A Translational Profiling Approach for the Molecular Characterization of CNS Cell Types

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SUMMARY

The cellular heterogeneity of the brain confounds efforts to elucidate the biological properties of distinct neuronal populations. Using bacterial artificial chromosome (BAC) transgenic mice that express EGFP-tagged ribosomal protein L10a in defined cell populations, we have developed a methodology for affinity purification of polysomal mRNAs from genetically defined cell populations in the brain. The utility of this approach is illustrated by the comparative analysis of four types of neurons, revealing hundreds of genes that distinguish these four cell populations. We find that even two morphologically indistinguishable, intermixed subclasses of medium spiny neurons display vastly different translational profiles and present examples of the physiological significance of such differences. This genetically targeted translating ribosome affinity purification (TRAP) methodology is a generalizable method useful for the identification of molecular changes in any genetically defined cell type in response to genetic alterations, disease, or pharmacological perturbations.

INTRODUCTION

A century ago, Ramón y Cajal used Golgi staining to show that distinct cells called neurons are a major component of the mammalian central nervous system (CNS) (Ramón y Cajal et al., 1899). Today, hundreds of neuronal subtypes have been defined, but a molecular description of each subtype has been hampered by the same problems faced by Ramón y Cajal and his contemporaries: neuronal subtypes are highly heterogeneous and intermixed. Numerous attempts to extend microarray analysis of gene expression to defined cell populations in the CNS have relied upon the physical enrichment of target cell populations with laser-capture microdissection (LCM) or fluorescence-activated cell sorting (FACS) of acutely dissociated primary neurons. Unfortunately, these studies have been limited by stresses introduced during cellular isolation procedures, adaptations that occur upon loss of tissue-intrinsic signals, and the technical challenges associated with RNA purification from fixed tissue. To circumvent these problems, we have developed a direct, rapid affinity purification strategy for isolation of polysomal RNA from genetically targeted cell types.

We describe here a new translating ribosome affinity purification (TRAP) methodology, which readily and reproducibly identifies translated mRNAs in any cell type of interest. This methodology involves expression of an EGFP-L10a ribosomal transgene, which enables tagging of polysomes for immunofluorescence-activated cell sorting (FACS) of acutely dissociated primary neurons. Unfortunately, these studies have been limited by stresses introduced during cellular isolation procedures, adaptations that occur upon loss of tissue-intrinsic signals, and the technical challenges associated with RNA purification from fixed tissue. To circumvent these problems, we have developed a direct, rapid affinity purification strategy for isolation of polysomal RNA from genetically targeted cell types.

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RESULTS

A Genetically Targeted Translational Profiling Methodology

Because all mRNAs translated into protein are at one point attached to a ribosome or polyribosome complex (polysome), we reasoned that an affinity tag fused to a ribosomal protein would allow isolation of bound mRNAs. We therefore screened fusions of ribosomal proteins with enhanced green fluorescent protein (EGFP) for efficient incorporation into polysomes to provide an immunoaffinity tag for all translated cellular mRNAs (schematic, Figure 1A). EGFP was chosen because preliminary screens using small epitope tags were unsatisfactory and because visualization of EGFP fluorescence provides a simple assay for proper expression and localization of the fusion protein. After dozens of candidate ribosomal protein fusions were tested, EGFP fused to the N terminus of the large-subunit ribosomal protein L10a (EGFP-L10a) was chosen because its nucleolar and cytoplasmic localization was consistent with incorporation into intact ribosomes and because immunoelectron microscopy data demonstrated its presence on polysomes (Figure S1 available online and data not shown). Prior to the production of bacTRAP transgenic mice, preliminary studies in HEK293T cells transfected with EGFP-L10a achieved rapid and specific immunoaffinity purification of polysomes (Figure 1B), overall copurification of ~10% of untagged ribosomal proteins and ribosomal RNA from cultures in which ~30% of cells expressed EGFP-L10a, and recovery observed for translated, and not untranslated, mRNAs (Table S1 and Figure S2). As further validation of the technique, measurements of the well-documented shift in translational efficiency of Ferritin mRNA in response to iron treatment were comparable with the TRAP methodology or traditional polysome gradient methods (Figure S2).

To genetically target expression of EGFP-L10a to defined CNS cell populations, we generated BAC transgenic mice. To tag polysomes in striatonigral and striatopallidal cells of the mouse striatum, we used homologous recombination in bacteria to place EGFP-L10a under the control of either the Drd2 receptor (striatopallidal) or Drd1a receptor (striatonigral) loci in the appropriate BACs. Striatonigral MSNs send projection axons directly to the output nuclei of the basal ganglia, i.e., the substantia nigra and the internal segment of the globus pallidus (the entopeduncular nucleus in rodents), whereas striatopallidal MSNs send projection axons to the external segment of the globus pallidus. Mouse lines bearing the TRAP transgene (EGFP-L10a) were generated and screened by immunohistochemistry for appropriate expression of the transgene, as judged by known Drd1a and Drd2 receptor expression patterns. The Drd2 bacTRAP line CP101 showed highest transgenic EGFP-L10a expression in the dorsal and ventral striatum, olfactory tubercle, and hippocampus, as well as in the substantia nigra pars compacta and ventral tegmental area, as expected because of Drd2 autoreceptor expression in dopaminergic cells (Figure 2A). The Drd1a bacTRAP line CP73 showed highest transgenic EGFP-L10a expression in the dorsal and ventral striatum, olfactory bulb, olfactory tubercle, and cortical layers 5 and 6 (Figure 2C). As expected for a ribosomal

Figure 1. The TRAP Methodology

(A) Schematic of affinity purification of EGFP-tagged polysomes (originating from the target cell population; green polysomes) with anti-GFP antibody-coated beads.

(B) Transmission electron micrographs of anti-GFP-coated magnetic beads after incubation with extracts taken from HEK293T cells transfected with an empty vector (left panel) or the EGFP-L10a construct (right panel); images were acquired at 50,000× magnification, with inserts enlarged by a factor of 2.3×.
protein fusion, EGFP fluorescence localized to the nucleoli and cytoplasm (Figure 2B). EGFP direct fluorescence coincident with enkephalin immunohistochemical detection (striatopallidal cell marker) was observed in striatal cells from the Drd2 bacTRAP line but not the Drd1a bacTRAP line (Figures 2B and 2D), verifying correct BAC-mediated cell-type expression. Velocity sedimentation analysis of polysome complexes isolated from striatal extracts of both bacTRAP lines confirmed incorporation of the EGFP-L10a fusion protein into functional polysomes in vivo (Figure S3 and data not shown).

We next developed procedures for rapid extraction and immunoaffinity purification of the EGFP-tagged polysome complexes from intact brain tissue, which proved substantially more challenging than from transfected cells in culture. However, after several optimization steps (see the Experimental Procedures), highly purified RNA was consistently obtained from bacTRAP mouse brain tissue (Figure 3). Key steps of the purification protocol include rapid manual dissection and homogenization of the tissue, inclusion of magnesium and cycloheximide in the lysis buffer to maintain ribosomal subunits on mRNA during purification, inhibition of endogenous RNase activity, solubilization of rough endoplasmic reticulum-bound polysomes under nondenaturing conditions, use of high-affinity anti-EGFP antibodies, and the addition of high-salt washes after immunoaffinity purification to reduce background.

Translational Profiling of Striatonigral and Striatopallidal MSNs
Translational profiling analysis was performed with immunoaffinity-purified mRNA from adult striatonigral or striatopallidal bacTRAP mice. After two rounds of in vitro transcription, biotin-labeled antisense RNA (cRNA) was used to interrogate Affymetrix GeneChip Mouse Genome 430 2.0 arrays. Replicate bacTRAP samples collected from each line gave nearly identical genome-wide translational profiles (average Pearson correlation of 0.982 and 0.985 for striatonigral and striatopallidal samples, respectively). For each cell type, data were collected from three independent biological replicates, each prepared from a cohort of seven animals. Analysis of immunoaffinity-purified samples revealed no bias for mRNA length or abundance (Figure S4). Comparative analysis of these data (see the Experimental Procedures) revealed that all of the well-characterized, differentially expressed MSN markers (Gerfen, 1992) were enriched with the TRAP translational profiling approach: dopamine receptor 2 (Drd2) (36.6), adenosine 2a receptor (Adora2a) (13.2), and enkephalin (Penk) (7.5) were enriched in the striatopallidal bacTRAP sample, whereas dopamine receptor D1A (Drd1a) (3.9), substance P (Tac1) (3.6), and dynorphin (Pdyn) (5.6) were enriched in the striatonigral bacTRAP sample (Figure 3C and Table S2). We also confirmed four striatopallidal-enriched mRNAs (Adk, Plxdc1, BC004044, and Hist1h2bc), as well as six striatonigral-enriched mRNAs (Slic35d3, Zfp521, Ebf1, Stmn2, Gnb4, and Mrn1) reported in a microarray study of FACS-isolated MSNs (Lobo et al., 2006) (Table S2). We further identified ~70 additional striatopallidal-enriched transcripts and more than 150 additional striatonigral-enriched transcripts (Table S2). To initially verify our data, we performed quantitative PCR assays using independent biological bacTRAP Drd1a and Drd2 samples and a different cDNA amplification procedure (see the Supplemental Experimental Procedures). Differential translation of Eya1, Isl1, Gng2, and Cryn in striatonigral MSNs and Gpr6, Lhx8, Gpr88, Trpc4, and Tpm2 in striatopallidal MSNs was confirmed (Tables S3 and S4). These genes were selected because they represent both highly and moderately enriched messages.

Given the apparent enhanced sensitivity of our translational profiling method, we were next interested in large-scale

Figure 2. Expression of EGFP-L10a in Drd1a and Drd2 bacTRAP Lines
(A) Immunohistochemistry to EGFP in adult sagittal sections from the Drd2 bacTRAP line CP101.
(B) Characterization of Drd2 bacTRAP line CP101 striatal MSN cells: direct EGFP fluorescence (left panel with high-magnification image insert), enkephalin immunohistochemical staining (middle panel), and merge (right panel, with 20 μm scale bar).
(C) Immunohistochemistry to EGFP in adult sagittal sections from the Drd1a bacTRAP line CP73.
(D) Characterization of Drd1a bacTRAP line CP73 striatal MSNs: direct EGFP fluorescence (left panel), enkephalin immunohistochemical staining (middle panel), and merge (right panel).
validation of the data with publicly available gene expression databases. Since in situ hybridization (ISH) alone cannot distinguish MSN subtypes, we pooled data from Drd1a and Drd2 bacTRAP experiments and compared them to data collected from the total RNA of one whole brain (minus striatum) (Table S5). This analysis would be expected to identify most MSN-enriched transcripts, which can be evaluated in the ISH databases, whether or not they are differentially expressed in striatopallidal or striatonigral projection neurons. The analysis resulted in detection of several thousand translated mRNAs enriched in striatum relative to whole brain, including all previously well-known striatal-enriched genes: Ppp1r1b/ Darpp-32 (Walaas and Green-gard, 1984), Ptnp5/Step (Lombroso et al., 1993), Arpp-19 (Girault et al., 1990), Arpp-21/RCS (Quimet et al., 1989), Gnl/Golf (Herve et al., 1993), Rhes/Ras2d (Falk et al., 1999), Rgs9 (Gold et al., 1997), Adcy5 (Glatt and Snyder, 1993), Gng7 (Watson et al., 1994), Rasgrp2 (Kawasaki et al., 1998), Pde1b (Polli and Kincaid, 1992), Pde10a (Fujishige et al., 1999), Gpr88 (Mizushima et al., 2000), Rab9 (Krezel et al., 1999), and Strn4 (Castets et al., 1996), as well as the transcription factors Foxp1, Foxp2 (Ferland et al., 2003), Ebf1 (Lobo et al., 2006), and Zfp503/Notz (Chang et al., 2004) (Table S5). Of the first 100 genes appearing in our MSN-enriched dataset, 26 were present in both of two major gene expression databases (the GENSAT/Brain Gene Expression Map [BGEM] and Allen Brain Atlas [ABA] ISH databases [http://www. ncbi.nlm.nih.gov/projects/gensat/; http://www.stjudebgem.org/]) [Gong et al., 2003; Lein et al., 2007; Magdaleno et al., 2006], and enriched striatal expression is evident for 22 of these genes (Table S5; Figure S5). Thus, TRAP translational profiling can provide a sensitive tool for discovery of large sets of translated messages in defined CNS cell populations.

To group striatonigral- and striatopallidal-enriched genes according to biological function, we looked for statistically over-represented associations with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway terms (Tables S6–S11). GO terms delineate the known molecular functions, biological processes, and cellular localizations (components) for a particular gene (Ashburner et al., 2000), whereas KEGG pathways summarize known molecular interaction and reaction networks (Kanehisa, 1997). Some differentially translated mRNAs immediately predicted physiological differences between striatonigral and striatopallidal cells. For example, among striatopallidal-enriched mRNAs is Gpr6 (Lobo et al., 2007), which encodes a G protein-coupled receptor for the lysophospholipid sphingosine 1-phosphate (S1P) (Ignatov et al., 2003). In heterologous expression systems, S1P activation of Gpr6 receptors induces intracellular Ca2+ release. As intracellular Ca2+ is a crucial regulator of neuronal physiology, we investigated whether striatopallidal enrichment of Gpr6 reflects a differential response to S1P. To this end, BAC Drd2 striatopallidal or BAC Drd1a striatopallidal MSNs (expressing soluble EGFP) were identified in brain slices, patch clamped (Day et al., 2006), loaded with Alexa 594 to visualize dendrites, and loaded with Fluo-4 to monitor intracellular Ca2+ concentration (Figure 4A). A second pipette was then brought into close physical proximity to a dendrite, 60–80 μm from the soma, and used to apply S1P (Figures 4A and 4B). With the somatic membrane potential clamped at −70 mV, focal application of S1P consistently and reversibly increased

Figure 3. Protein and mRNA Purification from bacTRAP Lines
(A) Representative purification of EGFP-tagged L10a and copurification of untagged ribosomal protein L7 from Drd1a bacTRAP animals but not wild-type littermates (D1, samples from Drd1a bacTRAP mice; WT, samples from wild-type littermates; IN, 1% input; UB, 1% unbound; IP, 6.5% immunoaffinity-purified sample). EGFP-L10a signal is only present in the D1 IP lane because the IP samples were more concentrated relative to IN and UB. (B) Representative purification of 18S and 28S rRNA from Drd1a bacTRAP transgenic animals (green) but not wild-type littermates (red) as detected by Bioanalyzer PicoChips (Agilent Technologies). 28S rRNA runs at 47 s, 18S rRNA runs at −43 s, and the Picochip marker peak runs at −23 s. (C) Normalized expression values from Affymetrix Mouse Genome 430 2.0 arrays are plotted for Drd1a and Drd2 bacTRAP samples. The middle diagonal line represents equal expression, and lines to each side represent 1.5-fold enrichment in either cell population. Axes are labeled for expression in powers of 10. The probesets of well-studied genes known to be differentially expressed are represented in blue.
dendritic Ca\(^{2+}\) levels in BAC Drd2 striatopallidal neurons (Kruskal-Wallis ANOVA, p < 0.01, n = 6) but not in BAC Drd1a striatonigral neurons (Kruskal-Wallis ANOVA, p > 0.01, n = 4) (Figures 4C and 4D). Depletion of intracellular Ca\(^{2+}\) stores with the Ca\(^{2+}\)-ATPase inhibitor thapsigargin abolished this response to S1P (Kruskal-Wallis ANOVA, p > 0.01, n = 4; Figures 4C and 4D). This striatopallidal-specific S1P response would be predicted to result in a decrease in threonine 34 (T34) phosphorylation of the centrally important regulatory protein DARPP-32 because of activation of the Ca\(^{2+}\) and calmodulin-dependent phosphatase calcineurin (Nishi et al., 1999) and/or inhibition of adenyl cyclase type 5 (AC5; Adcy5) (Ishikawa et al., 1992; Glatt and Snyder, 1993). Indeed, a decrease in T34 DARPP-32 phosphorylation was seen after 5 min of S1P treatment of striatal slices (1.04 ± 0.17 normalized units at 0 min; 0.58 ± 0.24 normalized units at 5 min after S1P addition; one-tailed Mann-Whitney test, p = 0.05, n = 12). These data demonstrate the utility of TRAP translational profiling in identifying previously unrecognized functional differences in signaling responses between cell types.

**Profiling of Cocaine-Induced Translational Changes**

On the basis of the data above, it seemed likely that TRAP translational profiling could identify molecular responses to genetic, pharmacologic, or environmental changes in single cell types. To test this idea, we investigated changes in mRNA translation of MSNs upon pharmacological perturbation of dopaminergic signaling using cocaine, a competitive inhibitor of the dopamine transporter, which acts as a psychostimulant by elevating synaptic dopamine levels (Ritz et al., 1987; Di Chiara and Imperato, 1988). Adult mice were treated acutely or chronically with cocaine or saline and used for translational profiling of striatonigral (Drd1a) and striatopallidal (Drd2) MSNs. From this analysis, we identified hundreds of genes whose expression was increased or decreased in each cell type in response to cocaine (Tables S12 and S13). It is often difficult to directly compare gene expression data across drug administration experiments from published studies because of differences in strain background, dosing regimen, and assay sensitivity or array platform; in spite of this, we were able to identify various genes whose expression has been reported to be affected by cocaine administration, including: Cartpt (Douglass et al., 1995) (up in acute striatonigral; Table S12), Fosb (Hope et al., 1992) (up in acute striatonigral and striatopallidal and chronic striatonigral; Tables S12 and S13), Homer1 (Brakeman et al., 1997) (up in acute striatonigral and striatopallidal, chronic striatonigral and striatopallidal; Tables S12 and S13), Per2 (Yufnerov et al., 2003) (up in acute striatopallidal and striatonigral).
Figure 5. Cocaine Treatment Increases the Frequency of Small-Amplitude GABAergic mIPSCs in BAC Drd1a Striatonigral Medium Spiny Neurons

(A and B) Representative spontaneous mIPSCs traces from BAC Drd1a striatonigral neurons (expressing soluble EGFP under the Drd1a promoter) taken from mice treated for 15 days with saline (A) or cocaine (20 mg/kg/day) (B).

(C) Bar graph summary of mean mIPSC frequency showing a significant increase in BAC Drd1a striatonigral neuron mIPSCs frequency after cocaine treatment (saline = 0.83 ± 0.07 Hz, n = 22; cocaine = 1.04 ± 0.05 Hz, n = 26; p < 0.05, one-tailed t test).

(D) Bar graph summary showing that the number of small-amplitude mIPSCs (<75 pA) in equal length records (7 min) increased in BAC Drd1a striatonigral neurons after cocaine treatment (saline = 79.4 ± 8.2, n = 22; cocaine = 104.2 ± 6.3, n = 26; p < 0.05, one-tailed t test).

(E) Representative variance-mean current plots from saline-treated (black symbols) and cocaine-treated (red symbols) BAC Drd1a neurons suggesting that the cocaine-induced small-amplitude events arise from synapses that have fewer GABAA receptors (N) per synapse but receptors with an unchanged unitary receptor conductance (γ) (saline N = 33, γ = 31 pS; cocaine N = 29, γ = 31 pS; see Figures S6C and S6D for means).

(F and G) Representative spontaneous mIPSCs traces from BAC Drd2 striatopallidal neurons after saline treatment for 15 days (F) and after cocaine treatment for 15 days (G).

(H) Bar graph summary of mean mIPSC frequency in saline- and cocaine-treated Drd2 neurons, showing no effect of treatment condition (saline = 0.72 ± 0.06 Hz, n = 12; cocaine = 0.78 ± 0.07 Hz, n = 16; p > 0.05, one-tailed t test).

(I) Bar graph summary showing that the number of small-amplitude mIPSCs (<75 pA) in equal length records (7 min) was not altered by treatment condition in BAC Drd2 neurons (saline = 68.1 ± 9.7, n = 12; cocaine = 71.7 ± 7.5, n = 16; p > 0.05, one-tailed t test).

(J) Representative variance-mean current plots showing that cocaine treatment did not change in the number of receptors per synapse or the unitary receptor conductance in BAC Drd2 neurons (saline N = 34, γ = 31 pS; cocaine N = 33, γ = 30 pS; see Figures S7C and S7D for means). All error bars represent standard error.
alter this value (saline $\gamma = 31.1 \pm 1.6$, n = 22; cocaine $\gamma = 30.8 \pm 1.2$, n = 26; p > 0.05, one-tailed t test; Figure 5E; Figure S6C).

However, cocaine treatment did significantly reduce the estimated average number of receptors per synapse (saline N = 33.1 \pm 1.4, n = 22; cocaine N = 29.3 \pm 1.0, n = 26; p < 0.05, one-tailed t test; Figure 5E; Figure S6D). The most parsimonious interpretation of these results is that cocaine treatment leads to (1) the addition of small, dendritic GABA$_A$ synapses, resulting in an increase in mIPSC frequency, and (2) a decrease in the average number of receptors per synapse, due to dendritic synapses having fewer receptors than larger somatic synapses.

In contrast to Drd1a-expressing striatonigral neurons, Drd2-expressing striatopallidal neurons displayed no change in the frequency (saline = 0.72 \pm 0.05 Hz, n = 12; cocaine = 0.78 \pm 0.07 Hz, n = 16; p > 0.05, one-tailed t test; Figure 5H), amplitude (submodal mIPSCs, saline = 68.1 \pm 9.7, n = 12; cocaine = 71.7 \pm 7.5, n = 16; p > 0.05, one-tailed t test; Figure 5I), supramodal mIPSCs, saline = 59.1 \pm 6.5, n = 12; cocaine = 63.3 \pm 4.7, n = 16; p > 0.05, one-tailed t test; data not shown) and kinetics (Figures S7A and S7B) of GABA$_A$ mIPSCs after chronic cocaine treatment. Likewise, nonstationary noise analysis suggested that the average receptor conductance and number of receptors per synapse did not change in Drd2-expressing striatopallidal neurons after cocaine treatment (Figure 5J and Figures S7C and S7D). Taken together, these results demonstrate the physiological relevance of the selective cocaine-induced upregulation of mRNAs associated with GABA$_A$ receptors specific to Drd1a-expressing striatonigral neurons.

Although alterations in GABA release probability could also cause changes in mIPSC frequency, cocaine treatment altered the amplitude distribution of GABA$_A$ mIPSCs selectively in Drd1a-expressing striatonigral neurons, which would not be expected after a blanket increase in GABA release probability. The preferential increase in small-amplitude mIPSCs and the suggestion from nonstationary noise analysis that the average number of receptors per synapse fell in striatonigral neurons after cocaine treatment are consistent with the addition of small dendritic synapses, as opposed to larger somatic synapses (Kubota and Kawaguchi, 2000). Dendritic GABAergic synapses arise either from recurrent collaterals of other MSNs or from interneurons (Tepper et al., 2004). Definitively sorting out whether one or both of these classes contribute to the change in synaptic properties will require paired cell recordings and ultrastructural analysis (e.g., Koos et al., 2004; Kubota and Kawaguchi, 2000). Dendritic GABAergic synapses from the top 1000 enriched probesets from this analysis (Tables S22–S25) showed that the translational profiles of striatonigral and striatopallidal cells are more similar to each other than to translational profiles of either brain stem cholinergic cells or Purkinje cells (Figures 7E–7H).

**DISCUSSION**

TRAP translational profiling combines genetic targeting of an EGFP-L10a ribosomal fusion protein to specific cell populations; simple, rapid, affinity purification of mRNA; and microarray technology to identify translated mRNAs in situ. Unlike traditional approaches, it combines coincident detection of all translated mRNAs with cell-type specificity; thus, one quantitative TRAP experiment can replace thousands of high-throughput qualitative in situ hybridization runs and, further, can be repeated easily for each experimental condition. Second, the information obtained is cell type-specific while abrogating the physiologic adaptations and mRNA degradation that can occur during the lengthy cell separation procedures used in other approaches that sever neuronal axons and dendrites and disrupt tissue-intrinsic signaling. Third, because polysomes are stabilized with cycloheximide in the extraction buffers and during the affinity purification steps, redistribution of the affinity tag within the mRNA pool during polysome isolation is prevented, obviating the problems inherent in using less stable mRNA-binding proteins. Fourth, the use of established bacTRAP lines ensures that mRNA translation profiles can be reproducibly obtained and directly compared from the same cell population across experimental conditions. Crossing of these and other established bacTRAP lines to mouse models of various diseases...
should greatly facilitate the elucidation of the molecular basis of disease phenotypes. Fifth, the use of EGFP as the affinity tag greatly facilitates anatomic studies of candidate bacTRAP mouse lines, as well as electrophysiological studies of each cell type. Finally, the profiling of only translated mRNA pools will more accurately reflect actual protein levels in a cell than does conventional gene expression profiling, which relies on total mRNA pools.

The TRAP approach thus overcomes limitations of previous mRNA-tagging methods that have targeted other RNA-binding proteins, of which only poly(A)-binding protein (PABP) has been used in vivo for tissue-specific studies (Roy et al., 2002; Kunitomo et al., 2005), and which requires crosslinking of the tagged PABP to mRNA (and its inherent artifacts) because of its loose association with mRNA. The use of tagged ribosomal proteins to purify translating polysomes from yeast as well as plant cells has recently been reported (Inada et al., 2002; Zanetti et al., 2005), although in these cases, cell-specific targeting to obtain cell-specific translational profiles was not attempted.
From our own experience, we know that adapting this methodology to cell-specific targeting is not trivial, particularly for applications in the mammalian CNS, with its far more difficult conditions of low expression, limited material, and contamination by blood, myelin, and other biological factors. As a result of the optimization reported here, the TRAP translational profiling methodology is now extremely robust to these conditions and broadly applicable (Doyle et al., 2008, accompanying paper).

Furthermore, the TRAP approach was able to identify all previously known, well-studied striatonigral- and striatopallidal-enriched genes (with one exception: the striatonigral-enriched muscarinic receptor M4 [Chrm4] [Ince et al., 1997], for which probesets on the Affymetrix Mouse 430 2.0 GeneChips gave very little signal; however, real-time PCR analysis of Chrm4 expression demonstrated clear enrichment of Chrm4 mRNA in our striatonigral bacTRAP cell sample [Table S3]). These results stand in distinction to a recent microarray study of FACS-sorted MSNs (Lobo et al., 2006), in which well-known positive-control genes (e.g., Chrm4, Pdyn, Drd1a, and Drd2) were not identified as differentially expressed on microarrays, and 16 of the 21 mRNAs reported in that study as striatopallidal-enriched in adult MSNs could not be confirmed in our analysis. Furthermore, we report here hundreds of distinguishing transcripts not identified in that study.

More importantly, our translational profiling analysis of striatal MSN subtypes has identified novel physiological differences between striatonigral and striatopallidal cells, which may provide new therapeutic targets for various neurological diseases associated with pathophysiology in the striatum. As one example, we demonstrate that striatopallidal cells selectively express Gpr6 and that they correspondingly display a cell type-specific release of intracellular Ca2+ in response to sphingosine 1-phosphate. A second example of the distinct properties of these medically important cell types is provided by our studies of psychostimulant drug action, which give molecular and physiological evidence for upregulation of GABAB receptor subunits in striatopallinal neurons after chronic cocaine administration.

The results presented here demonstrate that the TRAP translational profiling methodology provides an enabling technology for studies of the biology of specific cell types in even the most heterogeneous cell populations, such as those that occur in the CNS. We have identified numerous distinguishing molecular characteristics among four distinct neuronal populations, including two closely related, critical cell types and have demonstrated that the TRAP methodology can be employed to analyze physiological adaptations of specific cell types in vivo. An accompanying study (Doyle et al., 2008) has demonstrated the general applicability of the TRAP methodology by characterizing additional CNS cell types, each of which exhibits an enormously complex and cell-type-specific molecular phenotype.

### EXPERIMENTAL PROCEDURES

#### Generation of Mouse Lines

BAC transgenic mice were produced according to published protocols (Gong et al., 2003), with the exception that the EGFP-L10a transgene was used in place of EGFP.

#### Purification of mRNA from bacTRAP Mice

For striatonigral or striatopallidal translated mRNA purification, mice were decapitated, and the striata of seven bacTRAP transgenic mice were quickly manually dissected. Pooled striatal tissue was immediately homogenized in ice-cold polysome extraction buffer (10 mM HEPES [pH 7.4], 150 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 100 μg/ml cycloheximide, protease inhibitors, and recombinant RNase inhibitors) with a motor-driven Teflon glass homogenizer. Homogenates were centrifuged for 10 min at 2000 × g, 4°C, to pellet large cell debris, and NP-40 (EMD Biosciences, San Diego, CA) and 1.2-Diheptanoyl-sn-Glycero-3-Phosphocholine (DHPC; Avanti Polar Lipids, Alabaster, AL) were added to the supernatant at a final concentration of 1% and 30 mM, respectively. After incubation on ice for 5 min, the clarified lysate was centrifuged for 10 min at 13,000 × g to pellet unsolubilized material. Goat anti-GFP (custom made) -coated protein G Dynal magnetic beads (Invitrogen, Carlsbad, CA) were added to the supernatant, and the mixture was incubated at 4°C with end-over-end rotation for 30 min. Beads were subsequently collected on a magnetic rack, washed three times with high-salt polysome wash buffer (10 mM HEPES [pH 7.4], 350 mM KCl, 5 mM MgCl2, 1% NP-40, 0.5 mM dithiothreitol, and 100 μg/ml cycloheximide) and immediately placed in Trizol-LS reagent (Invitrogen) and chloroform to extract the bound RNA and mRNA from polysomes. After extraction, RNA was precipitated with sodium acetate and Glycoblue (Ambion, Austin, TX) in isopropanol overnight at −80°C, washed twice with 70% ethanol, resuspended in water, and further purified with an Rneasy Micro Kit (QiAGEN, Valencia, CA) with in-column DNase digestion. For the purification of translated mRNAs from brain stem cholinergic motor neurons (from the Chat bacTRAP line) or cerebellar Purkinje neurons (from the Pcp2 bacTRAP line), nearly identical purifications as outlined above were performed, with a few minor modifications (see the Supplemental Experimental Procedures).

#### Microarray Data Normalization and Analysis

Three biological replicates were performed for each experiment. Quantitative PCR reactions were performed to validate array results with an independent biological source and amplification methodology (see the Supplemental Experimental Procedures). Striatonigral and striatopallidal GeneChip CEL files were imported into Genespring GX 7.3.1 (Agilent Technologies, Santa Clara, CA) and processed with the GC-RMA algorithm, and expression values on biological source and amplification methodology (see the Supplemental Experimental Procedures). Striatonigral and striatopallidal GeneChip CEL files were imported into Genespring GX 7.3.1 (Agilent Technologies, Santa Clara, CA) and processed with the GC-RMA algorithm, and expression values on each chip were normalized to that chip’s 50th percentile. Data were converted to log2 scale and filtered to eliminate genes with intensities in the lower range. A moderated two-tailed paired t test was performed with the Limma package from the Bioconductor project (http://www.bioconductor.org/). The p value of the moderated t test was adjusted for multiple hypothesis testing, controlling the false discovery rate (FDR) with the Benjamini-Hochberg procedure. We then selected all genes that had an FDR less than 0.1 (10%) and fold change larger than 1.5. Other comparisons were performed as described in detail in the Supplemental Experimental Procedures.

#### ACCESSION NUMBERS

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO SuperSeries accession number GSE13394 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13394).

#### SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, and 26 tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01365-2.

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