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Molecular mapping of striatal subdivisions in juvenile *Macaca Mulata*

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Abstract

The striatum of the primate brain can be subdivided into three distinct anatomical subregions: caudate (CAU), putamen (PUT), and ventral striatum (VS). Although these subregions share several anatomical connections, cell morphological, and histochemical features, they differ considerably in their vulnerability to different neurological and psychiatric diseases, and these brain regions have significantly different functions in health and disease. In order to better understand the molecular underpinnings of the different disease and functional vulnerabilities, transcriptional profiles were generated from the CAU, PUT, and VS of five juvenile rhesus macaques (*Macaca mulatta*) using human cDNA neuromicroarrays containing triplicate spots of 1227 cDNAs. Differences in microarray gene expression were assessed using *z* score analysis and 1.5-fold change between paired subregions. Clustering of genes based on dissimilarity of expression patterns between regions revealed subregion specific expression profiles encoding G-protein-coupled receptor signaling transcripts, transcription factors, kinases and phosphatases, and cell signaling and signal transduction transcripts. Twelve transcripts were examined using quantitative real-time PCR (qPCR), and 81% demonstrated alterations similar to those seen with microarray analysis, some of which were statistically significant. Subregion specific transcription profiles support the anatomical differentiation and potential disease vulnerabilities of the respective subregions.

Keywords

Microarray; Genomics; Striatum; Monkey; Quantitative PCR; Caudate; Putamen; Ventral striatum; Nucleus accumbens

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Introduction

The striatum is the main input structure of the basal ganglia integrating information from various cortical and subcortical inputs to generate behavioral output. The striatum has received considerable attention based on its involvement in sensory–motor integration, motor planning and execution (Lidsky et al., 1985; Marshall et al., 1971), learning and memory (Jog et al., 1999; Jueptner et al., 1997; Phillips and Carr, 1987), and reward (Koob, 1999; Koob and Nestler, 1997; MacLean, 1978; Marsden, 1984). Historically, the primate striatum has been subdivided primarily by the projections of the mesencephalic dopamine system relative to the internal capsule into three main areas: caudate (CAU), putamen (PUT), and ventral striatum (VS), which includes the nucleus accumbens (Haber and Gdowski, 2004; Joel and Weiner, 2000). Alternatively, the striatum can be subdivided functionally based on corticostriatal connections; referred to as motor, associative and limbic striatum (Joel and Weiner, 1994; Joel and Weiner, 2000; Parent, 1990; Parent and Hazrati, 1993, 1995). Additionally, numerous other organizational criteria and nomenclature exist for regions of the striatum based on patch/striasome matrix divisions (Gerfen et al., 1985), dopaminergic projections (i.e., mesolimbic, nigrostriatal), and functional gradients (rostral–caudal and medial–lateral) (Karachi et al., 2002; Morel et al., 2002; Piggott et al., 1999). While these recent, connection-based divisions provide important insights into basal ganglia functioning, morphological subdivisions of PUT, CAU and VS remain important for use in studies without tract tracing techniques and for comparisons to behavioral studies where regional anatomical tissue dissections of the striatum are more common.

Differences in specific connectivity undoubtedly contribute to differences in the functional output in these striatal regions; however, differential abundances of protein and transcripts likely mediate many of the functional differences and selective vulnerabilities to neurological and psychiatric diseases between these regions (Calabresi et al., 2000; Elsworth et al., 2000; Hermel et al., 2004; Martinez et al., 2004; Miller et al., 1999). For example, CAU/PUT and VS are differentially affected by the neuropathology of schizophrenia (Bachus and Kleinman, 1996), Parkinson disease (Gibb, 1997), and substance abuse (O'Donnell and Grace, 1998) as well as by medications used to treat these diseases (Knable and Weinberger, 1994; Onn and Grace, 1995). However, the molecular mechanisms underlying the diversity of function between the morphologically similar subregions of the striatum remains poorly understood. Several studies have described different gene and protein expression between the subregions with the greatest differences between the VS and CAU/PUT (Bouthenet et al., 1991; Glass et al., 1997; Joseph et al., 2003; Mijster et al., 1997; Piggott et al., 1999; Richtand et al., 1995). Most likely, regional differences in the functional output of the basal ganglia result from a combination of differences in connectivity and differences in the abundances of specific transcripts and proteins.

To date, molecular assessments of striatal regions have largely focused on individual candidate genes primarily involved in functions known to be affected during different pathogenic states. The advent of high throughput transcriptional profiling allows the integrated examination of multiple transcripts, thus providing a more comprehensive understanding of the molecular basis of striatal organization. Anatomical comparisons have been reported previously for rodent hippocampal (Zhao et al., 2001) and midbrain regions

(Greene et al., 2005). In the present study, cDNA microarrays and quantitative real-time PCR were used to examine differences in the molecular profiles between the CAU, PUT, and VS of preadolescent rhesus monkeys. The goal of the present study was to identify transcripts that were differentially expressed between the subregions of the striatum as a foundation for understanding the functional differentiation of the striatal subregions and the differential disease vulnerabilities of these regions. Computational analyses were used to determine statistical significance and partition the data into groups of genes with similar expression patterns.

Materials and methods

Animals use

Five rhesus macaques (4 males, 1 female) ranging from 11 to 15 months were used in this experiment. Monkeys were housed individually with standard enrichment, including social enrichment, human interaction, variety in diet, and age appropriate objects as dictated by the Animal Welfare Act and the Emory University Policy for Environmental Enhancement. Food and water were available ad libitum. The monkeys had no previous experimental history and were obtained from Yerkes National Primate Center (Emory University, Atlanta, GA). Monkeys were sedated with 5 mg/kg telazol and euthanized with 100 mg/kg sodium pentobarbital.

Preparation of striatal tissue

The brain was immediately removed and placed in 4°C PBS for 5 min. Four-millimeter coronal slabs were cut through the extent of the striatum. The CAU, PUT, and VS were dissected from the appropriate blocks, and the samples were immediately frozen and stored at –80°C. The postmortem interval (PMI) was less than 15 min for all subjects in this study. Experiments were conducted with the approval of the Emory University Institutional Animal Care and Use Committee as promulgated by the National Institutes of Health.

RNA isolation and microarray procedures

Isolated tissue samples from CAU, PUT, and VS for each animal were pulverized under liquid nitrogen. Approximately 50–100 mg of tissue specimens from each monkey from each region were placed in Trizol[®] (Invitrogen Corporation, Carlsbad, CA) and disrupted using a PowerGen tissue homogenizer. Samples were incubated at 25°C for 5 min to allow complete dissociation of nucleoprotein complexes. Chloroform was added, and samples were centrifuged for 15 min for phase separation. The aqueous phase was retained and total RNA was precipitated with isopropyl alcohol and linear acrylamide (5 µg) and washed with 75% ethanol. The RNA pellet was re-suspended in RNase-free water and stored at –80°C. RNA was quantified using spectrophotometric analysis, and the integrity of total RNA for each sample was assessed using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Lab Chip according to the manufacturer's protocol. Samples in which 28S and 18S bands were clearly present, the 28 S/18 S ratio was greater than 1.0, and a RIN number (Agilent Bioanalyzer) greater than 6.0 were included in the study. On average, 50–75 mg of tissue yielded approximately 30–60 µg of quality total RNA.

To synthesize cDNA probes from total RNA template, oligo-dT₂₄ primer, 10 mM DTT, 5× CyScript reverse transcriptase buffer, dCTP mix, Cy5-dCTP, and 200 U of reverse transcriptase (CyScript; Amersham) were added to total RNA. In addition 1.75 ng of cab, rbcL, and LTP4 RNAs (Stratagene #252201, #252202, #252203) were added to each RNA sample as internal controls. The mix was incubated at 42°C for 90 min. Afterwards, 2 U RNase H and 2 U RNase A were added to the mix and incubated at 37°C for 15 min to remove the RNA template. Labeled cDNA probes were purified using QIAquick PCR purification kit (Qiagen), and quality was analyzed by spectrophotometry (A₂₆₀ and A₂₈₀ optical density readings). Measurements were used to determine [1] nanograms of probe synthesized ($A_{260} \times 37 \times \text{vol of sample } (\mu\text{l}) = \text{ng of probe}$), [2] picomoles of dye incorporated ($A_{650} \times \text{volume of sample } (\mu\text{l}) / 0.25 = \text{pmol of Cy5}$), and [3] number of labeled nucleotides per 1000 nucleotides or frequency of incorporation (FOI) ($\text{FOI} = \text{pmol of dye incorporated} \times 324.5 / \text{ng of probe}$). Probe quality was deemed sufficient if picomole of dye incorporated was ≥ 10 and FOI was ≥ 5 . For each sample, labeled cDNA was added to the hybridization buffer and hybridized to Human Neuroarray V2 (Emory University Microarray Facility; www.microarray.emory.edu) containing 1227 UniGen set, sequence verified human cDNAs and cab, rbcL, LTP4 arabidopsis cDNAs (Stratagene) designed by S.E. Hemby. Each cDNA on the array was spotted in triplicate. Arrays were incubated overnight at 42°C in a hybridization solution of formamide, 20× SSC, 10% SDS, 1M DTT, 2% BSA, sterile water, and sonicated salmon sperm DNA. Post-hybridization washes consisted of 2× SSC, 0.1% SDS solution (1 min, 5 min), 0.1× SSC, 0.1% SDS solution (10 min), 0.1× SSC solution (15 s, 2 min, 2 min, 1 min), and 0.01× SSC solution (15 s). Slides were dried and scanned using a confocal laser scanner (ScanArray 5000 Packard BioChip Technologies, Billerica, MA) to determine hybridization intensity of each spot.

Quality control

Array images from the scanner were analyzed with Gene Pix 4.0 (Axon Instruments, Foster City, CA). Array spots considered “present” if 35% of the spot width was at least one standard deviation above local background, and the spot was not one of the spiked controls. Only arrays with $\geq 50\%$ of the spots identified as “present” were included in analysis, as a control for hybridization efficiency. Each cDNA on the array was spotted in triplicate, and a mean value for each gene was generated. For genes with at least two of the three replicates flagged “present”, the background subtracted signals for those replicates were averaged. For each set of replicates, the standard error of the mean was calculated to assess signal reliability. Signal was considered undetectable for those genes with less than two “present” replicates, and the signal value was reported as “null”.

Cluster analysis

Gene Spring 7.2 was used for the generation of heat maps, self-organizing maps (SOM) and ontological clustering. Array data from each subject from each region were normalized per chip and per gene by median gene polishing. Gene tree and condition tree analyses were performed with a separation ratio of 1.0 and a minimum distance of 0.001. Self-organizing map (SOM) clustering was employed to elucidate common patterns of expression over time. SOM clustering is a data mining and visualization method originally developed (Kohonen, 1990, 2000) and applied to the analysis of gene expression data from microarrays (Fasulo

and Hemby, 2003; Tamayo et al., 1999; Toronen et al., 1999). The clustering method is similar to k means clustering (Kaeck et al., 2002) but differs in that genes are divided into groups based on expression patterns and relationships between groups are illustrated by two-dimensional maps.

The values used for SOM analysis were the mean expression levels for each of the 816 “present” genes in each region. For each gene, the mean of the values across the regions was normalized to zero with a standard deviation of one. This normalization enables the examination of the *shape* of expression patterns rather than the absolute levels of expression (Tamayo et al., 1999). Parameters for SOM clustering were as follows: 2 rows $\times$ 3 columns, 816,000 iterations—representing 1000 samplings of each gene and a neighborhood radius of 4. The number of nodes was derived empirically, such that a large number of nodes yielded redundant clustering patterns, whereas too few nodes resulted in high error rates and included genes in cluster patterns that were inaccurate.

Z statistic analysis

Density data were exported into Excel for calculation of z statistic and fold change for each transcript. Raw intensity data for each gene (mean of triplicates) were $\log_{10}$ transformed and used for the calculation of z score. Z score transformation was calculated as described in Cheadle et al. (2003) according to the following equation: $z \text{ score} = (\text{intensity}_x - \text{mean intensity}_{x1 \dots xn}) / \text{SD}_{x1 \dots xn}$. Comparisons between subregions for each animal were made by comparing z scores and calculating a z ratio, determined as the difference between the mean of the observed gene z score per subregion and divided by the standard deviation of all the differences for that comparison. Z ratios were generated for each of the set of comparisons: VS-CAU, VS-PUT, and PUT-CAU respectively. The z statistic can be converted to a significance value based on a two-tailed z statistic critical value, such that if $z = \pm 1.96$, then $\alpha = 0.05$; if $z = \pm 2.58$ then $\alpha = 0.01$. In addition, fold change was calculated for transcript comparison between regions. Z ratios were only calculated for genes with detectable signal in 4 of 5 subjects for each subregion. A z statistic ± 1.96 and a fold change 1.5 were used as the operational definition of differential expression of transcripts.

Quantitative PCR

Total RNA from each subject was reverse transcribed in a 20 μ l reaction using OmniScript Reverse Transcription Kits (Qiagen #): total RNA (2 μ g) was added to oligo-dT primer, 10 mM DTT, 10 $\times$ OmniScript reverse transcriptase buffer, dNTP mix (5 mM), and 200U of reverse transcriptase (OmniScript, Qiagen). cDNA was diluted 1:10 or 1:200 with DNase/RNase-free water depending on the abundance of the target gene. The cDNA was amplified in a 384-well format using an ABI Prism 7900 Sequence Detection System. 0.5 μ l aliquots of TaqMan Expression Assay (20X; Supplemental Data 1), 5.5 μ l 2 $\times$ TaqMan Universal PCR Mastermix (Applied Biosystems, #4304437), and 4.5 μ l diluted cDNA or water for a no template control (NTC) were mixed together for each sample for each target gene, and an aliquot was placed into a single well of a 384-well PCR plate (Applied Biosystems #4309849). Each sample, including NTC was run in triplicate. Thermocycling conditions in the Applied Biosystems 7900HT were as follows: (1) one cycle 2 min at 50°C, (2) on cycle 10 min at 95°C, and (3) 50 cycles: 15 s at 95°C and 1 min at 60°C. Fluorescence was

measured during the 60° step for each cycle. The reactions were quantified by the standard curve method (as described in User Bulletin #2, Applied Biosystems) using SDS2.1 software, where the threshold cycle (Ct) for the target cDNA for each sample was selected and interpolated into a standard curve generated from the Ct values of the PCR product of interest in a 2-fold dilution series of cDNA standards. The Qty mean for each gene was calculated by the software using the triplicate wells for each gene. The expression level of each gene of interest was normalized to the expression level of the endogenous reference (human 18S) in each sample. The software calculated a Qty mean value for the endogenous control in the same manner as described for the target gene, and relative expression was expressed as Qty mean (mean quantity for triplicates of each gene as determined from the standard curve for that gene)/18S Qty mean (mean quantity for triplicates of 18S for each sample as determined from the 18S standard curve). One-tailed paired *t* tests were performed on the quantity mean values relative to 18S to determine the significant differences between the relative quantities for each gene between subregional pairs. The null hypothesis was rejected if $P < 0.05$.

Results

The goal of this study was to identify gene expression pattern differences in the subregions of the primate striatum in drug naïve, healthy animals. Samples from each subregion from each animal were individually labeled and hybridized to glass arrays. To assure quality and reproducibility of the custom-made Human cDNA Neuroarrays, a pooled sample of striatal tissue was reverse transcribed and hybridized to five separate arrays. The average Pearson's correlation coefficient ($\pm$ SEM) for these arrays was $R = 0.87 \pm 0.02$ with a range of $R = 0.78$ – 0.97 (Supplemental Data 2). Array quality was assessed by the percent of “present” spots quality control measure described in the Materials and methods. The mean ($\pm$ SEM) percent of spots that were called present spots for the arrays was 70% (± 0.05). Quality control measures described in the Materials and methods section were applied, and the remaining arrays yielded a mean ($\pm$ SEM) percent of present spots for the arrays of 77% (± 2) indicating that hybridization sensitivity was consistent across arrays. In summary, these data indicate that (1) monkey RNA can hybridize to human arrays and, (2) the reproducibility of our array platform, thus differences observed reflect underlying differences in gene expression.

Z score analysis

In the current study, 816 of the 1227 transcripts represented on the array met or exceeded inclusion criteria in at least 4 of 5 subjects in all three subregions. The raw intensity data for each gene were $\log_{10}$ transformed and used for the calculation of *z* score. The *z* transformation corrects data within a given hybridization by expressing the signal intensity values for each gene as a unit of standard deviation from the normalized mean of zero (Cheadle et al., 2003). Comparisons between subregions for each animal were made by comparing *z* score values yielding a *Z* score ratio for that particular transcript. The *z* score ratio for each regional comparison for each transcript was calculated for each animal separately using a within subject design and then mean ratios were calculated.

Zratios for each of the 816 genes that met inclusion criteria were compared for each pair of subregions: VS-CAU, VS-PUT, and PUT-CAU respectively. In addition, fold change was calculated for each pair of subregions. Genes were compiled into a list of VS enriched genes, PUT enriched genes, and CAU enriched genes based on z ratios and fold change values (Table 1). Forty-nine transcripts were more abundant in the CAU (CAU > PUT; 29; CAU > VS: 20), twenty eight transcripts from a variety of functional classes were more abundant in the PUT (PUT > CAU: 8; PUT > VS: 20), and twelve transcripts were more abundant in the VS (VS > CAU: 9; VS > PUT: 3). Genes identified as significantly different between groups include genes from a number of gene families including transcriptional regulators, structural proteins, signaling molecules, and receptors (Table 1).

Clustering analysis

A two-way clustering approach was used to detect groups of correlated genes and brain regions from the 816 “present” transcripts (Fig. 1; Supplemental Data 3). Transcripts that appear close to each other in the tree exhibit a strong correlation across the brain regions relative to one another, while the proximity of brain regions indicate similarities of gene expression patterns. Data were plotted using a color code to help with visualization, with gene intensity (abundance) varying from high (red) to low (blue). The intensity of each gene was normalized so that the relative variation in intensity was emphasized rather than the absolute intensity. The two-way clustering method applied to the gene expression data set yielded a matrix that appears to bear patterns reflecting underlying organization in the present data set. Areas of high or low intensity correspond to groups of tens to hundreds of transcripts whose expression is coordinated to a substantial degree across brain regions.

SOM clustering was used to detect patterns of gene expression across the brain regions within the data set (Fig. 2, see Supplemental Data 3 for transcripts corresponding to each node). It is important to note that these patterns reflect the normalized data only and cannot address issues of comparative levels of expression or statistical significance but rather are a tool for data mining and visualization of the data. Filtering of the present data set was employed to remove genes that changed less than 5% across all brain regions, and normalization was used to enable examination of the *shape* of expression patterns rather than the absolute levels of expression (Tamayo et al., 1999). The SOM algorithm was then performed and 6 distinct patterns of expression were generated. Each cluster contains genes with similar expression patterns (Supplementary Data 3). For example, pattern A consists 154 genes in the following pattern: VS > CAU, PUT; pattern B contains 131 genes in the following pattern: VS, CAU > PUT; pattern C contains 135 genes (CAU > PUT, VS); pattern D contains 139 genes (VS, PUT > CAU; pattern E describes a regulation of 152 (PUT > VS, CAU); and pattern F contains 105 genes CAU, PUT > VS). Eight transcripts were not assigned to a node—indicating that there were no calculable regional differences based on the SOM calculation. The clusters and the respective gene expression patterns correlate with the organization identified by the gene trees.

Quantitative PCR analysis

Microarray analysis identified a number of transcripts exhibiting regionally enriched expression patterns (Table 1). Of these, 12 transcripts were selected for verification using

TaqMan real-time PCR expression assays (Supplemental Data 1) normalized to an endogenous control (18S ribosomal RNA). Relative expression was determined using the relative standard curve method described in the Applied Biosystems User Bulletin #2. In addition, three of the transcripts (GSR, TFAP2 γ , and MOBP) were expressed at relatively low abundance in these samples, and the threshold levels (Cts) were at or above 35 cycles. Since Ct values of 35 approach the sensitivity limits of the real-time PCR detection system, there is inherently more variability with Ct values above 35 (Applied Biosystems Application Note: Amplification Efficiency of TaqMan Gene Expression Assays), which may explain why trends were observed in a direction similar to microarray analysis.

CAU enriched transcripts

qPCR was used to examine five of the 29 transcripts that showed enrichment in the CAU compared with the VS using array analysis. These include the cannabinoid receptor 1 (CNR1), cytochrome *c* oxidase subunit VIIb (COX7B), glutathione reductase (GSR), transcription factor AP2 γ (TFAP2C), and zinc finger 263 (ZNF263). For these five transcripts, the fold change in differential expression obtained by qPCR was similar to that obtained by the array analysis. Furthermore, mRNA levels were significantly greater in the CAU versus the VS for three of the five transcripts: CNR1 ($P=0.002$), COX7B ($P=0.019$), and TFAP2C ($P=0.006$). The remaining two transcripts showed trends toward significance: GSR ($P=0.078$) and ZNF263 ($P=0.088$) (Fig. 3).

qPCR was also used to examine three of the 20 transcripts that showed differential expression between the CAU and PUT using array analysis: COX7B, TFAP2C, and ZNF263. Two of the three transcripts, TFAP2C and ZNF263, exhibited a similar directional fold change obtained by qPCR as obtained by array analysis, though these changes were not significant in the qPCR analysis. COX7B mRNA abundance was found to be greater in PUT than CAU (1.14-fold) in contrast to the array data; however, this change was not significant. In summary, of the eight regional comparisons of the five different transcripts, seven showed the same pattern of regional variation in the microarray and qPCR analyses, though there were differences in the size of the differences observed.

PUT enriched transcripts

We used follow-up qPCR to examine three of the 20 transcripts that showed differential expression between the PUT and VS using array analysis. These include actin-related protein 2/3 complex, subunit 2 (34 kDa) (ARPC2), CNR1, and myelin-associated oligodendrocyte basic protein (MOBP). All three transcripts showed similar magnitudes of differential expression between microarray analysis and qPCR; moreover, all were statistically significantly different: ARPC2 ($P=0.021$), CNR1 ($P=0.011$), and MOBP (0.022) (Fig. 4).

We used follow-up qPCR to examine three of the eight transcripts that showed greater abundance in the PUT compared to the CAU using array analysis. These include BCL2-associated X protein (BAX), gamma-aminobutyric acid A receptor β 1 (GABRB1), and MOBP. All three transcripts showed greater abundance in the PUT compared to the CAU by qPCR, as they did in the microarray analysis. However, the degree of fold change was

different between the two techniques, and none of the transcripts were different in a statistically significant manner between the regions. In summary, each of the six regional comparisons of the five transcripts showed the same pattern of regional variation in the microarray and qPCR analyses, though there were some differences in the size of the differences observed.

VS enriched transcripts

We used follow-up qPCR to examine two of the nine transcripts that showed differential expression between the CAU and VS using array analysis: CCAAT/enhancer binding protein alpha (CEBPA) and gamma-aminobutyric acid A receptor, $\beta 3$ (GABRB3). Interestingly, qPCR analysis revealed differential expression in the opposite direction as indicated by microarray analysis, in that both transcripts were expressed in greater abundance in the CAU than VS with the abundance of CEBPA mRNA in the CAU significantly greater than in the VS ($P = 0.049$). Of the three transcripts exhibiting greater abundance in the VS than PUT following array analysis, none were selected for validation by qPCR.

Discussion

In this study, cDNA microarrays were used to investigate normative differences in the expression of 1227 mRNAs between three subregions of the striatum in rhesus monkeys. The use of high-density microarrays enables the assessment of the coordinated expression of multiple transcripts simultaneously which ultimately determine the brain region phenotype. Using DNA microarray technology in this regard provides a unique format to describe and cluster global transcriptional characteristics underlying anatomical differentiation between brain subregions. A number of previous studies have employed this technique to investigate similar questions in different brain subregions, demonstrating the utility of this approach (Sandberg et al., 2000; Zhao et al., 2001). Previous studies of the striatum have used in situ hybridization and ribonuclease protection assays to examine a few transcriptional differences within the striatum on a gene by gene basis (Honrubia et al., 2000; Huntley et al., 1992; Kultas-Ilinsky et al., 1998; Mijster et al., 1997; Rappaport et al., 1993; Richtand et al., 1995; Svenningsson et al., 1998; Wullner et al., 1997). The work presented here demonstrates the first large-scale molecular profiles for distinguishing subregions of the striatum in primates. From studies such as those presented here, we can construct genomic databases that are more similar to the human primate transcriptome which may provide important information for understanding the molecular basis of the functional striatal subregions.

Given the relative paucity of annotated gene sequences of non-human primates, assessing gene expression in non-human primates by cross-species hybridization using human microarrays has been shown to be a useful approach for determining gene expression profiles (Caceres et al., 2003; Enard et al., 2002; Lachance and Chaudhuri, 2004; Miyahara et al., 2003; Redmond et al., 2003; Wang et al., 2004). Furthermore, such an approach is intuitively appealing due to the high degree of sequence homology between monkeys and humans and the similarity of expression patterns using oligo and cDNA arrays (Redmond et al., 2003). The use of a long to full-length cDNAs as probes on the array platform limits the

influence of sequence differences between rhesus monkey and human and provides greater probability of hybridization between the complementary sequences between rhesus monkey mRNA and human cDNA. However, the length of cDNAs may decrease discriminability of the assay due to cross hybridization of mRNAs, thus increasing the Type I error. Conversely, a more stringent assay (e.g., one base-pair mismatch discrimination) would not allow for minor dissimilarities in sequence between human and monkey and would likely result in under-representation of recognizable transcripts therefore increasing Type II error. The determination of “true” differential expression is multifaceted requiring several levels of evaluation. In the present study, *Z* score statistic, fold change, and ontological clustering were used to assess statistical differences, the robustness of change, and the functional relevance of such changes, respectively. In addition, qPCR was used to determine the reliability of such changes. It should be noted that the human TaqMan primer/probes were designed using the human sequence and therefore represent a more stringent assessment of mRNA levels than the cDNA microarray platform-one factor likely contributing to discrepancies between microarray and qPCR data. qPCR was selected as the confirmatory assay due to the specificity and quantitative nature of this assay. Other studies have chosen in situ hybridization (ISH) which provides important information to guide future studies for assessing functional striatal gradients as well as discrete cell populations within these regions using laser capture microdissection; however, the ISH method is neither quantifiable nor representative of regional assessment of gene expression analysis, two main concerns of the present study.

The striatum is a region which shows relative cellular homology and in which differences in cortical and thalamic innervation contribute to a functional differentiation in the striatal subregions (Haber and Gdowski, 2004). Given the significant differences between rodent and primate prefrontal cortex (Preuss, 1995) and the significant prefrontal corticostriatal projections in monkeys, it is particularly relevant to study primate striatal profiles to enable extrapolation to humans in the future. Differences in thalamocortical projections originating in the medial–dorsal, ventral–anterior and ventral–lateral nuclei exist (Haber and Gdowski, 2004), and mesencephalic dopamine projections in primates suggest more complex patterns of projections (Francois et al., 1999; Lynd-Balta and Haber, 1994; Williams and Goldman-Rakic, 1998), further emphasize the relevance of non-human primate anatomical models.

A variety of transcripts from different functional classes were differentially expressed in striatal subregions; however, among the more interesting findings was the enriched expression of the cannabinoid 1 receptor (CNR1) in the CAU and PUT. Understanding the differential expression of CNR1 is of particular interest, given recent evidence that endogenous signaling at CNR1 modifies with glutamatergic and dopaminergic signaling in striatal cells (Robbe et al., 2003; Rodriguez De Fonseca et al., 2001). Data suggest that CNR1 is co-localized with D₁ or D₂ receptors and can interact at the G protein level (Meschler and Howlett, 2001). Present results demonstrate highest abundance of CNR1 mRNA in the PUT and CAU, which is parsimonious with the idea that both dopamine and CB1 receptors are important to movement. Indeed, there is evidence for endocannabinoid system involvement in PD, a disease associated with the selective degeneration of DA neurons projecting to the PUT and CAU (Fernandez-Ruiz et al., 2002; Van der Stelt and Di Marzo, 2003; Van der Stelt et al., 2002) as well as haloperidol-induced catalepsy (Marchese

et al., 2003). With regard to CNR1 receptor binding, levels are greater in the striatum compared to nucleus accumbens of rodents and human postmortem tissue (Mailleux and Vanderhaeghen, 1992; Mailleux et al., 1992). However, it should be noted that juvenile monkeys were used in the present study, and thus, extrapolation to particular disease states which occur post-adolescence may be tenuous.

A number of transcripts encoding transcriptional regulators were also differentially expressed between striatal subregions. For example, TFAP2C was more abundant in CAU than either of the other two striatal subregions. TFAP2C is known to play a role in mammalian development (Werling and Schorle, 2002) and regulate the adenosine deaminase mRNA expression (Werling and Schorle, 2002). Inasmuch as adenosine deaminase is an important regulator of dopaminergic and cholinergic release in the striatum (Golembiowska and Zylewska, 2000; Preston et al., 2000), TFAP2C induced regulation of this transcript may exert considerable influence on striatal neurochemical function. However, additional studies are warranted to determine the role of TFAP2C regulation of adenosine deaminase in the primate striatum. Another transcription factor, ZNF263, also exhibited the highest mRNA levels in the CAU. ZNF263 is a member of the zinc finger family and contains a KRAB-A domain (Yokoyama et al., 1997), indicating involvement in a variety of cell functions including maintenance of the nucleolus, cell differentiation, cell proliferation, apoptosis, and neoplastic transformation (Urrutia, 2003). The possible involvement of this transcript in the neurodegeneration of specific striatal subregions in Parkinson and Huntington disease and the neuroprotective effects of atypical antipsychotics (Jann, 2004; Joyce, 2001) merits further study.

As noted previously, significant differences were also observed in structural proteins like ARPC2 and MOBP. Expression of both of these transcripts has been examined in conjunction with other diseases. ARPC2, also called Arp2/3 plays a critical role in actin polymerization and has been implicated in Down's syndrome (Weitzdoerfer et al., 2002) and may be involved in dendritic spine abnormalities associated with schizophrenia (Irie and Yamaguchi, 2004). Less is known about the function and regional localization of MOBP. As the name implies, MOBP encodes a component of myelin, is highly abundant, and is selectively expressed in the CNS. Studies indicate that myelin acts during the late stages of myelin formation and is likely involved in sheath compaction (Holz and Schwab, 1997). Studies in mouse and rat indicate the presence of multiple splice variants that are differentially localized in oligodendrocytes (McCallion et al., 1999; Montague and Greer, 1999). The discordant sequences of the variants between rat and mouse suggest species-specific expression and regulation and indicate it is reasonable to speculate that similar MOBP splice variants exist in human and non-human primates and thus warrant further investigation. Nonetheless, this is the first study to our knowledge indicating differential regional expression of MOBP in the primate brain. MOBP has been implicated in the pathophysiology of cocaine abuse in the VS (Albertson et al., 2004) and has been associated with psychiatric disorders in other brain regions (Aston et al., 2005; Flynn et al., 2003; Tkachev et al., 2003). While it is difficult to speculate on functional relevance of these transcriptional changes between striatal subregions, this study has identified a number of transcriptional regulators and other molecules that may be relevant to functional differences between the subregions.

It is important to note that the monkeys in the present study were all juveniles. It is not unlikely that the patterns of gene expression elucidated here may show developmental variations. Additional studies in different age groups will be needed to address this issue; however, these data in a cohort of animals of very similar ages will provide a useful data set to contrast future studies in adult and aged animals. Given the plethora of gender-specific gene expression patterns, differences in gender will certainly contribute to differences in regional transcriptomes in the striatum as well as other regions throughout the brain. While expression levels and patterns for the female monkey included in the present study were similar to those exhibited by the four male monkeys, the sample size precludes detailed examination of gender differences. In addition, a limit of this study is that molecular alterations were not assessed at a cellular level, and it is therefore difficult to relate these findings to the established functional domains of the striatum. Future studies using ISH and LCM are necessary to assess specific cellular changes within these functional domains to more closely understand the link between function, connectivity, and molecular alterations therein. However, this does not negate the importance of the findings of this study, which provide the first examination of differential gene expression in primate striatum subregions with a large-scale view of the genome. This and further work will provide information relevant to differential susceptibility of these subregions to various neuropsychiatric and neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.expneurol.2005.11.028.

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Fig. 1.

Two-way clustering approach using condition and gene tree algorithms in Gene Spring 7.2. The 816 “present” genes from human cDNA microarray data were clustered by region using the Condition and Gene Tree algorithms in Gene Spring 7.2. “Present” genes are genes increased or decreased 1.5-fold in 4 of 5 subjects in all regions. Relative transcript abundance indicated by spectrum where blue denotes low abundance and red denotes high abundance. Lines on the left axis indicate the degree of similarity of expression between regions. Lines on top of map indicate the similarity of regions, in the present study, CAU and PUT are more similar to each other than either is to VS.

Fig. 2.

Self-organizing map of 816 genes relative to one another in striatal brain regions. Six maps were found to incorporate the shape of expression patterns within the present data set. Each transcript was sampled 1000 times for a total of 816,000 iterations. Transcripts corresponding to each cluster can be found in Supplemental Data 3.

Fig. 3.

Real-time PCR assessment of select transcripts identified as differentially expressed in the CAU by microarray analysis. Bars illustrate the mean ($\pm$ SEM) expression of target genes relative to an 18S endogenous control for each individual subject. The panels depict mRNA levels that were less abundant in VS (left) and PUT (right). Black bars indicate mean ($\pm$ SEM) mRNA expression in the CAU, and the gray bars indicate mRNA expression in the VS in the left panel and PUT in the right panel. Significant differences in relative gene expression are denoted by an * ($P < 0.05$) or ** ($P < 0.01$).

Fig. 4.

Real-time PCR assessment of select transcripts identified as differentially expressed in the PUT by microarray analysis. Bars illustrate the mean ($\pm$ SEM) expression of target genes relative to an 18S endogenous control for each individual subject. The panels depict mRNA levels that were less abundant in VS (left) and CAU (right). Black bars indicate mean ($\pm$ SEM) mRNA expression in the PUT, and the gray bars indicate mRNA expression in the VS in the left panel and CAU in the right panel. Significant differences in relative gene expression are denoted by an * ($P < 0.05$) or ** ($P < 0.01$).

Table 1

Subregion specific changes in striatum

Accession number	Description	Array fold change	qPCR fold change	P value
<i>Caudate enriched</i>				
Caudate > VS				
NM_014203	Adaptin, alpha A	2.22		
R98254	Amyloid P component, serum	3.19		
AA417881	Bleomycin hydrolase	1.90		
R20626	Cannabinoid receptor 1 (brain)	2.44	4.07	0.002
W45688	Caspase 6, apoptosis-related cysteine protease	5.91		
AA621315	Catenin (cadherin-associated protein), alpha-like 1	1.88		
AA985354	Cerebellar degeneration-related protein (34 kDa)	1.78		
AI672462	Chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	2.62		
AI311067	Cyclin-dependent kinase 7 (homolog of <i>Xenopus</i> MO15 cdk-activating kinase)	1.99		
AA463492	Cytochrome <i>b</i> -245, beta polypeptide (chronic granulomatous disease)	1.90		
AA629999	Cytochrome <i>c</i> oxidase subunit VIIb	1.83	2.62	0.019
AI016456	Cytochrome P450, subfamily IID, polypeptide 6	2.02		
AA776176	Gamma-aminobutyric acid (GABA) A receptor, alpha 1	2.19		
AA779105	Gamma-tubulin complex protein 2	2.05		
AA777289	Glutathione reductase	2.28	1.41	0.078
AA464525	Interleukin 1 receptor, type I	2.14		
T65739	Interleukin 7 receptor	2.08		
AI359441	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	3.06		
AA973152	Phosphodiesterase 10A	2.15		
AA281784	Phosphoinositide-3-kinase, catalytic, delta polypeptide	2.21		
AA974913	Potassium voltage-gated channel, shaker-related subfamily, member 4	2.01		
H96775	Protein kinase, AMP-activated, alpha 1 catalytic subunit	1.94		
AI345015	Similar to gamma-glutamyltransferase 1 precursor	1.95		
N92711	TATA box binding protein (TBP)-associated factor, RNA polymerase II, I, T528 kDa	4.20		
<i>Caudate enriched</i>				
Caudate > VS				
AA399334	Transcription factor AP-2 gamma (activating enhancer-binding protein 2 gamma)	3.01	4.20	0.004
AA043458	Zinc finger protein 137 (clone pHZ-30)	2.03		
R01941	Zinc finger protein 200	1.90		
AA421783	Zinc finger protein 263	2.90	2.90	0.088
Caudate > Putamen				
AI341924	Apolipoprotein F	1.69		
H18306	Bassoon (presynaptic cytomatrix protein)	1.67		
AI672462	Chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	2.02		

Accession number	Description	Array fold change	qPCR fold change	P value
AA629999	Cytochrome <i>c</i> oxidase subunit VIIb	1.66	0.88	0.398
AA779105	Gamma-tubulin complex protein 2	1.73		
AI123272	Guanine nucleotide binding protein (G protein), q polypeptide	1.53		
R68021	Inositol 1,4,5- triphosphate receptor, type 2	1.65		
AA485668	Integrin, beta 4	1.61		
AA182847	Mitogen-activated protein kinase kinase kinase 5	2.15		
AI359441	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	2.06		
AA974913	Potassium voltage-gated channel, shaker-related subfamily, member 4	1.91		
H96775	Protein kinase, AMP-activated, alpha 1 catalytic subunit	1.53		
AA071526	Protein phosphatase 1, regulatory subunit 10	1.53		
R59164	Protein phosphatase 2, regulatory subunit B (B56), alpha isoform	1.56		
AI024435	Synapsin III	1.58		
AA399334	Transcription factor AP-2 gamma (activating enhancer-binding protein 2 gamma)	1.88	1.51	0.107
R01941	Zinc finger protein 200	1.75		
AA421783	zinc finger protein 263	1.67	1.14	0.376
<i>Putamen enriched</i>				
Putamen > VS				
H25917	Actin-related protein 2/3 complex, subunit 2 (34 kDa)	2.90	2.84	0.021
R20626	Cannabinoid receptor 1 (brain)	4.69	5.36	0.011
W73874	Cathepsin L	2.38		
AA985354	Cerebellar degeneration-related protein (34 kDa)	2.74		
<i>Putamen enriched</i>				
Putamen > VS				
AA629719	Cytochrome <i>c</i> oxidase subunit VIIc	2.30		
AI016456	Cytochrome P450, subfamily IID polypeptide 6	2.80		
AA035450	Inositol 1,4,5-triphosphate receptor, type 1	1.99		
AA621034	Myelin-associated oligodendrocyte basic protein	2.39	4.18	0.022
AA993687	Myotrophin	2.11		
H38086	<i>N</i> -ethylmaleimide-sensitive factor	3.14		
AI650675	Neuromedin B	2.50		
AI017154	Neurotensin receptor, type 2	1.93		
AA634267	Niemann-Pick disease, type C1	2.19		
AI681004	Nuclear factor related to kappa B binding protein	2.24		
AA281784	Phosphoinositide-3-kinase, catalytic, delta polypeptide	2.74		
N62620	Potassium channel, subfamily K, member 1 (TWIK-1)	1.89		
R91438	Protein phosphatase 1, catalytic subunit, alpha isoform	1.96		
N66208	Protein phosphatase 1, regulatory (inhibitor) subunit 8	2.10		
N49856	Solute carrier family 6 (neurotransmitter transporter, betaine/ GABA), member 12	3.07		
H46254	Solute carrier family 6 (neurotransmitter transporter, GABA), member 1	1.96		

Accession number	Description	Array fold change	qPCR fold change	P value
Putamen > Caudate				
AI241388	Activating transcription factor 1	2.06		
AI565203	BCL2-associated X protein	3.10	1.11	0.405
W73874	Cathepsin L	2.67		
R24969	Gamma-aminobutyric acid (GABA) A receptor, beta 1	2.12	1.07	0.442
AI025126	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9 (22 kDa, B22) (NDUFB9)	2.51		
AA621034	Myelin-associated oligodendrocyte basic protein	2.12	1.72	0.096
R20770	Syntaxin binding protein 3	1.96		
N21624	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	2.62		
<i>VS enriched</i>				
Putamen > Caudate				
AA706974	ADP-ribosylation factor domain protein 1, 64 kDa	1.55		
AA456474	Apolipoprotein C-II	1.58		
AA775957	ATPase, +/-K+ transporting, alpha 3 polypeptide	1.82		
H80712	Caspase 10, apoptosis-related cysteine protease	1.97		
AI669881	CCAAT/enhancer binding protein (C/EBP), alpha	2.35	0.27	0.049
R43452	Gamma-aminobutyric acid (GABA) A receptor, beta 3	1.90	0.64	0.109
AA102670	Gamma-aminobutyric acid (GABA) A receptor, pi	1.81		
AA486393	Interleukin 10 receptor, beta	1.67		
N50549	TATA box binding protein	1.73		
VS > Putamen				
AA456474	Apolipoprotein C-II	1.59		
AA456474	Protein phosphatase 2 (formerly 2A), regulatory subunit B, alpha isoform	1.53		
N50549	TATA box binding protein	1.64		

Compilation of differentially expressed transcripts between CAU, PUT and VS. Differential expression as determined by microarray analysis was designated as transcripts exhibiting z ratios ± 1.96 and fold change greater than 1.5-fold between two regions. Fold change in mRNA abundance between regions is indicated for microarrays and qPCR analysis as well as P values for qPCR statistical analysis.