for 30 min at room temperature and then incubated with the primary antibody for 24 h (72 h for DARPP-32 staining) at 4°C in PBS-serum. After 4 washes in PBST, sections were incubated with biotinylated secondary antibodies of the appropriate species (Vector; 1:1000) followed by the ABC complex according to manufacturer's instructions (Vectastain Elite kit, Vector). The bound peroxidase was revealed by incubating the sections with a solution containing 3,3'-diaminobenzidine (DAB) and H₂O₂ (DAB detection kit, Vector).

Image capture and analysis

Tissue sections were photographed by using a Zeiss Axioskop microscope fitted with a Zeiss AxioCam digital camera. Images were captured by using AxioVision AC software (Zeiss) and assembled in Adobe Photoshop. Some images were modified to adjust contrast and/or brightness. For Supplementary Material 1, a series of z-stack images were collected on a Zeiss ApoTome system and assembled in Adobe Photoshop.

Stereological Analyses

Unbiased stereological analysis was performed using a Zeiss Axioskop equipped with an automatic stage and coupled to a computer running StereoInvestigator software (MicroBrightField Inc., Colchester, VT). Researchers were unaware of the disease state of the tissue during counting. The Cd and Pt regions were drawn in each section in the series based on cytoarchitectonic landmarks (Figure 1). Analyses of the associative, sensorimotor and limbic functional territories were based upon previously published studies (Bernácer et al., 2007; Morel et al., 2002; Parent and Hazrati, 1995). Analyzed regions were defined as follows (Figure 8A): Associative region: the Cd (exclusive of a very small dorso-lateral portion) and the ventral portion of the Pt rostral to the anterior commissure; sensorimotor region: the dorso-lateral portion of the Cd and the dorsal half of the Pt anterior to the anterior commissure and the entire Pt posterior to the anterior commissure; limbic region: the nucleus accumbens (NA) and adjacent ventral portion of the Cd and Pt, as well as a ventral small portion of the Pt posteriorly to the GPe.

Regional volumes were estimated by planimetry, computed by adding the cross-sectional areas of the nucleus of interest in each section and multiplying this number by the section interval and by the measured section thickness. Nuclear profiles of stained cells were counted using the optical fractionator probe. Sampling grids were randomly superimposed over the sections using StereoInvestigator. Tri-dimensional sampling boxes with 3 out of 6 exclusion borders (Gundersen et al., 1988; West, 1993) were automatically placed by StereoInvestigator at each grid intersection point. The total number of cells was calculated by the formula:

$$N = \Sigma Q * t/h * 1/asf * 1/ssf$$

where ΣQ is the total number of nuclei counted, t the mean section thickness, h the height of the optical disector, asf is the area sampling fraction, and ssf is the section sampling fraction (West, 1993). The density for each cell type was calculated by dividing the total number of cells by the total volume sampled. Sampling grids and magnifications were adjusted for each staining in order to obtain a relatively constant number of cells sampled and a coefficient of error (CE Gundersen) of ≤ 0.2 . For PV sections, the sampling grid measured $2500 \times 2500 \mu\text{m}$, and the counting frame measured $700 \times 500 \times 15 \mu\text{m}$ in the X, Y and Z axes, respectively (objective: 10x) as previously described (Kalanithi et al., 2005). For CR sections the sampling grid measured $2500 \times 2500 \mu\text{m}$, and the counting frame measured $500 \times 500 \times 15 \mu\text{m}$ (objective:

10x). For ChAT-stained sections, the sampling grid measured $2500 \times 2500 \mu\text{m}$, and the counting frame measured $1000 \times 700 \times 15 \mu\text{m}$ (objective: 10x). For cresyl violet-stained sections, the sampling grid measured $3300 \times 3300 \mu\text{m}$, and the counting frame measured $130 \times 130 \times 15 \mu\text{m}$ (objective: 40x oil-immersion). Finally, for DARPP-32-stained sections, the sampling grid measured $3300 \times 3300 \times 15 \mu\text{m}$, and the counting frame measured $130 \times 130 \times 15 \mu\text{m}$ (objective: 40x oil-immersion). Counting frames measured $15 \mu\text{m}$ in the Z dimension and were placed $1 \mu\text{m}$ from the surface, to avoid variability caused by differential penetration of antibodies. In Supplementary Material 1, we present an analysis of CR staining in the Z plane, demonstrating full penetration of antibody within and beyond $15 \mu\text{m}$ from the surface. Stereological analyses for PV+ neurons was carried out in parallel by two researchers for three out of five TS brains and three out of five of the NC brains. Comparison of these counts yielded an inter-person variability of $<3\%$.

Statistical Analyses

All statistical analyses were performed using SPSS 16.0.1 for Macintosh (Chicago, Illinois). Repeated measures analyses of variance (ANOVA) were carried out for multiple comparisons. Given the small sample size, we confirmed all results with Mann Whitney U tests.

Results

The density of the three major striatal interneurons types (PV+, CR+ and ChAT+ interneurons), of DARPP-32+ medium spiny neurons (MSNs) and of cresyl violet-stained total neurons was estimated by stereological methods in the striatum (Cd and Pt) of NC and TS individuals. Using the anterior edge of the GPe as a landmark, density estimates were separately obtained for anterior and posterior portions of the Cd and Pt (denominated, respectively, Cd head and Pt rostral and Cd body and Pt caudal), excluding the NA (see Figure 1). Most of the anterior striatum defined in this way corresponds to the associative region, whereas the posterior mostly corresponds to the sensorimotor region (for a more precise delimitation of these functional regions, see below). Consistent with our previous data that included three of the five subjects used in the present study (Kalanithi et al., 2005), we found a 55.7% decrease in the density of PV-immunoreactive cells in the striatum of TS individuals (Figure 2 A, B; Figure 3; Table 3). The density of PV+ cells in the NC and TS striatum was, respectively, 331.4 ± 32.7 and 137.8 ± 32.7 cells/ mm^3 (mean $\pm$ SEM) and the difference between NC and TS was highly significant ($F(1, 8) = 14.085, p = 0.006$, ANOVA). There was no interaction between diagnosis (NC, TS) and area (Cd and Pt) or subarea (anterior and posterior), indicating that the decrease in PV+ interneurons in TS was equally significant in all regions of the striatum. This was confirmed with a non-parametric test, the Mann-Whitney U Test ($p < 0.0005$).

To determine if additional populations of neurons other than PV+ neurons are also altered in TS brains, we examined other classes of interneurons. The most abundant population of interneurons in the striatum is a class of medium sized aspiny interneurons ($10\text{--}20 \mu\text{m}$) expressing CR (Figure 4 A-D, arrowheads). There was no difference in the number of medium-sized CR+ interneurons between NC and TS individuals (Figure 5 C; $F(1, 5) = 0.056, p = 0.822$, ANOVA). Cell size and morphology did not appear to differ between NC and TS patients (Figure 4 A-D). However, approximately 10% of all CR+ cells are a distinct population of neurons with a much larger soma size ($24\text{--}42 \mu\text{m}$) and a more complex, multipolar pattern of dendritic branching. These latter cells are morphologically identical to ChAT+ cells (Figure 4 C, D arrows). This is consistent with previous studies showing that about 80% of the large-sized CR+ neurons co-localize ChAT, the acetylcholine synthetic enzyme (Cicchetti et al., 1998). The density of large-size CR+ interneurons, as determined by stereological analyses, was 236.0 ± 14.5 and 111.1 ± 14.5 cells/ mm^3 (mean $\pm$ SEM) in the striatum of NC and TS individuals, respectively (Figure 4 A-D; Figure 5 A, B; Table 3). A statistically significant

52.9% decrease of large-size CR+ interneurons was present in TS ($F(1, 8) = 36.994, p = 0.001$, ANOVA) and was confirmed with the Mann-Whitney U Test ($p < 0.0005$). Although the average decrease in large-size CR+ neurons was larger in the anterior portion than in the posterior portion of the striatum (62.9% versus 41.5%, respectively), neither the area (Cd/Pt) nor the subarea (anterior/posterior) showed any statistically significant interaction with the diagnosis. Thus, as for PV+ neurons, the decrease in large-size CR+ interneurons in TS was equally significant in all regions of the striatum.

To confirm the deficit of cholinergic interneurons, we examined ChAT immunoreactive neurons in the striatum. The density (cells/mm³, mean $\pm$ SEM) of ChAT+ cells in the striatum of NC and TS individuals was 213.6 ± 22.9 and 108.1 ± 22.9 , respectively (Figure 6 A-D; Figure 7 A, B; Table 3). The data revealed a highly significant 49.4% decrease in ChAT+ neuron density in the TS striatum ($F(1, 8) = 10.641, p = 0.011$, ANOVA). Again, area (Cd/Pt) and subarea (anterior/posterior) did not interact with diagnosis. The overall difference between NC and TS was again confirmed with the Mann-Whitney U Test ($p < 0.0005$). Qualitatively, no obvious differences in the soma size of cholinergic neurons nor in the arborization of individual cells were detected. The overall level of ChAT+ immunoreactivity within the neuropil appeared to be decreased, likely due to the decreased cell number (Figure 6 A-D). Together with the previous results, these data suggest that there is a combined decrease in PV+ and cholinergic interneurons in the striatum of TS patients, as compared to NC.

To understand whether these changes extended to more ventral, limbic portions of the basal ganglia, we assessed neuron density in the NA. PV neurons were too sparse in this region to be reliably counted. There was no significant change in large-size CR+ interneurons in the NA (Table 3; $p = 0.86$, Mann Whitney U test). Similarly, no difference in ChAT+ neurons was detected between NC and TS individuals in this region (Table 3; $p = 0.114$, Mann Whitney U test).

To understand whether these changes were specific to PV+ and cholinergic neurons or were attributable to a more general loss of total neurons within the striatum, we stained adjacent serial sections with cresyl violet. Neurons were distinguished from glia based on somatic size and nuclear morphology. The density of cresyl violet stained neurons (cells/mm³, mean $\pm$ SEM) in the striatum of NC and TS individuals was $45,709 \pm 2,083$ and $38,352 \pm 1,980$, respectively, indicating no significant difference between NC and TS ($F(1, 8) = 4.357, p = 0.070$). Furthermore, the density of cresyl violet stained neurons in the Cd head, the area with the strongest differences with respect to the PV and cholinergic interneurons, was almost identical in TS and NC (Table 3; $p = 0.841$, Mann Whitney U test). However, the TS samples showed a trend to a decrease in cresyl violet stained neurons in the Cd body and in the Pt (Table 3, Supplementary Material 2). Although we cannot exclude that these trends may become significant with the addition of more brain samples to the study, the decrease in density of PV+ and cholinergic interneurons is clearly not attributable to a generalized neuronal loss, particularly in the Cd head.

To obtain an independent confirmation of the specificity of changes in PV+ and cholinergic neuron density, we immunostained the sections for DARPP-32, a protein expressed by the majority of MSNs. The density of DARPP-32+ neurons in the head of the Cd was $21,843 \pm 3,038$ cells/mm³ and $17,337 \pm 1,075$ cells/mm³ in NC and TS brains, respectively ($p = 0.4$, Mann Whitney U test). Hence, TS patients demonstrated no significant change in MSNs in the head of the Cd. Collectively, the data suggest that cell losses were specific for PV and cholinergic neurons in the Cd head in this group of TS individuals.

To further evaluate physiological implications of these changes for cholinergic circuitry, we investigated the distribution of large-sized CR+ interneurons (i.e., cholinergic interneurons) in

the three main functional subdivisions of the striatum (receiving associative, sensorimotor and limbic afferents). For this analysis we used the StereoInvestigator program to accurately delineate in our serial tissue sections the associative, sensorimotor and limbic territories according to previous studies (Figure 8A) (Bernácer et al., 2007; Morel et al., 2002; Parent and Hazrati, 1995) and analyzed large-sized CR immunoreactive cell density in both control and TS brains. A statistical comparison of diagnosis versus regional effects by repeated measures ANOVA revealed significant main effects of diagnosis ($F(1, 8) = 25.852$; $p = 0.001$) and region ($F(2, 16) = 5.335$; $p = 0.017$) and a significant interaction of diagnosis versus region ($F(2, 16) = 5.631$; $p = 0.014$). Indeed, in NC there was a gradient in the density of cholinergic neurons, with highest values in the associative and lowest in the limbic regions (Figure 8B). Differences between associative and limbic and associative and sensorimotor cholinergic neuron densities were both statistically significant in NC (Sidak post-hoc test, $p = 0.016$ and $p = 0.031$, respectively); in contrast, no significant difference in cholinergic neuron density was present among subregions in the TS striatum. A comparison of TS with NC region by region by the Sidak post-hoc test indicated that the associative region showed the most pronounced decreases in cholinergic neuron density (Figure 8B; 60.0% decrease, $p < 0.0005$) followed by the sensorimotor region (Figure 8B; 44.7% decrease, $p = 0.008$) and that the limbic region was not significantly different between TS and NC individuals (Figure 8B; $p = 0.402$). The last finding is also in accordance with the lack of observable changes in cholinergic markers in the NA described above.

Since there was a statistically significant difference in age between NC and TS using the Mann-Whitney U Test, we re-ran the ANOVA using age as a covariate for each of the individual main variables. In no case the effect of age was statistically significant (PV, $p = 0.236$; CR-large, $p = 0.747$; ChAT, $p = 0.543$; CR-large in functional subdivisions, $p = 0.485$), suggesting that age does not account for the variance between NC and TS. To further understand whether there was a significant correlation between age and interneuron density, we then performed regression analyses. In both NC and TS, we found no significant correlation between age and the density of any cell type in the striatum (PV-NC, $p = 0.655$; PV-TS, $p = 0.703$; CR-large-NC, $p = 0.204$; CR-large-TS, $p = 0.593$; ChAT-NC, $p = 0.858$; ChAT-TS, $p = 0.29$).

Discussion

Our data indicate that cholinergic interneurons may be reduced in the striatum of patients with severe and persistent TS, as indicated by two cellular markers, CR and ChAT. The largest decrease was observed in the more rostral associative territory, an area comprising the head of Cd and the adjacent rostral Pt. In more posterior sensorimotor portions of the Cd and Pt, differences in cholinergic cells were smaller and more variable, but still statistically significant. In contrast, the limbic territory, which includes the NA and adjacent anterior/ventral striatum, as defined by its connections with allocortical areas and the medial prefrontal cortex (PFC) and its continuity with the NA and shell of the amygdala (Haber and McFarland, 1999; Heimer, 2000; Holt et al., 1997), failed to show significant changes. We cannot exclude that with the analysis of more brain samples, a statistically significant difference in cholinergic cells in the NA might emerge. Nevertheless, one of the most remarkable and robust findings of the current study is that the gradient in cholinergic neuron density found in normal subjects (highest in associative regions, intermediate in sensorimotor and lowest in limbic regions) is completely abolished in TS. These data strongly implicate associative and sensorimotor regions of the basal ganglia in the pathophysiology of TS or in long-term adaptive compensatory changes to this disorder.

We also report a strong deficit in PV+ interneurons in the Cd and Pt of TS patients, which confirms and extends previous data (Kalanithi et al., 2005). The average decrease in PV+ neuron number was strongest in the rostral Pt, although no statistically significant interaction

between diagnosis and regions was found, suggesting that PV+ cells are equally depleted in the whole striatum. We cannot exclude that the addition of more patients may reveal a statistically significant difference between subregions. PV+ cell bodies and fibers are enriched in the sensorimotor territory, extend into the more rostral associative territory, but are very sparse in the anterior/ventral limbic portions of the basal ganglia (Morel et al., 2002). Indeed, PV+ cells were too sparse in the NA to reliably assess their differences.

In contrast to PV and cholinergic interneurons, CR+ medium-sized interneurons, which represent the largest population of interneurons within the striatum, were not affected in this group of TS individuals. A count of total neuron densities in the striatum also revealed no statistically significant difference between TS and NC. PV and cholinergic neurons each represents less than 1% of the total neuronal population, and thus losses in these cells are not expected to have a significant impact on total neuron number. Together, the data suggest that there is a selective decrease in PV+ and cholinergic interneurons, rather than a generalized decrease in total neuron number in the striatum of TS individuals.

The main limitation of this study is the small sample size, and consequently the limited power to detect additional differences between the control and the patient groups and to control for sample variables. One of these variables is age, as TS are significantly younger in our sample (possibly because of frequent accidental deaths in this group). However, detailed statistical analyses suggested that age could not account for the significant differences between patients and controls.

Another variable is that most TS patients have been treated with dopamine D2 receptor blocking drugs. The possibility that the cellular changes may be the consequence of this treatment cannot be completely ruled out at present, nevertheless, it must be noted that long-term neuroleptic treatment in rats (4-12 months) did not decrease striatal levels of the GABA-synthesizing enzymes, neuropeptides and calcium binding proteins, including PV or CR, despite clear behavioral evidence of a movement disorder (Jolkkonen et al., 1994; Mithani et al., 1987).

Inconsistent results have been reported in the literature regarding the effect of chronic antipsychotic treatment on the cholinergic system. While some studies have reported that short-term neuroleptic treatment in rodents (40-90 days) leads to decreases in ChAT activity and ChAT-immunostained neurons in several brain regions (Mahadik et al., 1988; Terry et al., 2003), long-term treatment (6-12 months) produces more variable results, including increases, decreases, or no significant effect on ChAT activity, ChAT-immunoreactive neurons, acetylcholine levels and other indices of cholinergic function (Grimm et al., 2001; Lohr et al., 2000; Mithani et al., 1987; Murugaiah et al., 1982; Rupniak et al., 1986; Terry et al., 2007). These discrepancies may be explained by region-specific effects (the decrease in ChAT+ cells being more prominent in ventral striatum and NA) (Grimm et al., 2001), length of administration and types of drugs used (typical neuroleptics being more likely to cause changes in cholinergic function than the atypical risperidone and clozapine) (Friedman et al., 1983; Terry et al., 2003). Recent studies have reported that the density of cholinergic neurons in the limbic striatum is decreased in patients with schizophrenia (Holt et al., 1999) and that the lowest density of ChAT+ neurons was found in two schizophrenic individuals that had not been treated with neuroleptics. This is in agreement with pharmacological data suggesting that dopamine D2 receptor antagonists increase cholinergic neuron activity, choline utilization and acetylcholine levels in the striatum (Pedata et al., 1980; Rupniak et al., 1986). In aggregate, the above findings are not consistent with the idea that the decrease in ChAT+ cells observed in the present study is attributable to neuroleptic treatment.

Chronic exposure of macaque monkeys to typical and atypical antipsychotic drugs has been shown to be associated with decreased brain volume (Dorph-Petersen et al., 2005), which may

be caused by a reduction in number of glial cells (Konopaske et al., 2008). However, our changes in density values cannot be explained by such an effect, as this would have produced a generalized increase in density across all neuron types examined. Thus, changes in volumes due to chronic neuroleptic treatment are unlikely to be the explanation for the selective changes in PV+ and ChAT+ neuron density that we have found in TS individuals.

A similar decrease in cholinergic neuron markers has been described in the Cd of schizophrenic patients, albeit the NA was also affected in this population (Holt et al., 2005; Holt et al., 1999). As the Cd nucleus receives direct inputs from the dorsolateral PFC, the data are in line with the PFC hypometabolism and functional deficit in this disorder. Disruptions in the PFC-Cd circuitry may also be pivotal for long-term outcome in TS, since a favorable future outcome in children with TS is associated with increased Cd size (Bloch et al., 2005) and tic suppression is concomitant with activation of the PFC (Peterson et al., 1998). Thus, the cholinergic neuron alterations described here may be a manifestation of hypofunctional frontostriatal circuitry, a characteristic in common between severe TS and schizophrenia (Yoon et al., 2007).

The electrically coupled PV interneuron network is responsible for downregulating electrical activity in the striatum (Kawaguchi, 1993; Kita et al., 1990). The PV neurons receive a tonic input from the spontaneously active cholinergic interneurons (Koos and Tepper, 2002). Thus the cholinergic neurons, through their connection to the PV neurons, can exert widespread inhibitory influence over MSNs (Aosaki et al., 1994; Graybiel et al., 1994; Pakhotin and Bracci, 2007). The cholinergic neurons receive excitatory projections from the thalamus, and particularly the ventralis anterior (VA)/ventralis lateralis (VL) and centromedian-parafascicular nuclear complex (CM-Pf) (McFarland and Haber, 2000; McFarland and Haber, 2001). These thalamic nuclei in turn receive inhibitory projections from GPi neurons, a region exhibiting high-frequency tonic and oscillatory activity (Difiglia and Rafols, 1988). Collectively, these observations suggest that self-reinforcing, feedback inhibitory circuits involving the PFC and sensorimotor thalamus impinge on the ChAT-PV interneuron network in the striatum. The possible importance of this inhibitory network for tic-related motor activity is reinforced by neurosurgical experiments, in which patients with intractable TS found partial relief from their symptoms when thalamic nuclei of the CM-Pf complex were implanted with deep brain stimulation electrodes (Bajwa et al., 2007; Servello et al., 2008; Temel and Visser-Vandewalle, 2004). In TS, the combined loss or dysfunction of the cholinergic and PV+ cells in the associative and sensorimotor striatum may severely impair the ability of the PFC and thalamus to carry out the inhibitory modulation of MSNs, which may result in tics and deficits in procedural learning. The finding that deficits in procedural learning in both children and adults with TS are correlated with tic severity (Marsh et al., 2004) argues that a dysfunctional PV/cholinergic neuron network may well be a core feature of TS anatomy and not an epiphenomenon.

GABAergic and cholinergic neurotransmission powerfully modulate the activity of oscillating networks. The firing of striatal PV+ neurons precedes that of the other cell types in the circuit, suggesting that these cells pace the oscillations (Berke et al., 2004). Consequently, a dysfunction in the PV-cholinergic circuit may lead these networks to become dysrhythmic, producing a loss of control of sensory information and motor action (Leckman et al., 2006; Llinás et al., 2005; Llinás et al., 1999). This work suggests the intriguing notion that a dysfunction of PV-cholinergic neurons in the associative and sensorimotor regions of the basal ganglia may underlie the emergence of tics and other forms of disinhibited behavior characterizing TS symptomatology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grants R01NS054994 (F.M.V.), K05MH076273 (J.F.L.) and the Tourette Syndrome Association.

Other Acknowledgments

We wish to thank Dr. Daniela Brunner, PsychoGenics, for help with the statistical analyses. We thank Dr. Francine Benes and the personnel of the Harvard Brain Tissue Resource Center for help in tissue preservation and storage, and Drs. Brian Ciliax, Neal R. Swerdlow, as well as Sue Levi-Pearl and other members of the Tourette Syndrome Association Scientific Advisory Board and Tissue Committee for organizing tissue collection and providing essential suggestions and encouragements.

Abbreviations

TS	Tourette syndrome
CSTC	cortico- striatal-thalamo-cortical circuitry
Cd	caudate
Pt	putamen
GP	globus pallidus
GPI	GP pars interna
GPe	GP pars externa
NA	nucleus accumbens
SNr	substantia nigra reticulata
BG	basal ganglia
MSN	medium spiny neuron
PV	parvalbumin
NC	normal control
CR	calretinin
ChAT	choline acetyltransferase
DARPP-32	<i>dopamine- and cAMP regulated phosphoprotein</i> with an apparent Mr of 32,000
PFC	prefrontal cortex
VA	ventralis anterior
VL	ventralis lateralis
CM-Pf	centromedian-parafascicular nuclear complex

Literature cited

- Albin RL, Koeppe RA, Bohnen NI, Nichols TE, Meyer P, Wernette K, Minoshima S, Kilbourn MR, Frey KA. Increased ventral striatal monoaminergic innervation in Tourette syndrome. *Neurology* 2003;61(3):310–315. [PubMed: 12913189]
- Albin RL, Young AB, Penney JB. The functional anatomy of disorders of the basal ganglia. *Trends Neurosci* 1995;18(2):63–64. [PubMed: 7537410]
- Aosaki T, Tsubokawa H, Ishida A, Watanabe K, Graybiel AM, Kimura M. Responses of tonically active neurons in the primate's striatum undergo systematic changes during behavioral sensorimotor conditioning. *J Neurosci* 1994;14(6):3969–3984. [PubMed: 8207500]

- Bajwa RJ, de Lotbiniere AJ, King RA, Jabbari B, Quatrano S, Kunze K, Scahill L, Leckman JF. Deep brain stimulation in Tourette's syndrome. *Mov Disord* 2007;22(9):1346–1350. [PubMed: 17580320]
- Berke JD, Okatan M, Skurski J, Eichenbaum HB. Oscillatory entrainment of striatal neurons in freely moving rats. *Neuron* 2004;43(6):883–896. [PubMed: 15363398]
- Bernácer J, Prensa L, Gimenez-Amaya JM. Cholinergic interneurons are differentially distributed in the human striatum. *PLoS ONE* 2007;2(11):e1174. [PubMed: 18080007]
- Bernácer J, Prensa L, Gimenez-Amaya JM. Chemical architecture of the posterior striatum in the human brain. *J Neural Transm* 2008;115(1):67–75. [PubMed: 17726572]
- Bloch MH, Leckman JF, Zhu H, Peterson BS. Caudate volumes in childhood predict symptom severity in adults with Tourette syndrome. *Neurology* 2005;65(8):1253–1258. [PubMed: 16247053]
- Cicchetti F, Beach TG, Parent A. Chemical phenotype of calretinin interneurons in the human striatum. *Synapse* 1998;30(3):284–297. [PubMed: 9776132]
- DiFiglia M. Synaptic organization of cholinergic neurons in the monkey neostriatum. *J Comp Neurol* 1987;255(2):245–258. [PubMed: 3819015]
- Difiglia M, Rafols JA. Synaptic organization of the globus pallidus. *J Electron Microscop Tech* 1988;10(3):247–263. [PubMed: 2906997]
- Dorph-Petersen KA, Pierri JN, Perel JM, Sun Z, Sampson AR, Lewis DA. The influence of chronic exposure to antipsychotic medications on brain size before and after tissue fixation: a comparison of haloperidol and olanzapine in macaque monkeys. *Neuropsychopharmacology* 2005;30(9):1649–1661. [PubMed: 15756305]
- Friedman E, Gianutsos G, Kuster J. Chronic fluphenazine and clozapine elicit opposite changes in brain muscarinic receptor binding: implications for understanding tardive dyskinesia. *J Pharmacol Exp Ther* 1983;226(1):7–12. [PubMed: 6864552]
- Graybiel AM, Aosaki T, Flaherty AW, Kimura M. The basal ganglia and adaptive motor control. *Science* 1994;265(5180):1826–1831. [PubMed: 8091209]
- Graybiel AM, Canales JJ. The neurobiology of repetitive behaviors: clues to the neurobiology of Tourette syndrome. *Adv Neurol* 2001;85:123–131. [PubMed: 11530422]
- Grimm JW, Chapman MA, Zahm DS, See RE. Decreased choline acetyltransferase immunoreactivity in discrete striatal subregions following chronic haloperidol in rats. *Synapse* 2001;39(1):51–57. [PubMed: 11071709]
- Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B, et al. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 1988;96(10):857–881. [PubMed: 3056461]
- Gurney K, Prescott TJ, Wickens JR, Redgrave P. Computational models of the basal ganglia: from robots to membranes. *Trends Neurosci* 2004;27(8):453–459. [PubMed: 15271492]
- Haber SN, McFarland NR. The concept of the ventral striatum in nonhuman primates. *Ann N Y Acad Sci* 1999;877:33–48. [PubMed: 10415641]
- Hackney CM, Mahendrasingam S, Penn A, Fettiplace R. The concentrations of calcium buffering proteins in mammalian cochlear hair cells. *J Neurosci* 2005;25(34):7867–7875. [PubMed: 16120789]
- Heimer L. Basal forebrain in the context of schizophrenia. *Brain Res Brain Res Rev* 2000;31(23):205–235. [PubMed: 10719150]
- Holt DJ, Bachus SE, Hyde TM, Wittie M, Herman MM, Vangel M, Saper CB, Kleinman JE. Reduced density of cholinergic interneurons in the ventral striatum in schizophrenia: an in situ hybridization study. *Biol Psychiatry* 2005;58(5):408–416. [PubMed: 16023618]
- Holt DJ, Graybiel AM, Saper CB. Neurochemical architecture of the human striatum. *J Comp Neurol* 1997;384(1):1–25. [PubMed: 9214537]
- Holt DJ, Herman MM, Hyde TM, Kleinman JE, Sinton CM, German DC, Hersch LB, Graybiel AM, Saper CB. Evidence for a deficit in cholinergic interneurons in the striatum in schizophrenia. *Neuroscience* 1999;94(1):21–31. [PubMed: 10613493]
- Jolkkonen J, Jenner P, Marsden CD. GABAergic modulation of striatal peptide expression in rats and the alterations induced by dopamine antagonist treatment. *Neurosci Lett* 1994;180(2):273–276. [PubMed: 7535410]

- Kalanithi PS, Zheng W, Kataoka Y, DiFiglia M, Grantz H, Saper CB, Schwartz ML, Leckman JF, Vaccarino FM. Altered parvalbumin-positive neuron distribution in basal ganglia of individuals with Tourette syndrome. *Proc Natl Acad Sci U S A* 2005;102(37):13307–13312. [PubMed: 16131542]
- Kawaguchi Y. Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum. *J Neurosci* 1993;13(11):4908–4923. [PubMed: 7693897]
- Kita H, Kosaka T, Heizmann CW. Parvalbumin-immunoreactive neurons in the rat neostriatum: a light and electron microscopic study. *Brain Res* 1990;536(12):1–15. [PubMed: 2085740]
- Konopaske GT, Dorph-Petersen KA, Sweet RA, Pierri JN, Zhang W, Sampson AR, Lewis DA. Effect of chronic antipsychotic exposure on astrocyte and oligodendrocyte numbers in macaque monkeys. *Biol Psychiatry* 2008;63(8):759–765. [PubMed: 17945195]
- Koos T, Tepper JM. Dual cholinergic control of fast-spiking interneurons in the neostriatum. *J Neurosci* 2002;22(2):529–535. [PubMed: 11784799]
- Leckman JF. Tourette's syndrome. *Lancet* 2002;360(9345):1577–1586. [PubMed: 12443611]
- Leckman JF, Riddle MA, Hardin MT, Ort SI, Swartz KL, Stevenson J, Cohen DJ. The Yale Global Tic Severity Scale: initial testing of a clinician-rated scale of tic severity. *J Am Acad Child Adolesc Psychiatry* 1989;28(4):566–573. [PubMed: 2768151]
- Leckman JF, Sholomskas D, Thompson WD, Belanger A, Weissman MM. Best estimate of lifetime psychiatric diagnosis: a methodological study. *Arch Gen Psychiatry* 1982;39(8):879–883. [PubMed: 7103676]
- Leckman JF, Vaccarino FM, Kalanithi PS, Rothenberger A. Annotation: Tourette syndrome: a relentless drumbeat--driven by misguided brain oscillations. *J Child Psychol Psychiatry* 2006;47(6):537–550. [PubMed: 16712630]
- Llinás R, Urbano FJ, Leznik E, Ramirez RR, van Marle HJ. Rhythmic and dysrhythmic thalamocortical dynamics: GABA systems and the edge effect. *Trends Neurosci* 2005;28(6):325–333. [PubMed: 15927689]
- Llinás RR, Ribary U, Jeanmonod D, Kronberg E, Mitra PP. Thalamocortical dysrhythmia: A neurological and neuropsychiatric syndrome characterized by magnetoencephalography. *Proc Natl Acad Sci U S A* 1996;93(26):15222–15227.
- Lohr JB, Caligiuri MP, Manley MS, Browning JA. Neuroleptic-induced striatal damage in rats: a study of antioxidant treatment using accelerometric and immunocytochemical methods. *Psychopharmacology (Berl)* 2000;148(2):171–179. [PubMed: 10663432]
- Mahadik SP, Laev H, Korenovsky A, Karpiak SE. Haloperidol alters rat CNS cholinergic system: enzymatic and morphological analyses. *Biol Psychiatry* 1988;24(2):199–217. [PubMed: 3291956]
- Marsh R, Alexander GM, Packard MG, Zhu H, Wingard JC, Quackenbush G, Peterson BS. Habit learning in Tourette syndrome: a translational neuroscience approach to a developmental psychopathology. *Arch Gen Psychiatry* 2004;61(12):1259–1268. [PubMed: 15583117]
- McFarland NR, Haber SN. Convergent inputs from thalamic motor nuclei and frontal cortical areas to the dorsal striatum in the primate. *J Neurosci* 2000;20(10):3798–3813. [PubMed: 10804220]
- McFarland NR, Haber SN. Organization of thalamostriatal terminals from the ventral motor nuclei in the macaque. *J Comp Neurol* 2001;429(2):321–336. [PubMed: 11116223]
- Mithani S, Atmadja S, Baimbridge KG, Fibiger HC. Neuroleptic-induced oral dyskinesias: effects of progabide and lack of correlation with regional changes in glutamic acid decarboxylase and choline acetyltransferase activities. *Psychopharmacology (Berl)* 1987;93(1):94–100. [PubMed: 2888156]
- Morel A, Loup F, Magnin M, Jeanmonod D. Neurochemical organization of the human basal ganglia: anatomofunctional territories defined by the distributions of calcium-binding proteins and SMI-32. *J Comp Neurol* 2002;443(1):86–103. [PubMed: 11793349]
- Murugaiah K, Mann S, Theodorou AE, Jenner P, Marsden CD. Increased striatal acetylcholine after 14 months cis-flupenthixol treatment in rats suggests functional supersensitivity of dopamine receptors. *Life Sci* 1982;31(2):181–188. [PubMed: 6889662]
- Pakhotin P, Bracci E. Cholinergic interneurons control the excitatory input to the striatum. *J Neurosci* 2007;27(2):391–400. [PubMed: 17215400]
- Parent A, Hazrati LN. Functional anatomy of the basal ganglia. I. The cortico-basal ganglia-thalamo-cortical loop. *Brain Res Brain Res Rev* 1995;20(1):91–127. [PubMed: 7711769]

- Partida GJ, Lee SC, Haft-Candell L, Nichols GS, Ishida AT. DARPP-32-like immunoreactivity in AII amacrine cells of rat retina. *J Comp Neurol* 2004;480(3):251–263. [PubMed: 15515184]
- Pedata F, Sorbi S, Pepeu G. Choline high-affinity uptake and metabolism and choline acetyltransferase activity in the striatum of rats chronically treated with neuroleptics. *J Neurochem* 1980;35(3):606–611. [PubMed: 6108997]
- Peterson BS, Skudlarski P, Anderson AW, Zhang H, Gatenby JC, Lacadie CM, Leckman JF, Gore JC. A functional magnetic resonance imaging study of tic suppression in Tourette syndrome. *Arch Gen Psychiatry* 1998;55(4):326–333. [PubMed: 9554428]
- Peterson BS, Thomas P, Kane MJ, Scahill L, Zhang H, Bronen R, King RA, Leckman JF, Staib L. Basal Ganglia volumes in patients with Gilles de la Tourette syndrome. *Arch Gen Psychiatry* 2003;60(4):415–424. [PubMed: 12695320]
- Rico B, Cavada C. A population of cholinergic neurons is present in the macaque monkey thalamus. *Eur J Neurosci* 1998;10(7):2346–2352. [PubMed: 9749763]
- Robertson MM, Banerjee S, Kurlan R, Cohen DJ, Leckman JF, McMahon W, Pauls DL, Sandor P, van de Wetering BJ. The Tourette syndrome diagnostic confidence index: development and clinical associations. *Neurology* 1999;53(9):2108–2112. [PubMed: 10599790]
- Rupniak NM, Briggs RS, Petersen MM, Mann S, Reavill C, Jenner P, Marsden CD. Differential alterations in striatal acetylcholine function in rats during 12 months' continuous administration of haloperidol, sulpiride, or clozapine. *Clin Neuropharmacol* 1986;9(3):282–292. [PubMed: 3719573]
- Schwaller B, Buchwald P, Blumcke I, Celio MR, Hunziker W. Characterization of a polyclonal antiserum against the purified human recombinant calcium binding protein calretinin. *Cell Calcium* 1993;14(9):639–648. [PubMed: 8242719]
- Servello D, Porta M, Sassi M, Brambilla A, Robertson MM. Deep brain stimulation in 18 patients with severe Gilles de la Tourette syndrome refractory to treatment: the surgery and stimulation. *J Neurol Neurosurg Psychiatry* 2008;79(2):136–142. [PubMed: 17846115]
- Singer HS, Szymanski S, Giuliano J, Yokoi F, Dogan AS, Brasic JR, Zhou Y, Grace AA, Wong DF. Elevated intrasynaptic dopamine release in Tourette's syndrome measured by PET. *Am J Psychiatry* 2002;159(8):1329–1336. [PubMed: 12153825]
- Temel Y, Visser-Vandewalle V. Surgery in Tourette syndrome. *Mov Disord* 2004;19(1):3–14. [PubMed: 14743354]
- Terry AV Jr. Gearhart DA, Warner SE, Zhang G, Bartlett MG, Middlemore ML, Beck WD Jr. Mahadik SP, Waller JL. Oral haloperidol or risperidone treatment in rats: temporal effects on nerve growth factor receptors, cholinergic neurons, and memory performance. *Neuroscience* 2007;146(3):1316–1332. [PubMed: 17434684]
- Terry AV Jr. Hill WD, Parikh V, Waller JL, Evans DR, Mahadik SP. Differential effects of haloperidol, risperidone, and clozapine exposure on cholinergic markers and spatial learning performance in rats. *Neuropsychopharmacology* 2003;28(2):300–309. [PubMed: 12589383]
- West MJ. New stereological methods for counting neurons. *Neurobiol Aging* 1993;14(4):275–285. [PubMed: 8367009]
- Yang Z, You Y, Levison SW. Neonatal hypoxic/ischemic brain injury induces production of calretinin-expressing interneurons in the striatum. *J Comp Neurol* 2008;511(1):19–33. [PubMed: 18720478]
- Yoon DY, Gause CD, Leckman JF, Singer HS. Frontal dopaminergic abnormality in Tourette syndrome: a postmortem analysis. *J Neurol Sci* 2007;255(12):50–56. [PubMed: 17337006]

Figure 1.

Anatomical representation of striatal regions sampled by stereological analyses. A-F, Representative images of CR-immunostained sections taken from case 4454. C' and F' are larger magnifications of C and F, respectively. Contours for the caudate (Cd), putamen (Pt) and nucleus accumbens (NA) hand-drawn using StereoInvestigator are superimposed on each image. These are shown for illustrative purpose only, as sampling of sections was much more frequent than shown here. The approximate level of each image in the anteroposterior axis is indicated by a dashed line. The head of the Cd and the rostral Pt were defined as the region between their anteriormost border and the anterior boundary of the globus pallidus pars externa (GPe) (shown in C, C'). The Cd body was defined as the Cd region between the anterior edge of the GPe and the posterior edge of the globus pallidus pars interna (GPi) (shown in F, F'). Scale bar = 5mm.

Figure 2.

Unbiased stereological estimates of parvalbumin (PV)+ neuron densities in the caudate (Cd) (A) and the putamen (Pt) (B) of Tourette syndrome (TS) and normal control (NC) brains. Each small symbol represents a single subject. *N* = number of subjects. Across all regions (Cd, Pt), there was an overall strong significant decrease in PV+ neurons in TS ($F(1, 8) = 14.085$, $p = 0.006$, ANOVA), but no statistically significant difference between the regions and subregions (anterior, posterior).

Figure 3. Representative images of PV immunostaining in the head of the caudate of normal control (A, C) and Tourette syndrome (B, D) brains. Panels A and B are composites of several smaller panels. C and D are high magnifications of A and B, respectively. Scale bar in A is 100 μm for A and B; scale bar in C is 50 μm for C and D.

Figure 4. Representative images of CR immunostaining in the head of the caudate of normal control (A,C) and Tourette syndrome (B,D) brains. C and D are high magnifications of A and B, respectively. Arrows point to large-size CR+ interneurons and arrowheads point to the more abundant medium-size CR+ interneurons. Scale bar in A is 100 μ m for A and B; scale bar in C is 50 μ m for C and D.

Figure 5.

CR⁺ neuron densities in the caudate (Cd) and putamen (Pt) of Tourette syndrome (TS) and normal control (NC) brains using stereological analyses. (A,C) Caudate; (B) Putamen. Each small symbol in represents a single subject. *N* = number of subjects. **A,B:** Across all regions (Cd, Pt), there was an overall strong significant decrease in large-size CR⁺ interneurons in TS ($F(1, 8) = 36.994, p = 0.001$, ANOVA), but no statistically significant difference among the regions and subregions (anterior, posterior).

C: The density of medium-sized CR⁺ interneurons was not changed in the Cd of TS subjects. Note the different Y scales in C and A, B.

Figure 6. Representative images of ChAT immunostaining in the head of the caudate of normal control (A,C) and Tourette syndrome (B,D) brains. C and D are high magnifications of A and B, respectively. Arrows point to ChAT+ neurons. Scale bar in A is 100 μ m for A and B; scale bar in C is 50 μ m for C and D.

Figure 7.

Unbiased stereological estimates of ChAT+ neuron densities in the striatum Tourette syndrome (TS) and normal control (NC) brains. Each small symbol represents a single subject. (A) Caudate; (B) Putamen. N = number of subjects. Across all regions (Cd, Pt), there was an overall strong significant decrease in ChAT+ interneurons in TS ($F(1, 8) = 10.641, p = 0.011$, ANOVA), but no statistically significant difference among the regions and subregions (anterior, posterior).

Figure 8.

A: Functional subdivision of the basal ganglia in the coronal plane, based on previous studies (Bernácer et al., 2007; Morel et al., 2002; Parent and Hazrati, 1995). **a-d:** Drawings are shown in a rostrocaudal order. AS, associative; SM, sensorimotor; LI, limbic. **B:** The distribution of cholinergic interneurons density, as assessed by counts of large sized CR+ cells, in the functional territories of the striatum. Each small symbol represents a single subject. *N* = number of subjects. ANOVA showed an overall significant effect of diagnosis ($F(1, 8) = 25.852$; $p = 0.001$) and region ($F(2, 16) = 5.335$; $p = 0.017$) and a significant interaction of diagnosis versus region ($F(2, 16) = 5.631$; $p = 0.014$). * $p < 0.05$; ** $p < 0.025$, post-hoc tests with Sidak adjustment.

Table 1

Subject descriptive data. PMI= postmortem interval in hours; DCI = Diagnostic confidence index, a scale that measures the lifetime likelihood of having or of have ever had TS (Leckman et al., 1982); YGTSS= Worst Ever, Yale Global Tic Severity Scale (Leckman et al., 1989); ND = not determined; OTC= Over the counter.

Case #	Age	PMI	Sex	Hemisphere	DCI (0-100)	Age of onset	YGTSS (0-50)	Family History	Developmental History*	Psychotropic Medications	Cause of Death
Subjects with TS											
4187	54	11	M	L	95	7.5	49	Positive multiple cases	No evidence of any difficulties	Olanzapine; Clonazepam; Fluoxetine	Myocardial infarct
445	37	27	M	R	95	7.5	50	Positive paternal grandfather	One febrile seizure at 18 mos; possible Streptococcus infection	Prescription and OTC sedatives Haloperidol, Pimozide, Clonidine and Guanfacine	Accidental overdose
479	34	30	M	L	95	ND	49	Negative	Perinatal hypoxia & prolonged labor	Zispraside for 10 months prior to death; Clonazepam for 3 yrs; Pimozide for 10 yrs; Risperidone for 1 yr; Clonidine as a child	Myocardial infarct
562	42	21	M	R	48	8	36	Maternal negative Paternal unknown	Bedrest-3 mos bleeding	Haloperidol from 16-42 y.o.	Car accident
673	48	< 24	M	L	48	13	35	Maternal cousins OCD Paternal depression	No evidence of any difficulties	Lexapro, Clonazepam, Risperdal, Clonidine, Haloperidol for 1 yr, Fluvoxamine, Lorazepam, Lamictal, Geodon, Seroquel	Acute pneumonia, metastatic carcinoma
Normal Controls											
H001	47	21	M	R						None	Myocardial infarct
M96021	65	8.5	M	L						None	Sepsis
Y98-209	59	20	M	R						None	COPD
Y98-183	73	12	M	R						None	Aortic dissection
Y07-48	65	9	M	R						None	Sepsis

Table 2

Antibodies used in this study. For more detailed information, see Materials and Methods.

Antigen	Raised in	Immunogen	Source	Dilution
parvalbumin	mouse (monoclonal)	frog muscle parvalbumin	Sigma (P3088)	1:2500
calretinin	rabbit (polyclonal)	recombinat human calretinin	Swant (CR 7699/4)	1:2500
choline acetyltransferase	goat (polyclonal)	human placental enzyme	Chemicon (AB144P)	1:1000
DARPP-32	rabbit (polyclonal)	synthetic peptide (sequence CVEMIRRRRPTPAML) surrounding Thr34 of human DARPP-32	Cell Signaling (2302)	1:200

Table 3

Summary of neuronal density values for Parvalbumin (PV)+, Calretinin (CR)+, Choline Acetyltransferase (ChAT)+ and cresyl violet (CV)+ neurons assessed by unbiased stereological methods in serial sections of the caudate (Cd), putamen (Pt) and the nucleus accumbens (NA) (cells/mm³, mean $\pm$ SEM). *N* = number of subjects. Cd head/rostral Pt and Cd body/caudal Pt are defined as explained in the text and Figure 1. Across all regions (Cd, Pt) and subregions (anterior, posterior), TS individuals showed statistically significant decreases in PV+ interneurons ($F(1, 8) = 14.085, p = 0.006$, ANOVA), in large-sized CR+ interneurons ($F(1, 8) = 36.994, p = 0.001$, ANOVA), in ChAT+ interneurons ($F(1, 8) = 10.641, p = 0.011$, ANOVA), but no statistically significant difference in total neuron densities assessed by cresyl violet staining ($F(1, 8) = 4.357, p = 0.070$, ANOVA). No significant interactions were found between diagnosis (NC, TS) and region or subregion.

Caudate	Neuronal Density in the Striatum					
	Head			Body		
	NC	TS	% difference	NC	TS	% difference
PV	298.9 $\pm$ 58.0 (<i>N</i> = 5)	119.3 $\pm$ 22.0 (<i>N</i> = 5)	-60.1%	397.6 $\pm$ 52.5 (<i>N</i> = 5)	180.4 $\pm$ 57.8 (<i>N</i> = 5)	-54.6%
CR (large)	262.9 $\pm$ 30.3 (<i>N</i> = 5)	103.7 $\pm$ 20.3 (<i>N</i> = 5)	-60.5%	221.1 $\pm$ 31.6 (<i>N</i> = 5)	106.4 $\pm$ 26.8 (<i>N</i> = 5)	-51.9%
ChAT	248.9 $\pm$ 35.5 (<i>N</i> = 5)	115.8 $\pm$ 21.5 (<i>N</i> = 5)	-53.5%	203.3 $\pm$ 19.7 (<i>N</i> = 5)	112.0 $\pm$ 17.6 (<i>N</i> = 5)	-44.9%
CV ($\times$ 1000)	41.7 $\pm$ 5.2 (<i>N</i> = 5)	42.7 $\pm$ 3.2 (<i>N</i> = 5)	2.2%	54.1 $\pm$ 4.6 (<i>N</i> = 5)	41.1 $\pm$ 2.0 (<i>N</i> = 5)	-24.1%

Putamen	Rostral			Caudal		
	NC	TS	% difference	NC	TS	% difference
PV	291.6 $\pm$ 40.7 (<i>N</i> = 5)	105.3 $\pm$ 25.8 (<i>N</i> = 5)	-63.9%	257.4 $\pm$ 21.8 (<i>N</i> = 5)	146.3 $\pm$ 28.3 (<i>N</i> = 5)	-43.2%
CR (large)	237.2 $\pm$ 17.0 (<i>N</i> = 5)	92.4 $\pm$ 22.8 (<i>N</i> = 5)	-61.1%	223.0 $\pm$ 10.6 (<i>N</i> = 4)	137.6 $\pm$ 22.9 (<i>N</i> = 4)	-38.3%
ChAT	192.9 $\pm$ 27.6 (<i>N</i> = 5)	99.0 $\pm$ 16.2 (<i>N</i> = 5)	-48.7%	209.3 $\pm$ 40.1 (<i>N</i> = 5)	105.7 $\pm$ 29.5 (<i>N</i> = 5)	-49.5%
CV ($\times$ 1000)	44.0 $\pm$ 3.0 (<i>N</i> = 5)	37.0 $\pm$ 1.9 (<i>N</i> = 5)	-16.0%	42.5 $\pm$ 2.1 (<i>N</i> = 5)	35.0 $\pm$ 1.9 (<i>N</i> = 5)	-17.6%

Nucleus Accumbens				
	NC	TS	% difference	
PV	N/A	N/A		
CR (large)	224.5 ± 37.6 (N = 4)	159.6 ± 15.2 (N = 5)	-28.9%	
ChAT	307.8 ± 26.2 (N = 4)	196.3 ± 23.7 (N = 5)	-30.8%	