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Microarray analysis reveals distinctive signaling between the bed nucleus of the stria terminalis, nucleus accumbens, and dorsal striatum

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Olsen CM, Huang Y, Goodwin S, Ciobanu DC, Lu L, Sutter TR, Winder DG. Microarray analysis reveals distinctive signaling between the bed nucleus of the stria terminalis, nucleus accumbens, and dorsal striatum. *Physiol Genomics* 32: 283–298, 2008. First published October 2, 2006; doi:10.1152/physiolgenomics.00224.2006.—To identify distinct transcriptional patterns between the major subcortical dopamine targets commonly studied in addition we studied differences in gene expression between the bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAc), and dorsal striatum (dStr) using microarray analysis. We first tested for differences in expression of genes encoding transcripts for common neurotransmitter systems as well as calcium binding proteins routinely used in neuroanatomical delineation of brain regions. This a priori method revealed differential expression of corticotropin releasing hormone (*Crh*), the GABA transporter (*Slc6a1*), and prodynorphin (*Pdyn*) mRNAs as well as several others. Using a gene ontology tool, functional scoring analysis, and Ingenuity Pathway Analysis, we further identified several physiological pathways that were distinct among these brain regions. These two different analyses both identified calcium signaling, G-coupled protein receptor signaling, and adenylate cyclase-related signaling as significantly different among the BNST, NAc, and dStr. These types of signaling pathways play important roles in, amongst other things, synaptic plasticity. Investigation of differential gene expression revealed several instances that may provide insight into reported differences in synaptic plasticity between these brain regions. The results support other studies suggesting that crucial pathways involved in neurotransmission are distinct among the BNST, NAc, and dStr and provide insight into the potential use of pharmacological agents that may target region-specific signaling pathways. Furthermore, these studies provide a framework for future mouse-mouse comparisons of transcriptional profiles after behavioral/pharmacological manipulation.

mouse; brain; Ingenuity Pathways knowledge database; functional class scoring; gene ontology

DOPAMINERGIC TRANSMISSION plays a key role in many aspects of motivated behavior and plays important roles in pathophysiological states such as addiction and Parkinson's disease. Dopaminergic innervation of the central nervous system emanates predominantly from discrete midbrain and brainstem nuclei. The two major centers are the substantia nigra (SN) and the ventral tegmental area (VTA) (17); however, other nuclei, such as the periaqueductal gray (PAG), are also thought to be important contributors (4). These centers provide only partially

overlapping innervation of the forebrain. The primary subcortical structures innervated by these regions are the dorsal striatum (dStr), nucleus accumbens (NAc), and bed nucleus of the stria terminalis (BNST), for the SN, VTA, and VTA/PAG, respectively (13, 44). As with dopaminergic transmission in general, these brain regions have similarly been implicated in pathophysiological states ranging from Parkinson's disease to drug addiction (24, 25, 64, 118).

Among the dStr, NAc, and BNST, characteristics such as cell morphology and the presence of particular neuropeptides are similar (58, 59), although specific afferents and efferents are varied (20, 30, 48). A common feature of these three regions is that they integrate cortical and subcortical inputs to shape appropriate behavioral responses to stimuli (15, 63, 82). The dStr is primarily involved in motor function and initiation of movement (36, 50), the NAc is associated with the translation of motivational states into behavior (74), and the BNST has been associated with stress, anxiety and the expression of “fight or flight” responses (29).

The dStr, NAc, and BNST likely play distinct roles in addiction. The dStr is known to be involved in habit learning (38, 79), and drug-induced plasticity in the dStr has been proposed to underlie the compulsive nature of addiction (25, 34). Emerging evidence also suggests that the dStr may play an important role in relapse (27, 31, 110) and craving (101) after abstinence. The NAc has been associated with the acute reinforcing effects of both natural and drug reinforcers (10, 117, 119). It is also known to undergo extensive neuroadaptations (76, 114) and is crucial for the expression of reinstatement, an animal model of drug relapse (61). The BNST has been proposed to mediate stress and reward interactions, especially in regards to addiction (18, 104). Interruption of signaling within the BNST reduces the behavioral signs of morphine (5) and ethanol (89) withdrawal and prevents stress-induced drug-seeking in animals that previously self-administered cocaine (24). To further elucidate similarities and differences in these addiction-related brain regions, we performed microarray analysis on each tissue to compare transcriptional profiles between them.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) were housed in the Vanderbilt Animal Care Facilities in groups of four or five and were 12 wk old at the time of death. Male *Penk1*-EGFP BAC-transgenic hemizygous mice were bred from a founder line purchased from the Mutant Mouse Regional Resource

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Centers (<http://www.mmrc.org/>). Mice were maintained in temperature- and humidity-controlled rooms and kept on a 12-h light/dark cycle, with the lights on from 0600 to 1800 h. Food and water were available ad libitum. Experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and were approved by the Vanderbilt University Animal Care and Use Committee.

Tissue Dissection

Mice were briefly anesthetized with isoflurane and then rapidly decapitated. Brains were submerged in oxygenated (95% O₂-5% CO₂) ice-cold sucrose-artificial cerebrospinal fluid solution (in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl₂, 1 MgCl₂, 1.2 NaH₂PO₄, 10.0 glucose, and 26.0 NaHCO₃), and coronal brain slices (300 μ m) were made with a vibratome (Leica). Slices were transferred onto a glass Petri dish with a transfer pipette, and excess fluid was drained from the area surrounding the tissue. The Petri dish was placed on top of a chilled aluminum block, and tissue was brought to a semifrozen state before punches were taken. Punches (0.50 mm; Fine Science Tools, Foster City, CA) were taken from slices containing NAc (bregma $\sim$ 1.18), dStr (bregma $\sim$ 0.86) and BNST (bregma $\sim$ 0.14, see Fig. 1). Bilateral punches for each region were pooled into frozen sample tubes and stored at -80°C as identified from the atlas of Paxinos and Franklin (81). Tissue for quantitative reverse transcriptase polymerase chain reaction (qPCR) consisted of bilateral punches from three mice pooled together per sample. As a result, tissue from nine mice was used to obtain an n of 3 for qPCR.

Visualization of Penk1-driven EGFP expression

For visualizing enhanced green fluorescent protein (EGFP) expression, we obtained brain slices from a *Penk1*-EGFP mouse as described

Fig. 1. Diagram of punches taken from nucleus accumbens (NAc), dorsal striatum (dStr), and bed nucleus of the stria terminalis (BNST). We took 0.5 mm punches as illustrated from 300 μ m sections at the approximate coordinates shown. Figure adapted from Ref. 81.

Fig. 2. Pair-wise variance of all present genes. Difference vs. average (MA) plots of each pair-wise comparison. The percentage of genes differing by ≥ 2 log₂ units (¥) is displayed for each comparison.

and transferred them to glass-bottom culture dishes (MatTek, Ashland, MA). Slices were observed under a stereomicroscope equipped with a GFP filter set (SZX12; Olympus, Center Valley, PA) under identical illumination and exposure. Images were acquired with a

QICAM monochrome camera and QCapture software (QImaging, Burnaby, BC, Canada). Images were analyzed for fluorescence intensity using ImageJ (88). Briefly, a circular region of interest approximating the size of the punches was placed over each brain region, and the mean intensity of the region was measured. This was done for each hemisphere, and the mean of the two hemispheres was reported (see Fig. 5C). Following analysis, images were pseudocolored and overlaid with wireframes adapted from a mouse brain atlas (see Fig. 5, A and B) (81).

RNA Amplification, Target Synthesis, and Microarray Analysis

Amplification of RNA isolated from tissue was required to provide sufficient material for target synthesis (28, 109). Total RNA was isolated from the frozen tissue using Stat-60 (Tel-Test, Friendswood, TX). The RNA was resuspended in RNase-free water, and the concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE). To ensure that high-quality RNA was obtained, 1 μ l of each sample was analyzed on a Agilent Bioanalyzer 2100 using the RNA 6000 Pico Series II (Agilent technologies, Palo Alto, CA). Starting with 10 ng of total RNA, cRNA targets were generated by the two-cycle target labeling method (1, 22). Briefly, first-strand cDNA synthesis using a T₇(dT)₂₄ oligonucleotide was followed by second-strand cDNA synthesis (Invitrogen

Life Technologies, Carlsbad, CA). The double-stranded cDNA was ethanol precipitated, washed, and resuspended in water. In vitro transcription of the double-stranded cDNA was performed using MEGAscript T7 High Yield Transcription kit (Ambion, Austin, TX). Incubation was performed for 6 h at 37°C, and the sample was purified using a RNA-easy clean-up kit (Qiagen, Valencia, CA). For the second round of cDNA synthesis, cRNA and 200 ng random primers (Invitrogen) were denatured for 10 min at 70°C and cooled on ice for 2 min, followed by first-strand synthesis (Invitrogen). The mixture was incubated at 42°C for 1 h, followed by the addition of 2 units RNase H, and then incubated at 37°C for 20 min, followed by 95°C for 5 min. T₇(dT)₂₄ oligonucleotide (5 μ M) was added, and the mixture was incubated for 6 min at 70°C and then cooled on ice. Second-strand cDNA synthesis was performed as described above. Biotin-labeled cRNA was produced using the ENZO BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochemical, New York, NY) according to the manufacturer's instructions. Labeled cRNA was purified, and 20 μ g of cRNA was fragmented to a range of 35–200 bases in length. Samples were hybridized at 45°C for 16 h to Affymetrix mouse430_2 chips (containing 54k probe sets) according to standard GeneChip Expression assay protocol (1). After hybridization, the chips were washed and scanned using a GeneChip Scanner. The P (Present)- or A (Absent)-calls of the probe sets in the gene

Fig. 3. Expression profile of a priori genes in dStr, NAc, and BNST. The 30 most differential genes from the a priori list were displayed using dChip.

Fig. 4. In situ hybridization showing enrichment of corticotropin releasing hormone (*Crh*) in BNST. Coronal sections of mouse brain showing low expression of *Crh* in dStr and NAc (left) and high expression of *Crh* in the BNST relative to adjacent striatum (right). Images taken from the Allen Brain Atlas (www.brain-map.org). Image IDs: Crh_326_2040 (left) and Crh_294_2040 (right).

expression chips were determined by the Affymetrix GCOS v1.4. Chip quality, including RNA degradation, cDNA synthesis, hybridization, chip washing and scanning, was evaluated with GCOS v1.4, dChip and Bioconductor “affy” package. All RNA samples and chips adopted in current study passed the quality criteria (Supplemental Table S1 and Supplemental Fig. S1).¹ The intensities of probe sets were calculated by dChip software with Perfect-match/Mismatch difference model after invariant-set normalization (65). The microarray data have been submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus repository (series accession no. GSE5763).

qPCR Analysis

Total RNA was extracted from the frozen tissue using Stat-60 (Tel-Test). The RNA was suspended in RNase-free water, and its quality and quantity were determined using an Agilent-Bioanalyzer 2100. The total RNA was treated with DNase I (DNA-free kit; Ambion, Austin, TX) to remove any traces of DNA that could contaminate the samples and interfere with quantification. Following DNase treatment the RNA concentration was quantified using a NanoDrop spectrophotometer. cDNA synthesis was performed on equal amounts of total RNA per sample using random hexamers following the protocol provided by manufacturer (First strand cDNA Synthesis Kit; GE Healthcare Bio-Sciences, Piscataway, NJ). PCR amplification of a fragment of *Ppp1r13b* (Mm.313076) spanning a small intron did not show any evidence of genomic DNA contamination.

Genes that contributed to Ingenuity Pathway Analysis (IPA) results were considered for qPCR validation. We selected genes differentially

expressed between 1) BNST and NAc and 2) BNST and dStr and used qPCR assays approximating microarray probe location. The quantitative RT-PCR assays were selected using Universal Probe Library (www.universalprobelibrary.com, Roche Diagnostics, IN) (Supplemental Table S4). Most of the assays used span an intron while the remaining few targeted the 3′ untranslated region. The expression of these genes was measured using qPCR assays with cyclophilin D as a reference gene for comparative threshold quantification. This reference gene is not differentially expressed among three regions. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a LightCycler 480 System (Roche Diagnostics) and the standard protocol for the LC480 Probes Master (Roche Diagnostics). The efficiency of PCR amplification was performed for each of the assay using mouse (C57BL/6J) forebrain RNA. The expression of each gene was normalized by simultaneously assessing the reference gene in each experimental sample. Each experimental sample was assayed in duplicate. Standard curves for five 10-fold dilution steps between 2,500 and 0.25 ng of reverse-transcribed RNA samples were run for all primer pairs to determine the PCR efficiency under the experimental conditions for the reference and all selected genes. All PCR reactions including standard curves were performed in technical duplicates.

Data Analysis

For a priori analysis, we tested a list of genes encoding transcripts involved in common neurotransmitter systems as well as calcium binding proteins routinely used in neuroanatomical delineation of brain regions. We compiled this list prior to any analysis to include genes encoding prohormones, synthesis and degradation enzymes, transporters, and receptor subunits for common neurotransmitters (Supplemental Table S2). Only probe sets with P calls

¹ The online version of this article contains supplemental material.

in all three replicates of at least one brain region were used. Expression values for a priori transcripts from all three brain regions were analyzed by ANOVA followed by uncorrected Fisher's least significant difference comparisons, and the most significant genes were displayed by hierarchical clustering in dChip using the default clustering algorithm (65).

Transcriptional network and pathway analysis was performed on pair-wise comparisons between brain regions by two methods previously shown to give complementary results using different algorithms (51). First, functional class scoring (FCS) analysis was performed with the software downloaded from www.geneontology.org/GO.tools/microarray.shtml#ermine and implemented in a JAVA environment. FCS was used on a list of all transcripts that were expressed (determined by P calls) in 3/3 replicates for at least one region in the pair-wise comparison. The intensities of the expressed genes were analyzed by unpaired *t*-test followed by Benjamini-Hochberg procedure to correct *P* value (called *q*-value) for multiple tests using GeneSpring v7.0 (Agilent Technologies). For repeated occurrence of a gene (a gene was represented by 2 or more probe sets in the chips), only the best (minimum) *q*-value was used in FCS analysis (80). Second, IPA (<http://www.ingenuity.com>) was performed on differentially expressed genes ("focus genes") that met these criteria: 1) expressed in 3/3 replicates for at least one region in the comparison, 2) a fold difference ≥ 1.5 , 3) signal intensity difference ≥ 60 and 4) *q*-value < 0.065 . IPA uses a powerful database, the Ingenuity pathways knowledge base (IPKB), to reveal biological pathways that are significantly different among sample groups. The IPKB is a database curated by scientists that includes hundreds of thousands of modeled relationships between genes, proteins, anatomy, biological processes, and disease. The significance of a pathway is controlled by *P* value, which is calculated using the right-tailed (referring here to the overrepresented pathway) Fisher Exact Test for 2×2 contingency tables. This is done by comparing the number of "focus genes" that participate in a given canonical pathway, relative to the total number of occurrences of those genes in all networks or pathways stored in the IPKB. The significance threshold of pathways was set to 1.3 (derived by $-\log_{10} [P \text{ value}]$, whereas $P \leq 0.05$).

Validation of Ingenuity Results Using the Allen Brain Atlas

The top five canonical pathways revealed by IPA were subject to validation using an independent measure of gene expression. Individual genes contributing to differences in IPA (see Tables 2–5 and S3 for gene lists) were queried using the Allen Brain Atlas (www.brain-map.org), and images of coronal sections including BNST, NAc, and Str from in situ hybridization experiments were acquired (see Figs. 4 and 7 for representative images). For consistency, expression studies not available in coronal format were not used. From the acquired images, square regions of interest approximating the size and placement of our tissue punches (see Fig. 1) were analyzed for the number of labeled cells using ImageJ (88) by an experimenter blind to microarray results. Cell counts in pair-wise comparisons were represented as relative abundance values (RAVs, number of labeled cells in region 1/region 2) and compared with RAVs derived from microarray analysis. Regression analysis was performed on RAVs from the cell counts and microarray data (see Fig. 8A) and Pearson correlation coefficients were reported (Fig. 8B).

Validation of Ingenuity Results using qPCR

The mean crossing thresholds (CT) obtained for the technical duplicates of the different amplicons were statistically processed to calculate mean normalized expression (MNE) values that reflect the relative expression of the target gene compared with the reference gene by taking the efficiencies of the PCR reaction into account (100). MNE values were log10 transformed and compared by Bonferroni corrected *t*-tests within each pair-wise comparison (i.e., BNST vs. Str). Mean fold difference values were calculated using the expression

Fig. 5. Expression of enhanced green fluorescent protein (EGFP) under the control of the *Penk1* promoter. A and B: coronal sections of mouse brain showing expression of EGFP reporter under the control of the *Penk1* promoter. C: quantification of EGFP luminance for each region. Wireframe diagrams adapted from Ref. 81. Aca, anterior commissure; AcbC and AcbSh, nucleus accumbens core and shell; BST, bed nucleus of the stria terminalis; LD, lateral dorsal; LJ, lateral juxtacapsular; LP, lateral posterior; MA, medial anterior.

Table 1. *Functional class scoring analysis*

A. BNST vs. dStr				
ID	Name	Probes		P Value
		Total	Present	
GO:0006813	potassium ion transport	135	78	0.0029
GO:0007200	G protein signaling, coupled to IP3 second messenger (phospholipase C activating)	66	37	0.0041
GO:0007190	adenylate cyclase activation	35	25	0.0221
GO:0009190	cyclic nucleotide biosynthesis	15	13	0.0227
GO:0007610	behavior	153	97	0.0396
GO:0007411	axon guidance	104	57	0.0437
GO:0007189	G protein signaling, adenylate cyclase activating pathway	45	30	0.0443
GO:0009966	regulation of signal transduction	119	69	0.0465
GO:0009593	detection of chemical stimulus	31	13	0.0474
GO:0045055	regulated secretory pathway	102	52	0.0488
GO:0051057	positive regulation of small GTPase mediated signal transduction	7	5	0.0500
B. BNST vs. NAc				
ID	Name	Probes		P Value
		Total	Present	
GO:0006887	exocytosis	132	70	0.0151
GO:0050804	regulation of synaptic transmission	21	10	0.0356
GO:0001525	angiogenesis	58	41	0.0405
GO:0050769	positive regulation of neurogenesis	22	11	0.0426
GO:0007200	G protein signaling, coupled to IP3 second messenger (phospholipase C activating)	64	35	0.0440
GO:0006883	sodium ion homeostasis	12	6	0.0451
GO:0016337	cell-cell adhesion	158	100	0.0462
GO:0017157	regulation of exocytosis	29	15	0.0464
C. NAc vs. dStr				
ID	Name	Probes		P Value
		Total	Present	
GO:0009593	detection of chemical stimulus	30	13	0.0011
GO:0005513	detection of calcium ion	30	13	0.0023
GO:0030198	extracellular matrix organization and biogenesis	49	27	0.0254
GO:0030073	insulin secretion	17	9	0.0345
GO:0045898	regulation of transcriptional preinitiation complex formation	19	5	0.0406
GO:0030199	collagen fibril organization	12	9	0.0486

The most significant functional classes from pair-wise analysis between BNST and dStr (A), BNST and NAc (B), and NAc and dStr (C). Total probes refers to total probe sets in the chip. Present probes refers to probe sets in 3/3 chips in one or both of the compared brain regions. BNST, bed nucleus of the stria terminalis; dStr, dorsal striatum; NAc, nucleus accumbens.

Fig. 6. Ingenuity pathway analysis showing all canonical pathways significantly different in pair-wise comparisons. Pathways shown include all pathways significantly different in at least one pair-wise comparison.

in BNST (BNST vs. Str and BNST vs. NAc comparisons) or NAc (NAc vs. Str comparisons) as a reference.

RESULTS

Quality Control

The 260/280 ratio of total RNA in water was >1.7 , while the cRNA concentration was $>2.4 \mu\text{g}/\mu\text{l}$ for all samples. The chip quality was checked by GCOS v1.4, with RawQ <2 , Scaling factor <8.5 , Background <60 , percentage of P calls $>42\%$ for every sample (actual values in Supplemental Table S1). However, the range of actin 3'/5' and GAPDH 3'/5' was 3.2–9.6. This was caused by the 2nd round cDNA amplification protocol, whereas the oligo(dT) primers were used twice in the procedure, and thus significantly increased the amplification at 3' (22, 109). The chip quality was further validated by dChip. "Array outlier" was $<0.37\%$ and "single outlier" $<0.05\%$ (65). The affy package of Bioconductor was used to generate an RNA degradation plot for each array. On each chip, probe intensities were averaged by location in probe set, with the average taken over all probe sets (Supplemental Fig. S1). The large slopes confirmed the higher amplification efficiency in 3'. Importantly, the slopes are similar across all chips, indicating there was no RNA degradation and the efficiency of cDNA amplification synthesis was consistent among samples. There was no chip outlier inferred by the two-round cDNA amplification protocol in the current study.

Pair-wise Variance of All Expressed Genes

Expressed genes (P calls in 3/3 replicates) were represented as log₂ signal intensity. To compare consistency in gene expression between brain regions, replicate means were plotted in pair-wise Difference vs. Average (MA) plots (Fig. 2, A–C) (21). Briefly, the log₂ average signal intensity (A) was plotted on the abscissa and the log₂ difference between replicates (M) was plotted on the ordinate. These scatter plots illustrate pair-wise differences in signal intensity relative to the mean signal intensity for each expressed gene. From each MA plot, the percentage of genes having a signal intensity difference of >2 log₂ units (¥) was calculated and reported for each pair-wise comparison. Differences were greatest between BNST and dStr (Fig. 2A, ¥ = 1.91), while differences were minimal between NAc and dStr (Fig. 2C, ¥ = 0.30). This finding is not surprising as BNST and dStr are the most disparate in cell types, while dStr and NAc are highly similar (predominantly medium spiny neurons with a small population of cholinergic and GABAergic interneurons) (37, 58, 59).

A Priori Analysis of Common Neurotransmitter Systems

The expression profile of the most differential a priori genes was displayed in dChip (Fig. 3). Analysis of genes representing common neurotransmitter systems and calcium binding proteins revealed distinct patterns of expression, many of which are consistent with previously reported data (see below). Hierarchical cluster analysis on the individual samples correctly grouped replicates in all three regions. Samples also were divided into two further clusters (labeled 1 and 2, Fig. 3) with NAc and dStr residing in the same cluster. This is the same trend that was seen when all expressed genes were analyzed, as the percentage of genes with signal intensity >2 log₂ units (¥)

Table 2. Differentially expressed genes involved in cAMP-dependent signaling as revealed by Ingenuity pathway analysis

A. BNST vs. dStr		
Description	RAV	q Value
Glutamate receptor, metabotropic 4	2.6	0.002
Phosphodiesterase 10A	39.9	0.002
Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	1.9	0.003
Calcium/calmodulin-dependent protein kinase I	−2.9	0.004
GNAS complex locus	−2.5	0.004
Adenylate cyclase 2 (brain)	−1.6	0.005
Calmodulin 2 (phosphorylase kinase, delta)	2.2	0.005
Calcium/calmodulin-dependent protein kinase IV	9.9	0.005
Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	−7	0.005
Regulator of G protein signaling 4	6.1	0.005
A kinase (PRKA) anchor protein (yotiao) 9	2.3	0.006
Opioid receptor, kappa 1	4.1	0.006
Phosphodiesterase 7B	5.1	0.006
Rap guanine nucleotide exchange factor (GEF) 3	−2.3	0.006
B. BNST vs. NAc		
Description	RAV	q Value
Adenosine A1 receptor	1.7	0.044
Glutamate receptor, metabotropic 4	1.8	0.044
Phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	2.0	0.048
Dopamine receptor D2	2.7	0.052
5-Hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled)	−1.8	0.052
Opioid receptor, kappa 1	4.8	0.052
Protein kinase, cAMP-dependent, regulatory, type II, beta	2.7	0.056
Dopamine receptor D1	6.2	0.061
Adenylate cyclase 5	2.3	0.062
Phosphodiesterase 10A	6.4	0.063
Calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	−1.5	0.065
Rap guanine nucleotide exchange factor (GEF) 3	−1.5	0.065
C. NAc vs. dStr		
Description	RAV	q Value
Adenylate cyclase 1 (brain)	2.0	0.014
Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	−1.5	0.014
Phosphodiesterase 10A	7.0	0.014
Phosphodiesterase 1A, calmodulin-dependent	−6.0	0.014
Protein kinase, cAMP-dependent, regulatory, type II, alpha	−2.0	0.014
Regulator of G protein signaling 4	3.6	0.014
Rap guanine nucleotide exchange factor (GEF) 3	−1.5	0.017
Dopamine receptor D2	2.6	0.018
Phosphodiesterase 7B	2.7	0.022
Calmodulin 2 (phosphorylase kinase, delta)	1.5	0.025
Rap guanine nucleotide exchange factor (GEF) 4	1.7	0.025
A kinase (PRKA) anchor protein (yotiao) 9	2.6	0.026
Calcium/calmodulin-dependent protein kinase IV	3.5	0.026
Calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	2.3	0.029
Calcium/calmodulin-dependent protein kinase I	−2.3	0.031
Phosphodiesterase 1B, calmodulin-dependent	1.9	0.036
Mitogen-activated protein kinase kinase 1	−1.5	0.052
Adenosine A1 receptor	1.8	0.058
Regulator of G protein signaling 2, 24 kDa	1.5	0.062

Positive relative abundance value (RAV) indicates higher values in the 2nd brain region of each comparison.

was lowest between the NAc and dStr ($\Upsilon = 0.30$, Fig. 2). Expression levels of genes were grouped into four primary clusters (Fig. 3). The most differentially expressed genes in cluster A were corticotropin releasing hormone (*Crh*) and $\beta 1$ subunit of GABA_A receptor (*Gabrb1*), where a trend for the greatest enrichment in BNST relative to dStr. The BNST is known to have neurons that contain corticotropin releasing hormone (CRH/CRF) (83, 92). CRF projections from the amygdala are known to project to the NAc (92), although some medial NAc neurons have also been reported to contain CRF (57, 73). In situ hybridization data from the Allen Brain Atlas (3) illustrate the same trend of *Crh* expression being greatest in the BNST relative to the NAc or dStr (Fig. 4). Cluster B contained three genes that were lower in dStr relative to BNST and NAc. Two of these genes have to do with GABAergic signaling: the GABA transporter *Slc6a1* and the $\alpha 5$ GABA_A receptor subunit *Gabra5*. While GABAergic neurons are prevalent in the dStr, *Slc6a1* (GAT-1) is only reported to be present in 3–5% of striatal neurons (6). Additionally, striatal $\alpha 5$ GABA_A receptor subunit immunoreactivity is very low, although levels in the BNST were also reported to be low (84). Cluster C represents just over half of the genes, where the trend is for elevated expression in the dStr, moderate expression in the NAc, and low expression in the BNST. The most divergent region of this cluster also contains two genes involved

in GABAergic signaling, the GABA_A receptor subunits δ (*Gabrd*) and $\alpha 4$ (*Gabra4*). Immunoreactivity for the δ subunit was previously shown to be higher in NAc and dStr than BNST and $\alpha 4$ immunoreactivity ranked in the same order as the expression pattern in the cluster diagram: highest in dStr, moderate in NAc, and lowest in BNST (84). Analysis of EGFP expression from a *Penk1* reporter mouse revealed the same trend of expression as cluster analysis for *Penk1* (Figs. 3 and 5). Specifically, expression was highest in the dStr and lower in NAc and BNST. Cluster D represents genes enriched in NAc and includes prodynorphin (*Pdyn*) and the AMPA glutamate receptor 1 subunit (*Gria1*, GluR1). Prodynorphin immunoreactivity has been reported to be present in very high levels in NAc relative to dStr in rodents (112) and humans (52), although its presence has also been described in BNST of the rat (26). In the primate, GluR1 has been described as higher in the NAc than the dStr (70).

FCS Analysis of Brain Regions

BNST vs. dStr. FCS was performed using 19,557 probe sets found to be present in at least one of the two brain regions (see MATERIALS AND METHODS for details). Table 1A shows the 11 Gene ontology (GO) classes that were significantly different between the BNST and striatum (Str). The

Table 3. Differentially expressed genes involved in calcium signaling as revealed by Ingenuity pathway analysis

A. BNST vs. dStr			
Function	Description	RAV	q Value
Ion channel	glutamate receptor, ionotropic, kainate 1	−8.0	0.003
Ion channel	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	−3.5	0.003
Phosphatase	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform (calcineurin A alpha)	1.9	0.003
Ion channel	calcium channel, voltage-dependent, L type, alpha 1C subunit	1.8	0.004
Kinase	calcium/calmodulin-dependent protein kinase I	−2.9	0.004
Transporter	solute carrier family 8 (sodium/calcium exchanger), member 1	−8.0	0.004
Other	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	−7.0	0.005
Kinase	calcium/calmodulin-dependent protein kinase IV	9.9	0.005
Kinase	calcium/calmodulin-dependent protein kinase kinase 2, beta	4.6	0.005
Kinase	calmodulin 2 (phosphorylase kinase, delta)	2.2	0.005
B. NAc vs. dStr			
Function	Description	RAV	q Value
Other	tropomyosin 1 (alpha)	3.6	0.013
Ion channel	calcium channel, voltage-dependent, beta 2 subunit	1.7	0.014
Kinase	protein kinase, cAMP-dependent, regulatory, type II, alpha	−2.0	0.014
Ion channel	transient receptor potential cation channel, subfamily C, member 3	2.4	0.014
Transporter	ATPase, Ca ²⁺ transporting, plasma membrane 2	2.4	0.016
Ion channel	calcium channel, voltage-dependent, gamma subunit 4	2.2	0.017
Transporter	solute carrier family 8 (sodium/calcium exchanger), member 1	−5.8	0.018
Kinase	calcium/calmodulin-dependent protein kinase kinase 2, beta	3.0	0.024
Other	calmodulin 2 (phosphorylase kinase, delta)	1.5	0.025
Kinase	calcium/calmodulin-dependent protein kinase IV	3.5	0.026
Kinase	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	2.3	0.029
Kinase	calcium/calmodulin-dependent protein kinase I	−2.3	0.031
Kinase	calcium/calmodulin-dependent protein kinase ID	1.9	0.031
Ion channel	calcium channel, voltage-dependent, gamma subunit 2	2.5	0.033
Ion channel	calcium channel, voltage-dependent, beta 4 subunit	1.6	0.036
Transporter	ATPase, Ca ²⁺ transporting, plasma membrane 1	2.0	0.038
Ion channel	inositol 1,4,5-triphosphate receptor, type 1	2.0	0.039
Ion channel	glutamate receptor, ionotropic, N-methyl-D-aspartate 3A	−2.7	0.04
Other	calsequestrin 2 (cardiac muscle)	2.3	0.043
Ion channel	ryanodine receptor 1 (skeletal)	2.0	0.055

Positive RAV indicates higher values in the 2nd brain region of each comparison.

most significantly different class was potassium ion transport (GO:0006813), with 78 genes contributing to this result. Several of the GO classes were associated with G-coupled protein receptor (GPCR) signaling, including G protein signaling, coupled to IP3 second messenger (GO:0007200), adenylate cyclase activation (GO:0007190), cyclic nucleotide biosynthesis (GO:0009190), G protein signaling, adenylate cyclase activating pathway (GO:0007189), and positive regulation of small GTPase-mediated signal transduction (GO:0051057). This trend suggests that neurotransmission likely differs in fundamental ways between the BNST and dStr in several types of G-coupled protein receptor systems.

BNST vs. NAc. FCS was performed on 19,025 probe sets for the comparison of BNST and NAc. Six GO classes were identified as unique between these two regions (Table 1B). These were less specific than those identified in BNST vs. dStr. Some GO classes such as detection of chemical stimulus (GO:0009593) and calcium ion (GO:0005513) may be directly related to neural transmission, while other classes included diverse processes.

NAc vs. dStr. Of 19,557 probe sets analyzed, FCS analysis detected eight significant GO classes different between the NAc and dStr (Table 1C). Exocytosis was represented by two GO classes (GO:0006887 and GO:0017157). FCS also revealed differences between the NAc and dStr in cellular and/

or synaptic remodeling: angiogenesis (GO:0001525), positive regulation of neurogenesis (GO:0050769), and cell-cell adhesion (GO:0016337). The accumbens was also found to differ from the dStr in a similar pathway, extracellular matrix organization and biogenesis (GO:0030198, Table 1B).

IPA

In contrast to FCS analysis, IPA only analyzes genes differentially expressed in pair-wise compared brain regions, called "focus genes" (see MATERIALS AND METHODS). First, unpaired *t*-tests were performed on the genes present in at least one region (19,557, 19,025, and 19,557 genes for BNST/NAc, BNST/Str, and NAc/Str pair-wise comparisons, respectively.) From these comparisons, genes were found to be differentially expressed by stringent criteria (see MATERIALS AND METHODS for details) were submitted to IPA (177, 196, and 373 focus genes were found for BNST/NAc, BNST/Str, and NAc/Str pair-wise comparisons.) Figure 6 shows the pathways found to be significantly different in at least one pair-wise analysis using IPA. Similar to FCS analysis, cyclic adenosine monophosphate (cAMP)-mediated signaling, calcium signaling, and G-coupled protein receptor signaling pathways were the most distinct among the three regions studied. These types of signaling are prominent in many brain regions, but the data suggest that

Fig. 7. In situ hybridization showing enrichment of *Camk4* in dStr. Coronal sections of mouse brain showing high expression of *Camk4* in the dStr relative to NAc and BNST. Images taken from the Allen Brain Atlas (www.brain-map.org). Image IDs: Camk4_370_0303121839 (left) and Camk4_322_0303111783 (right).

there are differences in the specific gene products involved in these signaling pathways between the BNST, NAc, and dStr. For example, phosphodiesterases (PDEs) are unique to each region, the most marked result being that *Pde10a* is highly enriched in dStr relative to both BNST and NAc (Table 2b). The PDE isoform encoded by *Pde10a* is unique in that it hydrolyzes both cAMP and cGMP (32). It has been previously reported to be enriched in Str (32, 33), where it plays a critical role in modulating activity of striatal neurons (103). Transcripts of particular adenylate cyclase isoforms are also enriched in each brain region. For example, adenylate cyclase 2 (*Adcy2*) is enriched in the BNST relative to Str. This isoform

is stimulated by PKC (55) and by both $G_{s\alpha}$ as and $G_{\beta\gamma}$ subunits, where it is thought to serve as a coincidence detector stimulated by $G_{\beta\gamma}$ subunits in the context of activation by $G_{s\alpha}$ (16, 107). This suggests that adenylate cyclase activity within the BNST could be stimulated by a greater variety of GPCRs than as just $G_{s\alpha}$. IPA also identified *Adcy5* (adenylate cyclase 5) as being enriched in NAc relative to BNST (Table 2B) and *Adcy1* (adenylate cyclase 1) as enriched in dStr relative to NAc (Table 2C). Activity of the adenylate cyclase (AC) 5 isoform is inhibited by calcium/calmodulin, while AC1 is stimulated by calcium/calmodulin (for review, see Ref. 106). Also within the adenylate cyclase signaling pathway, regulator of G-coupled

Table 4. Differentially expressed genes involved in G-coupled protein receptor signaling as revealed by Ingenuity pathway analysis

A. BNST vs. dStr			
Function	Description	RAV	q Value
G protein-coupled receptor	glutamate receptor, metabotropic 4	2.6	0.002
G protein-coupled receptor	endothelin receptor type B	-3.9	0.003
Enzyme	GNAS complex locus	-2.5	0.004
Enzyme	adenylate cyclase 2 (brain)	-1.6	0.005
Kinase	v-akt murine thymoma viral oncogene homolog 2	2.3	0.005
Kinase	calcium/calmodulin-dependent protein kinase IV	9.9	0.005
Kinase	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	-7.0	0.005
Other	regulator of G protein signaling 4	6.1	0.005
G protein-coupled receptor	opioid receptor, kappa 1	4.1	0.006
Kinase	protein kinase C, beta 1	2.7	0.006
Other	Rap guanine nucleotide exchange factor (GEF) 3	-2.3	0.006
B. BNST vs. NAc			
Function	Description	RAV	q Value
G protein-coupled receptor	endothelin receptor type B	-2.6	0.026
G protein-coupled receptor	adenosine A1 receptor	1.7	0.044
G protein-coupled receptor	glutamate receptor, metabotropic 4	1.8	0.044
Enzyme	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	2.0	0.048
G protein-coupled receptor	dopamine receptor D2	2.7	0.052
G protein-coupled receptor	5-hydroxytryptamine (serotonin) receptor 7 (adenylate cyclase-coupled)	-1.8	0.052
G protein-coupled receptor	opioid receptor, kappa 1	4.8	0.052
Kinase	protein kinase, cAMP-dependent, regulatory, type II, beta	2.7	0.056
G protein-coupled receptor	dopamine receptor D1	6.2	0.061
Enzyme	adenylate cyclase 5	2.3	0.062
Kinase	calcium/calmodulin-dependent protein kinase (CaM kinase) II gamma	-1.5	0.065
Other	Rap guanine nucleotide exchange factor (GEF) 3	-1.5	0.065
C. NAc vs. dStr			
Function	Description	RAV	q Value
Enzyme	adenylate cyclase 1 (brain)	2.0	0.014
G protein-coupled receptor	endothelin receptor type B	-1.5	0.014
Enzyme	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	-1.5	0.014
Enzyme	phosphodiesterase 1A, calmodulin-dependent	-6.0	0.014
Kinase	protein kinase, cAMP-dependent, regulatory, type II, alpha	-2.0	0.014
Other	regulator of G protein signaling 4	3.6	0.014
Enzyme	phospholipase C, beta 1 (phosphoinositide-specific)	3.0	0.015
Other	Rap guanine nucleotide exchange factor (GEF) 3	-1.5	0.017
G protein-coupled receptor	dopamine receptor D2	2.6	0.018
Enzyme	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	1.9	0.023
Kinase	calcium/calmodulin-dependent protein kinase IV	3.5	0.026
Kinase	calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	2.3	0.029
Enzyme	phosphodiesterase 1B, calmodulin-dependent	1.9	0.036
Kinase	mitogen-activated protein kinase kinase 1	-1.5	0.052
Kinase	protein kinase C, beta 1	1.8	0.052
G protein-coupled receptor	adenosine A1 receptor	1.8	0.058
Other	regulator of G-protein signaling 2, 24 kDa	1.5	0.062

Positive RAV indicates higher values in the second brain region of each comparison.

signaling (RGS2 and RGS4, enzymes that attenuate signaling through G-coupled protein receptors, showed differential enrichment of transcripts (*Rgs2* and *Rgs4*, respectively). Isoforms of these enzymes have different target specificities, where RGS2 inhibits $G_{q\alpha}$ and AC5 (which had enriched transcript levels in NAc), but not AC1 or AC2 (enzymes whose transcripts were enriched in dStr and BNST, respectively) (102), and RGS4 inhibits $G_{i/o\alpha}$ (9, 111) and $G_{q\alpha}$ (47). RGS2 and RGS4 are intimately related to dopaminergic signaling, as transcription is regulated by D1 and D2 dopamine receptors, respectively (35, 54), and RGS4 polymorphisms have been associated with schizophrenia in human populations (43). Like adenylate cyclase, calcium/calmodulin-dependent protein kinase (CaMK) isoforms are also differentially enriched in all three brain regions and have different means of transduction. The CaMKs are a prevalent family of kinases that are crucial to neural function, representing ~1% of total brain protein (for review, see Refs. 41, 90). The most common is CaMKII, which phosphorylates numerous proteins and is involved in diverse processes such as neural plasticity, exocytosis, and gene transcription (90). Other CaMK isoforms can have unique substrates. For example, unlike the multifunctional CaMKs, CaMKIV is capable of phosphorylating cAMP-dependent protein kinase (PKA) substrates and the GTP-binding protein Rap1B (41, 91). It is also unique in that unlike most CaMKs it is monomeric and contains a nuclear localization sequence (90). CaMKIV transcript (*Camk4*) was enriched in dStr relative to BNST and NAc (Tables 2, A and C, and 3, A and B), a finding that was consistent with in situ hybridization data from the Allen Brain Atlas (Fig. 7). CaMKIV phosphorylates cAMP response element binding protein (CREB), stimulating CREB-mediated transcription, whereas CaMKII phosphorylates an additional site that prevents CREB activation (23, 105). Consistent with the observed differences in adenylate cyclases, PDEs, and RGSs, which also relate to G-coupled receptor signaling, IPA identified GPCR signaling as unique among all three brain regions (Fig. 6), while the majority of the genes contributing to this result were included in the more significantly different pathways (Table 4).

IPA also identified other canonical pathways that differed between brain regions (Fig. 6). Pair-wise comparisons between the dStr and both the NAc and BNST revealed chemokine

signaling as differentially regulated, although the majority of the genes contributing to this effect were included in the calcium signaling pathway (Supplemental Table S3). ERK-MAPK signaling was different between the dStr and NAc (gene list in Table 5), with eight of 13 significantly different transcripts higher in NAc than dStr. Differences in ERK-MAPK signaling between these two regions is not surprising, as neural ERK-MAPK signaling is highly associated with responses to stress (72, 99) and drugs of abuse (108), both of which are mediated to a greater extent by the NAc than the dStr (8, 11, 19). The gene most significantly contributing to this finding was histone H3.3B (*H3f3b*, Table 5). Histone 3.3B is a member of the H3.3 family of replacement histones which incorporate into the open chromatin of active genes in a replication-independent manner (2, 40, 46). Additionally, relative to the canonical H3, H3.3 variants have been shown to have two- to fivefold greater transcription-promoting modifications (i.e., acetylation and methylation) than the H3 (71), suggesting that an enrichment of H3.3 could be associated with greater induction of transcription in the NAc relative to dStr. Additional differences between regions found with IPA include dopamine signaling, IGF-1 signaling, and cardiac β -adrenergic signaling (Fig. 6).

In an effort to cross-validate our findings with independently obtained data, we downloaded microarray data from a publicly available database (<http://www.barlow-lockhart-brainmapnimhgrant.org/>) (120). Microarray data from this database were obtained using tissue from male C57BL/6J and 129S6/SvEvTac (two mice each) (120). This database includes expression data from two of the three brain regions investigated in the current study (Str and BNST). Data from Str and BNST were downloaded and filtered using the following criteria: 1) 3/4 P calls in at least one brain region, 2) fold difference ≥ 1.5 , and 3) signal intensity difference > 80 . Unpaired *t*-tests were performed for pair-wise comparison of Str and BNST. The Benjamini-Hochberg procedure was used to derive *q*-values, and false discovery rate was controlled at $< 1\%$. IPA performed on the Barlow-Lockhart data revealed significantly different pathways with a high degree of overlap with our data (Table 6). Importantly, the three most significantly different pathways identified using our own data were also among the top five pathways detected with the same analysis using indepen-

Table 5. Differentially expressed genes involved in mitogen-activated protein kinase/extracellular signal-regulated kinase signaling as revealed by Ingenuity pathway analysis

NAc vs. dStr			
Function	Description	RAV	<i>q</i> Value
Other	H3 histone, family 3B (H3.3B)	-1.8	0.014
Kinase	protein kinase, cAMP-dependent, regulatory, type II, alpha	-2.0	0.014
Transcription regulator	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-1.8	0.016
Other	Rap guanine nucleotide exchange factor (GEF) 3	-1.5	0.017
Other	talin 2	2.0	0.018
Kinase	ribosomal protein S6 kinase, 90 kDa, polypeptide 5	2.7	0.022
Enzyme	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	1.9	0.023
Other	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	-1.6	0.025
Other	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide	-1.7	0.041
Kinase	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	-1.9	0.050
Kinase	mitogen-activated protein kinase kinase 1	-1.5	0.052
Kinase	protein kinase C, beta 1	1.8	0.052
Phosphatase	protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform	1.8	0.063

Positive RAV indicates higher values in dStr.

Table 6. Rank order of significant pathways in the present study and from the Barlow-Lockhart database

Pathway	Present Study	Barlow-Lockhart
cAMP-mediated signaling	1	1
G protein-coupled receptor signaling	2	4
IGF-1 signaling	3	3
Glutamate receptor signaling	4	25
Calcium signaling	5	28
Insulin receptor signaling	6	6
Chemokine signaling	7	2
VEGF signaling	8	18
GM-CSF signaling	9	7
Cardiac β -adrenergic signaling	10	12

Ingenuity pathway analysis performed on BNST vs. dStr in the present analysis revealed 10 significant pathways. The analysis was repeated using publicly available microarray data from the same brain regions (available at <http://www.barlow-lockhart-brainmapnimhgrant.org/>).

dently acquired data (120). Additionally, six of the top 10 pathways IPA originally identified were also included in the top 10 pathways identified using the Barlow-Lockhart data. It is important to note that this consistency is observed despite the significantly different methodology used to obtain the datasets. The Barlow-Lockhart data were obtained from two different mouse strains (C57BL/6J and 129S6/SvEvTac) by different dissection methods (gross dissection of whole brain regions). The samples were also analyzed using a different microarray platform (Affymetrix GeneChip

MG_U74Av2) that has a less complete array of probe sets (12,422 vs. ~43,000).

To further cross-validate to our findings with independently obtained data, we analyzed cellular expression of genes from five most significantly different canonical pathways (Fig. 6) using publicly available in situ hybridization data from the Allen Brain Atlas (www.brain-map.org). Regression analysis on pair-wise comparisons significantly different in these Ingenuity pathways demonstrated a high degree of concordance between microarray and in situ hybridization data (Fig. 8, A and B). One factor contributing to differences, however, was that some of the pair-wise differences appeared greater in the degree of expression in cells rather than the number of cells that were labeled. This type of difference would be more likely to be detected by microarray than with in situ hybridization coupled with cell counting procedures. Nonetheless, there is consistency in relative abundance values despite the differences in quantification techniques (whole tissue RNA vs. number of cells positive for probe labeling).

As a final validation of our findings, we used qPCR as an additional measure of relative transcript abundance in the BNST, dStr, and NAc. Genes were selected from pair-wise analyses within the cAMP-dependent signaling pathway from IPA (Table 2, A–C). Relative abundance values obtained using qPCR showed trends of gene expression consistent with microarray data (Fig. 9). Additionally, all but one of the selected genes found to be significantly different in pair-wise analysis

Fig. 8. Regression analysis of differential gene expression reported by microarray and post hoc analysis from the Allen Brain Atlas. A: representative scatter plot of fold difference reported by microarray (x-axis) and Allen Brain Analysis (y-axis). Plot shown is for genes within the cyclic AMP signaling pathway in the BNST vs. striatum (Str) pair-wise analysis. B: R-values for pair-wise analyses found to be significant among the top 5 distinct canonical pathways reported by Ingenuity pathway analysis (see also Fig. 6). Pair-wise analyses not found to be significant by Ingenuity were not analyzed and are blacked out.

Fig. 9. qPCR validation of select genes from Ingenuity pathway analysis. Relative abundance values (RAV) of transcript between brain regions was calculated by microarray and qPCR. Positive RAV indicates higher values in the 2nd brain region of each comparison. Statistical analyses of pair-wise comparisons between brain regions for microarray and qPCR are in Table 7.

using microarray were also found to be significantly different using qPCR (Table 7).

DISCUSSION

The present data suggest that transcriptome differences across these regions may in part underlie previously observed signaling differences. The brain regions studied reflect neuroanatomical targets relevant to Parkinson's disease, Huntington's disease, drug abuse, and anxiety. Using a priori analysis, FCS, and IPA, we have identified differences in gene expression that contribute to differences in biochemical pathways crucial to neurotransmission.

A Priori Analysis

A priori analysis showed several trends consistent with neuroanatomical studies using other methods of detection. First, identification of genes within neuropeptide systems reflecting known differences among the three regions were investigated. Known localization of corticotrop releasing hor-

mone (CRH) signaling was recapitulated by high expression of transcripts for corticotrop releasing hormone (*Crh*) and CRH binding protein (*Crhbp*) in the BNST and NAc, but not Str (57, 73, 83). Levels of prodynorphin (*Pdyn*) and proenkephalin (*Penk1*) transcripts also reflected established patterns of the respective peptides. These peptides are unique in that they are expressed in a mutually exclusive manner within medium spiny neurons (the primary neuron type of the Str and NAc) (39). In the NAc, prodynorphin immunoreactivity is expressed in very high levels relative to the dorsal Str in rodents (112) and humans (52). Likewise, enkephalin expression is greater in the Str than the NAc (49, 85), and presence of enkephalin has been described in the BNST (62, 85, 86). A priori analysis in the present study also revealed the same pattern of elevated prodynorphin (*Pdyn*) in NAc relative to Str and higher levels of proenkephalin gene (*Penk1*) in Str relative to NAc, while levels of both peptides were lowest in BNST. This expression pattern was further confirmed in the *Penk1*-EGFP BAC-transgenic mouse.

A priori analysis also revealed expression patterns of noradrenergic receptors consistent with phenotypic data. The alpha2 adrenergic receptors alpha2a (*Adra2a*) and alpha2c (*Adra2c*) are highly similar in that they show >70% sequence homology and both couple to the same class of signal transduction mechanisms (45, 68), although they differ in the fact that alpha2a, but not alpha2c is prone to agonist-induced desensitization (66). Despite these similarities, genetic studies have shown divergent phenotypes controlled by *Adra2a* and *Adra2c* (for review see Ref. 60). Targeted deletion of *Adra2a* leads to a high-anxiety/depressive phenotype (97), while *Adra2c* deletion showed a resistance to depressive behavior (93). Both anxiety and depression have been linked to noradrenergic transmission within the BNST (75), where we found the highest expression of *Adra2a*. *Adra2c* null mutants also have a heightened locomotor response to amphetamine (94). Consistent with other reports (42, 77), we found *Adra2c* to be highly expressed in Str relative to other regions investigated, and the enhanced amphetamine locomotor response is consistent with altered striatal transmission (14).

Pathway Analysis

FCS and IPA revealed the most significantly different physiological classes and pathways based on gene expression values.

Table 7. qPCR validation of select genes revealed by Ingenuity pathway analysis

Gene	Comparison	Microarray Significance (Ingenuity <i>q</i> Value)	qPCR Significance
Adcy2	BNST vs. Str	0.005	n.s.
Akap9	BNST vs. Str	0.006	†
Gnas	BNST vs. Str	0.004	†
Pde7b	BNST vs. Str	0.006	†
Pde10a	BNST vs. Str	0.002	†
Rgs4	BNST vs. Str	0.005	†
Camk2 g	BNST vs. NAc	0.065	*
Drd2	BNST vs. NAc	0.052	†
Pde10a	BNST vs. NAc	0.063	†
Htr7	BNST vs. NAc	0.052	†
Adcy5	BNST vs. NAc	0.062	†
Pde7b	NAc vs. Str	0.022	†
Pde10a	NAc vs. Str	0.014	†
Drd2	NAc vs. Str	0.018	†
Rgs4	NAc vs. Str	0.025	†

Select genes from the cAMP-dependent signaling pathway identified by Ingenuity pathway analysis were analyzed by qPCR. Mean normalized expression values were compared using corrected *t*-tests. Significance from Ingenuity analyses (see also Table 2) is shown for comparison. **P* < 0.05, †*P* < 0.01. n.s., Not significant.

One overall trend observed was that numerous functional classes and pathways associated with synaptic plasticity, a cellular form of learning, are different between the brain regions investigated. FCS identified several plasticity related processes, including adenylyl cyclase activation (GO:0007190), regulation of synaptic transmission (GO:0050804), and detection of calcium ion (GO:0005513) as different between regions, and the top three pathways identified by IPA are all involved in synaptic plasticity (for review, see Ref. 69). While synaptic plasticity is associated with learning in general, plasticity within some brain regions is thought to be associated with enduring behavioral maladaptations such as drug seeking, depression, and chronic anxiety states (12, 53, 98, 115). Identification of exact molecular targets involved in region-specific plasticity is thus invaluable to the development of pharmaceuticals that may aid in the treatment of neurological disorders involving maladaptive learning.

While both FCS and IPA identified genes and pathways that play a role in plasticity as being different between the BNST, Str, and NAc, IPA revealed specific molecules that may regulate plasticity within individual regions. Several genes associated with inhibition of long-term potentiation (LTP) were revealed to be enriched in Str and NAc relative to the BNST. Specifically, phosphodiesterases (10A, 7B, and 4B) and protein phosphatase 3 were higher in Str or NAc than BNST. Recruitment of cAMP plays an important role in LTP in a number of systems (for review, see Ref. 69). In particular, inhibition of cAMP phosphodiesterases can facilitate LTP (7) and memory (87). The BNST also has a higher representation of genes associated with enhanced LTP than Str and NAc, including *Gnas*, *Rapgef3*, *Htr7*, and *Adcy2*. Consistent with these trends, data from our own lab also suggest regional differences in plasticity between the BNST and NAc. Specifically, a stimulus protocol effective in producing LTP in the BNST is ineffective in the NAc (96, 113). While LTP in several brain regions including the Str are dependent on L-type calcium channels (56, 78), LTP in the BNST is not (113). Similar to this electrophysiological finding, stimulation of L-type calcium channels induced robust activation of the transcription factor CREB in Str, but not in the BNST (67). These physiological phenomena were mirrored by IPA, which revealed a paucity of an integral component of the L-type calcium channel (*Cacna1c*) in BNST relative to Str (see Table 3). Furthermore, stimulation of the dopamine D1 receptor activates the transcription factor CREB (cyclic AMP response element binding protein) more effectively in the BNST than the dStr, even though there are fewer D1 receptors in BNST (67). Finally, the idea of the Str being more resistant to LTP is supported by IPA identification of the catalytic subunit of calcineurin, a negative regulator of LTP (116), as being elevated in Str relative to BNST.

Using this methodology, we successfully performed microarray analysis on individual brain regions with the precision of using 500 μ m diameter tissue punches from 300 μ m slices of fresh tissue. This technique yields high-quality RNA from discrete brain regions without the sample preparation, technical difficulty, or expense of laser capture microdissection (95). While the power to detect differences between brain regions may be reduced by low sample sizes (three replicates per brain region), our results were consistent with validation procedures. Cross-validation of genes identified in a priori analysis (*Crh*

and *Penk1*) and IPA using in situ hybridization data from the Allen Brain Institute (3) and a reporter mouse revealed similar anatomical specificity of enrichment. Additionally, independently obtained data analyzed with IPA, cell counting techniques, and qPCR yielded results highly consistent with those obtained by microarray analysis.

GRANTS

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