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FACS identifies unique cocaine-induced gene regulation in selectively activated adult striatal neurons

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Abstract

Numerous studies with the neural activity marker Fos indicate that cocaine activates only a small proportion of sparsely distributed striatal neurons. Until now, efficient methods were not available to assess neuroadaptations induced specifically within these activated neurons. We used fluorescence-activated cell sorting (FACS) to purify striatal neurons activated during cocaine-induced locomotion in naïve and cocaine-sensitized *cfos-lacZ* transgenic rats. Activated neurons were labeled with an antibody against β-galactosidase, the protein product of the lacZ gene. Cocaine induced a unique gene expression profile selectively in the small proportion of activated neurons that was not observed in the non-activated majority of neurons. These genes included altered levels of the immediate early genes *arc*, *fosB*, and *nr4a3*, as well as genes involved in p38 MAPK signaling and cell-type specificity. We propose that this FACS method can be used to study molecular neuroadaptations in specific neurons encoding the behavioral effects of abused drugs and other learned behaviors.

Keywords

cocaine sensitization; nucleus accumbens; neuronal ensembles; fluorescence activated cell sorting

INTRODUCTION

A major hypothesis of current neurobiological research on cocaine addiction is that chronic cocaine exposure causes long-lasting neuroadaptations within the striatum and other components of the mesolimbic dopamine reward circuit, leading to compulsive drug use and long-term relapse vulnerability (Nestler, 2001; Shaham and Hope, 2005; Kalivas, 2009; Bowers et al., 2010; Wolf and Ferrario, 2010). However, this hypothesis is based on numerous studies limited to measuring neuroadaptations in homogenates of brain regions or in neurons selected independently of their activation state during behavior.

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The distinction between neuroadaptations in activated versus non-activated neurons is important because they may play different roles in the physiological, behavioral, and psychological effects of cocaine. Numerous studies with the neural activity marker Fos indicate that cocaine activates only a small proportion of sparsely distributed neurons in the striatum. Following context-specific locomotor sensitization, we have shown that Fos is induced only when rats were injected in the drug-paired environment and not in a non-paired environment (Mattson et al., 2008; Koya et al., 2009). Most importantly, we have shown that these selectively activated neurons play a causal role in the learned association between cocaine and the drug-paired environment that mediates context-specific sensitization (Koya et al., 2009). Thus neuroadaptations within selectively activated striatal neurons may play unique and important roles in learning during context-specific sensitization and other drug-induced behaviors.

Until now, technical limitations have made it difficult to assess molecular alterations within the small numbers of sparsely distributed neurons selectively activated during behavior. Techniques such as laser capture microdissection (Kwon and Houpt, 2010) and double-label immunohistochemistry (Berretta et al., 1992; Gerfen et al., 1995; Peters et al., 1996) are capable of identifying molecular alterations selectively within activated neurons, but these techniques have very low throughput or are not quantitative. Fluorescence-activated cell sorting (FACS) can be an efficient tool for purifying adult neuronal populations for molecular analysis (Liberles and Buck, 2006; Lobo et al., 2006). However, FACS has not been used to purify neurons based on their activation state. In the current study, we used FACS to purify the interspersed cocaine-activated striatal neurons from transgenic *cfos-lacZ* rats and compared their unique patterns of gene expression with those in the non-activated majority of neurons.

METHODS

Animals

Female *cfos-lacZ* transgenic rats (Kasof et al., 1995; Koya et al., 2009) and female Sprague-Dawley rats (Charles River, Raleigh, NC, USA) were housed individually in standard plastic cages in a temperature- and humidity-controlled room. They were maintained on a 12:12 h reverse light:dark cycle (lights on at 20.00 h), and allowed free access to food and water. They were acclimatized to these housing conditions for a minimum of 7 days prior to drug treatments. Experimental procedures were approved by the NIDA Animal Care and Use Committee.

Drug treatments

For acute cocaine experiments, rats were injected with cocaine (30 mg/kg, i.p.; n=8) or saline (1 ml/kg; n=6) and placed in a round Plexiglass chamber (38 cm diameter). Rats were sacrificed 90 minutes after injections to obtain brain tissue. This treatment was repeated three times on separate days to obtain three biological replicates for microarray analysis. Additionally, three independent biological replicates were similarly produced for quantitative PCR (qPCR). For qPCR analyses of *arc, pdyn, adora2a*, only these three independent biological replicates were used. For qPCR analyses of *fosB, nr4a3, kcnc1, and map2k6*, we also used remaining RNA from each of the microarray samples.

For repeated cocaine experiments, female rats were sensitized with four injections of cocaine (15 mg/kg i.p.) administered once every second day. On injection days, each rat was placed in a 43 × 43 cm square Plexiglas locomotor activity chamber (Med Associates, St Albans, VT, USA) to habituate for 30 minutes. Rats were injected with cocaine and locomotor activity was recorded as distance traveled for 60 minutes. Following the fourth

repeated cocaine injection, rats remained in their home cage for 7–8 days. On test day, rats were habituated for 30 min in the locomotor activity chambers and then injected with cocaine (30 mg/kg i.p.) or saline vehicle. Rats were decapitated and brains were extracted ninety minutes after injections. The entire sensitization treatment was repeated three times over separate weeks to obtain three biological replicates of each group for qPCR analysis.

Cell dissociation

Striatal tissue was dissociated to obtain a single-cell suspension. Rats were decapitated and their striata extracted within two minutes. Each striatum was minced with razor blades on an ice-cold glass plate and placed in 1 ml of Hibernate A (Cat# HALF; Brain Bits, Springfield, IL). Striata were enzymatically digested in 1 ml of Accutase per striatum (Cat# SCR005; Chemicon, Billerica, MA) with end-over-end mixing for 30 min at 4°C. Tissue was centrifuged for two min at 425×g and resuspended in 300 μl of ice-cold Hibernate A. All subsequent centrifugation steps were performed at 4°C.

Digested striatal tissue was mechanically dissociated by trituration. Four striata were combined into one Eppendorf tube and triturated ten times with a large diameter Pasteur pipet (~1.3mm). Tubes were briefly placed on ice for large tissue pieces to settle; 600 μl of cloudy suspension containing dissociated cells were transferred to a 15 ml Falcon tube on ice. 600μL of fresh Hibernate A were added to the original Eppendorf tube and then trituration steps were repeated as above with medium and small diameter pipets (~0.8mm and ~0.4mm). Each cloudy suspension containing dissociated cells was pooled with the previous suspension.

Remaining cell clusters in the pooled cell suspension were removed by filtration through pre-wetted 100 μm and 40 μm cell strainers (Falcon brand, BD Biosciences, San Jose, CA). Small cellular debris in the suspension was reduced by centrifuging the filtrate for 3 min at 430×g through a three-step density gradient of Percoll (Cat# P1644; Sigma, St. Louis, MO). The cloudy top layer (approximately 2 ml) containing debris was discarded. Cells in the remaining layers were resuspended in the remaining Percoll solution and centrifuged for five min at 550xg. The pellet was resuspended in 1 ml of Hibernate A.

Immunolabeling and FACS

Dissociated cells were fixed and permeabilized by adding an equal volume of ethanol for a final concentration of 50% ethanol and kept on ice for 15 minutes with occasional mixing. Cells were centrifuged for two min at 425xg and resuspended in RNase-free phosphate-buffered saline (PBS). Cells were incubated with a biotinylated primary antibody against NeuN (1:1000 dilution, Cat# MAB377B, Chemicon) and a primary antibody against β-galactosidase ((β-gal; 1:10000 dilution, Cat# 4600-1409, Biogenesis, Poole, United Kingdom). Cells were rotated end-over-end in primary antibody for 30 minutes at 4°C and centrifuged for three min at 425xg. Cells were washed with 800 μl of PBS and resuspended in 700 μl of PBS. Cells were incubated with fluorescently-labeled streptavidin (Streptavidin-phycoerythrin, 1:1000 dilution, Cat# SA1004-1, Invitrogen, Carlsbad, CA) and secondary antibody (Alexa Fluor 488-labeled anti-goat IgG, 1:1000 dilution, Cat# A-11055, Invitrogen) and rotated end-over-end for 15 min. Cells were washed with 800 μl of PBS, centrifuged for three min at 425×g, and resuspended in 1 ml of PBS.

Immunolabeled cells were scanned and sorted at the Johns Hopkins Bayview Campus flow cytometry core facility. A FACS Aria (BD, Franklin Lakes, NJ) was used for cell sorting. Control samples were analyzed first to determine optimal criteria for sorting test samples. Specifically, fixed cells without antibody treatment were used to set the light scatter gate. Fixed cells treated with fluorescent secondary antibody, but no primary antibody, were then

used to set thresholds for endogenous tissue fluorescence and non-specific binding by streptavidin or secondary antibody. Next, control samples single-labeled with primary and secondary antibody for each protein (NeuN or β-gal) were used to compensate for fluorescent overlap in neighboring channels. Finally, test samples labeled with both fluorescent markers were analyzed and sorted. Sorted cells were collected into low-binding tubes (Cat# 022431081, Eppendorf, Westbury, NY) with 100 μl of PBS in each tube.

RNA extraction

After sorting, samples containing either βgal-positive or βgal-negative cells from cocaine-injected rats or all NeuN-positive cells from saline-injected rats were centrifuged for 8 min at 2650×g at 18°C. NeuN-negative cells from saline-injected rats were centrifuged for 8 min at 6000×*g* at 18°C. For microarray experiments, RNA was extracted with Trizol Reagent (Cat# 15596-026, Invitrogen) according to manufacturer's instructions. Quality and quantity of RNA were assessed using the Bioanalyzer Picochip (Cat# 5067-1513, Agilent Technologies, Palo Alto, CA). For qPCR experiments, RNA was extracted with RNEasy Micro kit (Cat# 74004; Qiagen, Valencia, CA) according to manufacturer's instructions with DNase treatment. Quantity and purity of RNA was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Microarray experiments

Microarrays were used to analyze RNA from NeuN-positive and NeuN-negative cells from saline-injected rats, and βgal-positive and βgal-negative neurons from cocaine-injected rats. As described above in the drug treatment section, microarrays contained three biological replicates for each of the four sample types. Five μl of total RNA from each sample were labeled using the Illumina TotalPrep RNA Amplification Kit (Cat# IL1791; Ambion, Austin, TX) in a two-step process of cDNA synthesis followed by *in vitro* RNA transcription with biotin-16-UTP. 0.75 μg of biotin-labeled cRNA was hybridized for 16 hours to Illumina Sentrix Rat Ref12_v1 BeadChips (Cat# BD-27-303; Illumina, San Diego, CA). Biotinylated cRNA hybridized to the chip was detected with Cy3-labeled streptavidin and quantified using Illumina's BeadStation 500GX Genetic Analysis Systems scanner. All labeling and analysis was done at the Johns Hopkins Bayview Medical Campus Lowe Family Genomics Core.

Real time quantitative PCR

Real-time quantitative PCR was used to validate FACS and microarray results. RNA was reverse-transcribed into cDNA using the RETROscript kit (Cat# AM1710, Ambion) with an oligo(dT) primer. Each 25 μl PCR reaction included 12.5 μl of Taqman Gene Expression Master Mix (Cat #4369514; Applied Biosystems, Foster City, CA), 1.1 μl each of 20 μM forward and reverse primers, 0.25 μl of 100 μM FAM-labeled probe, 5 μl of water, and 5 μl of 1 ng/μl cDNA. Primer and probe combinations [Table 1] were designed using the Roche Universal Probe Library Assay Design Center to be intron-spanning. PCR reactions were monitored using the Opticon Light Cycler (Biorad, Hercules, MD). The program began with 20 plate reads at 50°C for photobleaching, followed by 5 min at 95°C, and then 40 cycles of 20 sec at 94°C, 1 min at 60°C, and a plate read.

Standard curve reactions were first run for each gene to determine amplification efficiency [Table 1]. Expression levels for each amplified gene were calculated using efficiency^ΔC_q where $\Delta C_q = C_q$(experimental gene) − C_q(reference gene) for each biological replicate. *Gorasp2* was chosen as a reference gene because it was not differently expressed between neurons and glia in microarray analyses. It was further validated as a reference gene due to equal qPCR amplification across all samples when loading the same amount of template. Technical assay triplicate C_q values were averaged before calculating ΔC_q for each gene in a

sample. For each mRNA measured in qPCR, gene expression values were averaged across biological replicates. Then to reflect fold-change values, we divided this average by the average gene expression values of NeuN-positive cells from saline-injected rats. These values are indicated as means and standard errors in the graphs and tables.

Immunohistochemistry

Brain tissue was obtained from wild-type female Spraque-Dawley rats following the same acute and repeated cocaine treatments described above for the microarray and qPCR experiments. However 90 minutes after test day injections, rats were deeply anesthetized with isoflurane and perfused with 100 ml of PBS followed by 400 ml of 4% paraformaldehyde (PFA). Brains were post-fixed in PFA for 2 hours and transferred to 30% sucrose solution at 4°C for 2–3 days. Brains were frozen on powdered dry ice and kept at −80°C until sectioning. Coronal sections were cut 40 μm thick between Bregma 2.5 and −0.5 (Paxinos and Watson, 1998).

Sections were immunolabeled for Fos and Arc. Briefly, sections were washed three times in Tris-buffered saline (TBS) and permeabilized for 30 min in TBS with 0.2% Triton X-100. Sections were washed again in TBS and incubated in primary antibodies diluted in PBS with 0.3% Triton X-100 for 24 hours on a shaker at 4°C. We used a rabbit polyclonal against c-Fos antibody (1:500 dilution, Cat# sc-52, Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse monoclonal Arc antibody (1:100 dilution, Cat# sc-17839, Santa Cruz Biotechnology). Sections were washed three times in TBS and incubated in secondary antibodies diluted in PBS for 1.5 hours on a shaker at room temperature. We used Alexa Fluor 488-labeled donkey anti-rabbit antibody (1:200 dilution, Cat# A21206, Invitrogen) and Alexa Fluor 568-labeled goat anti-mouse antibody (1:200 dilution, Cat# A11004, Invitrogen). Sections were washed in TBS, mounted onto chrom-alum coated slides, and coverslipped with VectaShield hard-set mounting media.

Fluorescent images of Fos and Arc immunoreactivity in caudate-putamen and NAc (approximately 2.0 mm anterior to Bregma) were captured using a CCD camera (Coolsnap Photometrics, Roper Scientific Inc., Trenton, NJ) attached to a Zeiss Axioskop 2 microscope. Images for counting labeled cells were taken at 200x magnification; images for determining Fos and Arc colocalization were taken at 400x magnification. Labeled cells from four hemispheres per rat were automatically counted using IPLab software for Macintosh, version 3.9.4 r5 (Scanalytics, Inc., Fairfax, VA). Counts from all four hemispheres were averaged to obtain a single value for each rat.

Data analysis

Locomotor sensitization data were analyzed using one-way ANOVA with repeated measures for the main effect of time over 4 injection days. Statistical significance was set at $p < 0.05$. Microarray data were analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program based on SAS JMP 7.0, as previously described (Garg et al., 2009). Fluorescence intensity data from microarrays were subject to filtering by detection *p*-values and *Z* normalization. Sample quality was analyzed using scatterplots, principal components analysis, and gene sample *Z*-score-based hierarchical clustering to exclude possible outliers. ANOVA tests were then used to eliminate genes with larger variances within each comparison group. Genes were identified as differentially expressed if $p < 0.01$, absolute *Z*-ratio ≥ 1.5, and false discovery ratio (fdr) <0.07. For qPCR data, one-way ANOVA was used to compare data for each gene. Statistical significance was set at $p < 0.05$. For Fos and Arc immunohistochemistry, a t-test assuming unequal variance was used to calculate statistical significance, set at $p < 0.05$.

RESULTS

cfos-lacZ transgenic rats

The *cfos-lacZ* transgene in these rats is regulated by a *c-fos* promoter similar to that in the endogenous *c-fos* gene and is thus similarly activated in neurons by high levels of excitatory synaptic input (Shin et al., 1990; Labiner et al., 1993; Sgambato et al., 1997). The protein product of the *cfos-lacZ* transgene, β-galactosidase (βgal), is co-expressed with c-Fos in strongly activated neurons (Koya et al., 2009) and used to label activated striatal neurons in the following FACS purification procedure.

FACS purification of activated striatal neurons following single acute cocaine injections

Whole striata were obtained from *cfos-lacZ* rats 90 minutes following acute injections of 30 mg/kg cocaine or saline outside their home cage. Dissociated striatal cells from similarly treated rats were pooled prior to FACS purification for each of the biological replicates in subsequent microarray and quantitative PCR (qPCR) analyses. Dissociated striatal cells were initially analyzed according to their forward and side light-scattering characteristics [Figure 1a]. The majority of NeuN-labeled neurons were found within a strip of events along the lower part of the plot. Thus, all subsequent analyses were forward gated to include only events found within this strip.

Cells within this gate were separated according to their degree of immunolabeling with the neuronal marker NeuN and the neural activation marker βgal. In tissue obtained from cocaine-injected rats, approximately 15% of NeuN-labeled neurons had high levels of βgal [Figure 1b], which corresponded to approximately 100,000 βgal-labeled neurons per rat. All βgal-labeled cells co-expressed NeuN, indicating that only neurons expressed βgal. In contrast, very few NeuN-labeled neurons obtained from saline-injected rats expressed the activation markers βgal or Fos, which supports βgal and Fos immunoreactivity as markers of neural activation in cocaine-injected rats. The low numbers of βgal-expressing neurons from saline-injected rats did not provide enough cells for subsequent molecular analyses. Thus, we used only NeuN-labeling to separate neuronal from non-neuronal cells in all subsequent experiments with striatal tissue obtained from saline-injected rats. Overall, approximately 30% of all striatal cell bodies were NeuN-positive, which corresponded to approximately 600,000 striatal neurons per rat. When FACS-purified cells were examined with fluorescence microscopy, all cells were round and devoid of processes [Figure 2]. Microscopic observation confirmed that FACS-sorted cells were appropriately labeled for NeuN− and βgal-immunoreactivity. Samples of FACS-purified cells were found to be >90% pure when assessed with a second round of flow cytometry (data not shown).

We determined selectivity of our FACS purification procedure with microarray to analyze gene expression patterns of the FACS-purified cells. We compared gene expression in βgal-positive and βgal-negative neurons from cocaine-injected rats as well as NeuN-positive and NeuN-negative cells from saline-injected rats. Principal component and cluster analysis indicated that the primary differentiating factor was whether the cells were neurons or glia [Figure 3a]. The second differentiating factor was βgal expression used to separate activated from non-activated neurons. Genes were identified as differentially expressed if $p < 0.01$, absolute Z-ratio ≥ 1.5, and false discovery ratio (fdr) < 0.07. We found that 125 genes were increased and 467 genes were decreased in βgal-positive neurons relative to βgal-negative neurons obtained from the same striatal tissue of cocaine-injected rats [Figure 3b]. When the same βgal-positive neurons from cocaine-injected rats were compared to control samples of all NeuN-labeled neurons from saline-injected rats, 133 genes were increased and 890 genes were decreased in the βgal-positive neurons. Notably the two control groups--βgal-negative neurons from cocaine-injected rats and all NeuN-labeled neurons from saline-injected rats--

produced very similar gene expression patterns; gene expression was not significantly different between these control groups [Figure 3b].

Specific genes found in the different sample groups confirmed separation of activated from non-activated neurons, as well as neuronal from non-neuronal cells using FACS [Table 2]. βgal-positive neurons from cocaine-injected rats expressed higher levels of many neural activation marker genes relative to βgal-negative neurons from the same cocaine-injected rats or all NeuN-labeled neurons from saline-injected rats [Figure 3c]. These activity marker genes included *c-fos*, *junB*, *arc*, *egr* family (1, 2, 4), and *nr4a3* (Guzowski et al., 2005). Interestingly, these βgal-positive neurons also expressed significantly higher levels of the D1 dopamine receptor cell-type specific marker prodynorphin gene and significantly lower levels of the D2 dopamine receptor gene.

Microarray gene expression data were validated using qPCR. The activity marker genes *fosB*, *arc*, and *nr4a3* were expressed at higher levels in βgal-positive neurons from cocaine-injected rats relative to both βgal-negative neurons from the same cocaine-injected rats and to all neurons from saline-injected rats [Figure 4a; data and statistical information in Table 2]. βgal-positive neurons also expressed higher levels of the prodynorphin gene [Figure 4b]. While βgal-positive neurons had apparently lower levels of expression for several genes, including the A2A adenosine receptor, the Kv3.1 potassium channel, and map2k6/MEKK6 genes, only Kv3.1 and map2k6/MEKK6 expression levels were statistically different using qPCR [Figure 4c]. Overall, gene expression alterations assessed with qPCR were nearly identical to gene expression alterations assessed by microarray experiments.

To determine whether mRNA alterations in activated neurons were reflected at the protein level, we used immunohistochemical analysis of coronal brain sections from cocaine- and saline-injected wild-type female rats [Figure 5]. These sections did not undergo dissociation or FACS, and therefore immunohistochemical analysis was not affected by the cell dissociation found in the FACS procedure. In ventral striatum from cocaine-injected rats [Figure 5a], the neural activity markers Fos and Arc were co-expressed in the same cells: 85±4% of all Fos-positive cells were Arc-positive, while 82±3% of all Arc-positive cells were Fos-positive. Cocaine injections produced a 2.7-fold increase in Fos-labeled neurons and a 2.7-fold increase in Arc-labeled neurons. In dorsal striatum from cocaine-injected rats [Figure 5b], 80±2% of all Fos-positive cells were Arc-positive, while 86±3% of all Arc-positive cells were Fos-positive. Cocaine injections produced a 4.4-fold increase in Fos-labeled neurons and a 3.8-fold increase in Arc-labeled neurons.

FACS purification of activated striatal neurons from cocaine-sensitized rats

In the second FACS experiment, all rats were sensitized with repeated cocaine injections. Repeated cocaine administration in the locomotor box enhanced cocaine-induced locomotion: locomotion on day 7 was 180±18% of day 1 locomotion (main effect of time over 4 repeated injections, $F_{3, 363} = 24.9$, $p < 0.001$), which indicated significant psychomotor sensitization. Seven days later on test day, cocaine or saline was administered to rats in the same locomotor box. Ninety minutes later, striatal tissue including NAc was extracted and cells were FACS-purified using the procedure described above.

Cells within the same light scatter-gated strip of events as in the previous experiment were separated according to their degree of immunolabeling with the neuronal marker NeuN and the neural activation marker βgal. In tissue obtained from cocaine-injected rats, approximately 10% of NeuN-labeled neurons had high levels of βgal [Figure 6], which corresponded to approximately 60,000 βgal-labeled neurons per rat. All βgal-labeled cells co-expressed NeuN, indicating that only neurons expressed βgal. Again, tissue obtained from rats injected with saline on test day produced very few βgal- or Fos-expressing

neurons, and thus not enough cells for subsequent molecular analyses. Therefore, we used only NeuN-labeling to separate neuronal from non-neuronal cells obtained from saline-injected rats. Approximately 30% of all cell bodies in saline-injected rats were NeuN-positive, which in this experiment corresponded to approximately 400,000 striatal neurons per rat.

We used qPCR to compare gene expression in βgal-positive and βgal-negative neurons from cocaine-injected rats as well as all NeuN-positive neurons from saline-injected rats following cocaine sensitization. βgal-positive neurons from cocaine-injected rats expressed higher levels of three neural activity marker genes-- *fosB*, *arc*, and *nr4a3*--relative to βgal-negative neurons from the same cocaine-injected rats and to all neurons from saline-injected rats [Figure 7a; data and statistical information in Table 3]. βgal-positive neurons also expressed a higher level of the prodynorphin gene similar to that in the single cocaine injection experiment [Figure 7b]. In contrast, βgal-positive neurons had lower levels of expression for several genes [Figure 7c], including *Drd2* encoding the D2 dopamine receptor, and *Map2k6* encoding the kinase that activates p38 MAPK, similar to the single acute cocaine injection experiment. Finally, βgal-positive neurons had increased expression of *dusp1*, also known as mkp1 [Figure 7c], which encodes the phosphatase that inactivates p38 MAPK (Sgambato et al., 1998).

To determine whether mRNA alterations in activated neurons are reflected at the protein level, we used immunohistochemical analysis of coronal brain sections from wild-type cocaine-sensitized female rats following either cocaine or saline injections on test day. In ventral striatum from cocaine-injected rats, the neural activity markers Fos and Arc were co-expressed in the same cells: 90±2% of all Fos-positive cells were Arc-positive, and 83±2% of all Arc-positive cells were Fos-positive. Cocaine test injections produced a 2.3-fold increase in Fos-labeled neurons and a 2.2-fold increase in Arc-labeled neurons. In dorsal striatum from cocaine-injected rats, 93±2% of all Fos-positive cells were Arc-positive, and 90±3% of all Arc-positive cells were Fos-positive. Cocaine test injections produced a 4.4-fold increase in Fos-labeled neurons and a 4.5-fold increase in Arc-labeled neurons. Thus, very few non-Fos-labeled cells expressed Arc and very few non-Arc-labeled cells expressed Fos which supports our findings from sorted cells.

DISCUSSION

We developed a procedure for purifying and assessing molecular alterations within neurons defined only by their previous activation state during cocaine-induced locomotor activity. Data from the microarray, qPCR, and immunohistochemistry experiments independently confirmed that our FACS procedure identified unique molecular alterations within activated neurons, as defined by *c-fos* promoter induction of βgal expression. The main biological finding using this procedure was that immediate early genes (IEGs) are induced only in activated neurons, whereas IEG expression is unchanged or even decreased in the non-activated majority of neurons. Since many of these IEG neural activity markers are also transcription factors, it is likely that very different patterns of gene expression are subsequently induced within these activated neurons that may contribute to the physiological and behavioral effects of cocaine (for example, see (Curran and Franza, 1988; Nestler et al., 1993; McClung et al., 2004; Loebrich and Nedivi, 2009).

Differential IEG induction between activated and non-activated neurons supports the hypothesis that only a minority of neurons are strongly activated during cocaine-induced locomotion, while the majority of neurons are activated to a far lesser degree or not at all. Electrophysiological experiments in wild-type rats indicate that Fos protein expression correlates with high levels of excitatory synaptic input (Shin et al., 1990; Labiner et al.,

1993; Sgambato et al., 1997). We know that Fos and βgal expression are similarly regulated in the same neurons in *cfos-lacZ* rats (Koya et al., 2009). Thus we infer that increased βgal and IEG expression in only a small number of striatal neurons indicates that only a minority of striatal neurons receives high levels of excitatory synaptic input in response to cocaine administration.

Discrete activation of striatal neurons is consistent with the upstate-downstate hypothesis that describes opposing effects of dopamine on striatal neuronal activity. Cocaine-induced dopamine is thought to enhance differences between high activity and low activity neurons by enhancing ongoing electrophysiological activity of neurons that are already in a high activity state, while attenuating ongoing activity of the majority of neurons with lower activity (Wilson and Kawaguchi, 1996; Hernandez-Lopez et al., 1997; Nicola et al., 2000; O'Donnell, 2003; Nicola et al., 2004). The resulting increase in signal to noise ratio not only enhances selectivity during acute signaling but, as suggested in our study, may also contribute to induction of unique molecular neuroadaptations in only the high activity neurons. Meanwhile gene expression in βgal-negative neurons, which compose the majority of striatal neurons from cocaine-injected rats, appeared to be no different from gene expression in striatal neurons from saline-injected rats; this supports the hypothesis that the majority of striatal neurons remain in the downstate during cocaine exposure.

Components of the p38 MAP kinase signaling pathway were also differentially altered between activated and non-activated neurons. Gene expression of Map2k6/MEKK6, a kinase that activates p38, was decreased, while gene expression of Mkp1/Dusp1, a phosphatase that inactivates p38 (Sgambato et al., 1998), was increased in activated neurons but not in non-activated neurons. Thus overall p38 MAPK signaling, shown to be play a role in synaptic plasticity (Bolshakov et al., 2000; Izumi et al., 2008), may be attenuated in activated neurons relative to the non-activated majority of neurons.

FACS can also help identify the neuronal types that are activated after acute or repeated cocaine exposure. We found higher expression of the D1 neuronal marker gene prodynorphin in activated neurons, and lower expression of the D2 neuronal marker genes D2 dopamine receptor and A2A adenosine receptor in activated neurons. This suggests that Fos was induced primarily in D1 striatal neurons. While this agrees with many immunohistochemical studies that report Fos induction primarily in D1 neurons following psychostimulant injections in the *home cage* (Berretta et al., 1992; Cenci et al., 1992), it contradicts the equal activation of D1 and D2 neurons found following psychostimulant injections in a relatively *novel environment* outside the rat's home cage (Jaber et al., 1995; Badiani et al., 1999; Hope et al., 2006).

Since we used the same βgal marker to identify activated neurons during FACS as we used for our previously described Daun02 inactivation procedure (Koya et al., 2009), it is plausible that we are observing unique molecular alterations within neuronal ensembles necessary for the learned component of context-specific sensitization of cocaine-induced locomotion. We previously found that inactivation of only a small number of sparsely distributed neurons within a putative ensemble is sufficient to disrupt context-specific cocaine psychomotor sensitization (Koya et al., 2009). This finding was replicated using the same female *cfos-lacZ* rats and sensitization regimen used in the present study [http://irp.drugabuse.gov]. These neurons are not inherently more sensitive to all stimuli, since altering the environment or context where drug was administered activated a different set of striatal neurons following cocaine injections (Mattson et al., 2008; Koya et al., 2009). Selection of a specific striatal neuronal ensemble appears primarily dependent on the pattern of activity from cortical and subcortical glutamatergic afferents (Pennartz et al., 1994; O'Donnell, 2003), and these inputs are themselves determined by specific environmental

and interoceptive stimuli present during behavior. From this previous work along with our present data, we hypothesize that repeated pairing of cocaine with environmental stimuli during our sensitization regimen produces unique molecular alterations within repeatedly activated neuronal ensembles that may contribute to the learned associations underlying context-specific sensitization.

Overall, we demonstrated a novel application of FACS to isolate adult neurons selectively activated during acute cocaine-induced locomotor activity and repeated cocaine-induced locomotor sensitization. We propose that this approach could also be used to identify unique molecular neuroadaptations in neuronal ensembles encoding the behavioral effects of abused drugs and other learned behaviors. The causal roles of these unique molecular neuroadaptations in neural plasticity and behavior must eventually be examined by manipulating gene expression in only activated neurons; however this technique has yet to be developed.

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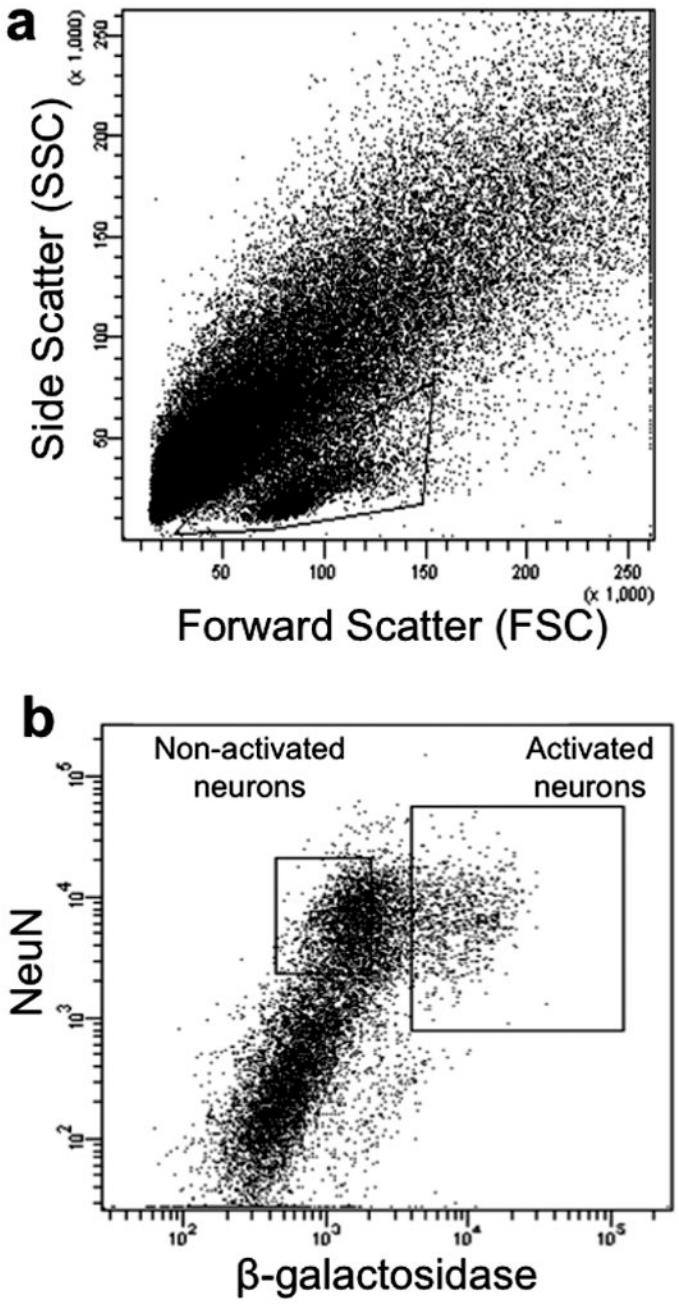

Figure 1. Fluorescence activated cell sorting of βgal-labeled neurons from rats treated with a single injection of cocaine

(a) Light scatter plot in which each dot represents one event (cell or debris). Forward scatter is a measure of size; side scatter is a measure of granularity. The “gate”, indicated by the solid-line box, encompasses all events that are analyzed on subsequent fluorescence plots. This gate allows analysis of cells and exclusion of debris. (b) Fluorescence plot in which each dot represents one event. NeuN antibody labels all neurons; β-galactosidase antibody labels activated neurons. Two collection gates, indicated by the solid-line boxes, encompass activated and non-activated neurons that were collected for further analysis.

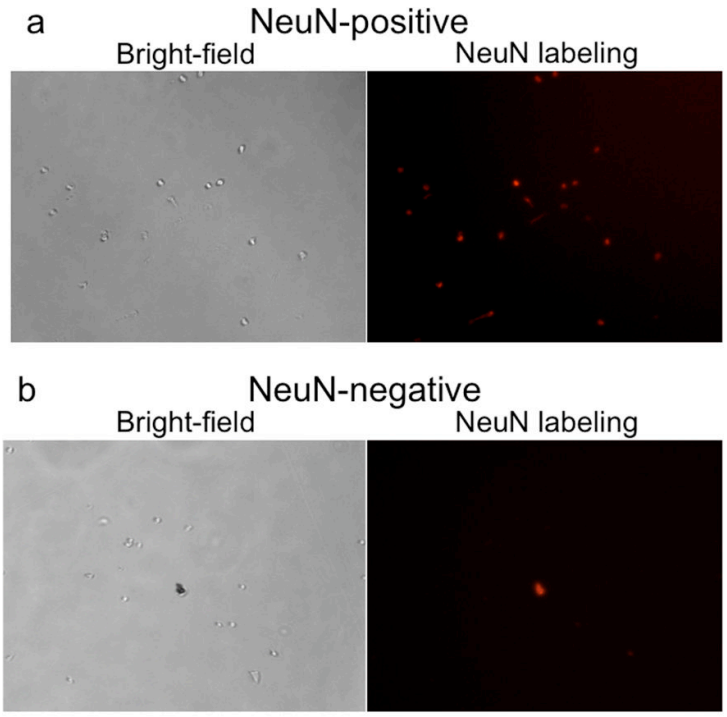

Figure 2. Microscope photos of FACS-purified cells and debris

Bright-field and fluorescence images of (a) NeuN-positive (neural) and (b) NeuN-negative (glial) cells after fluorescence activated cell sorting. All cells are round and devoid of processes. NeuN labeling is red. In (b) there is a piece of debris that is autofluorescent (shows up in every fluorescent light channel).

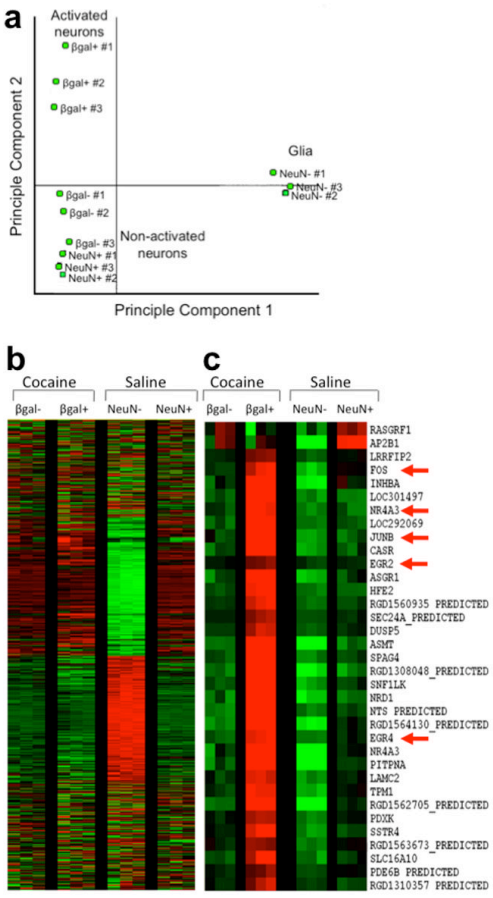

Figure 3. Microarray analysis of cells sorted from rat striata after a single injection of cocaine or saline

(a) Principle component analysis demonstrates that the primary differentiating factor among these samples was whether the cells were neurons or glia. The second differentiating factor was whether cells were activated (βgal-positive) or non-activated (βgal-negative and NeuN-positive). (b) Heat maps display expression of thousands of genes in the four samples: activated βgal-positive and non-activated βgal-negative neurons from cocaine-treated rats, and NeuN-positive neurons and NeuN-negative cells from saline-treated rats. Each row is one gene; each column is one sample; each group had 3 biological replicates. Green indicates decreased expression, and red indicates increased expression. Note that the NeuN-negative cells (glia) have a vastly different gene expression pattern than all 3 neuron groups. (**c**) Enlarged portion of heat map focused on *fos* and related IEGs. Many IEGs are upregulated in the activated βgal-positive neurons, including *nr4a3, junB, egr2,* and *egr4*.

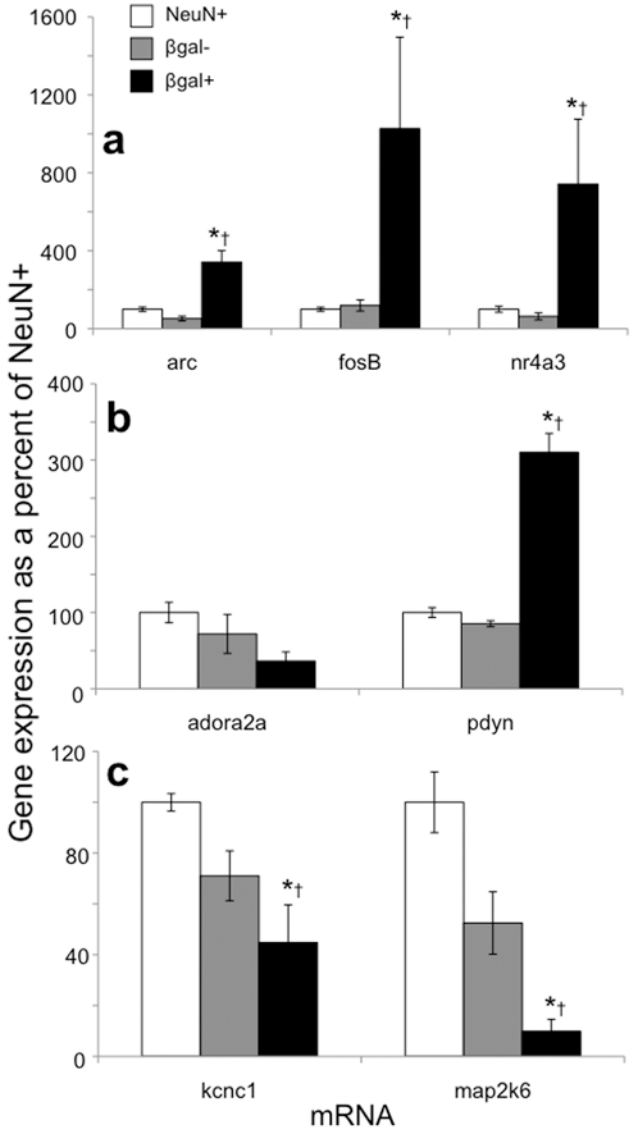

Figure 4. Quantitative PCR demonstrates a different gene expression profile for βgal-positive neurons after acute cocaine injections, as compared to βgal-negative neurons from the same rats, or to all neurons from saline-injected rats

qPCR assays confirm microarray results. (a) mRNA levels for the immediate early genes *arc*, *fosB*, and *nr4a3* are increased in βgal-positive neurons. (b) mRNA levels for the D1-type cell marker prodynorphin (*pdyn*) are increased in βgal-positive neurons, while only a trend is apparent for the A2A adenosine receptor (*adora2a*). (c) mRNA levels for the Kv3.1 potassium channel subunit (*kcnc1*) and the kinase that activates p38 MAPK (*map2k6*) are decreased in βgal-positive neurons. For expression values, n, and p values, see Table 2. * indicates $p<0.05$ versus NeuN-positive neurons from saline-injected rats. † indicates $p<0.05$ versus βgal-negative neurons from cocaine-injected rats.

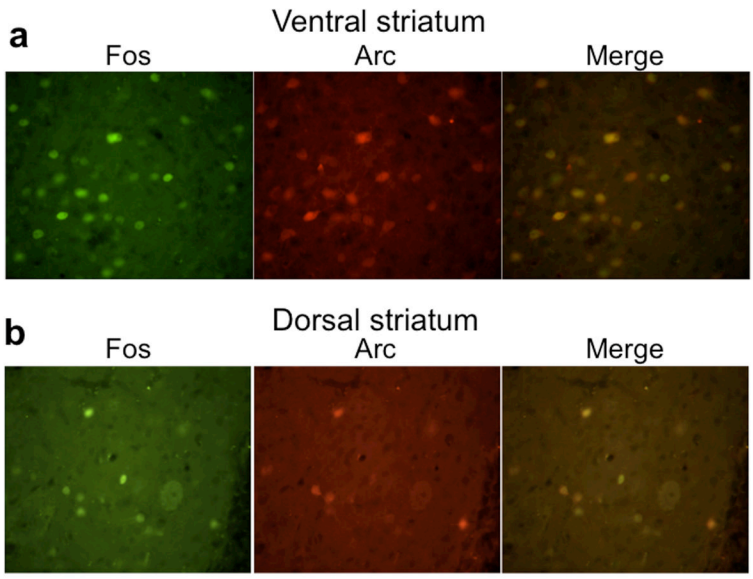

Figure 5. Fos and Arc are co-expressed in (a) ventral striatum and (b) dorsal striatum after a single injection of cocaine

Fos-expressing nuclei are labeled green; Arc-expressing neurons are labeled red. Right-hand panels merge the Fos and Arc panels.

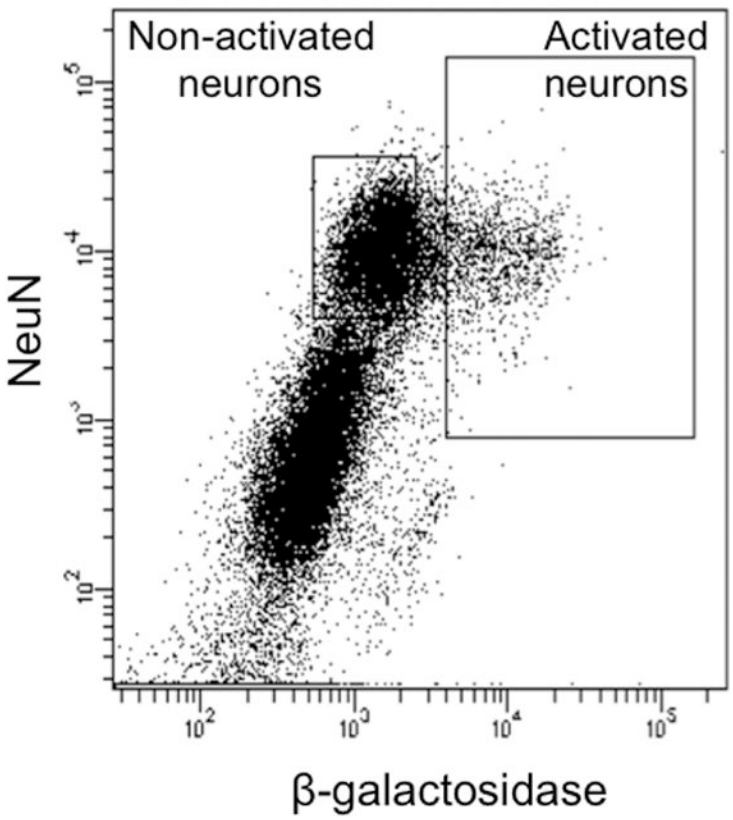

Figure 6. Fluorescence activated cell sorting of βgal-labeled neurons from sensitized rats challenged with cocaine 7 days after repeated cocaine treatment

Fluorescence plot in which each dot represents one event. NeuN labels all neurons; β-galactosidase labels activated neurons. Two collection gates (solid line boxes) encompass activated and non-activated neurons that were collected for further analysis.

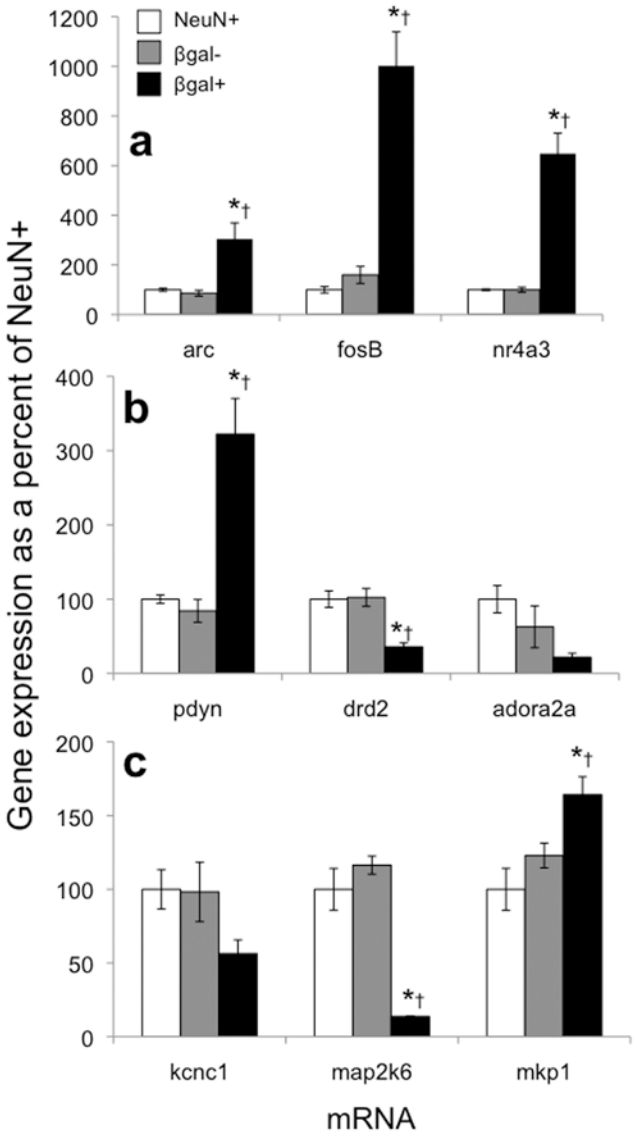

Figure 7. Quantitative PCR demonstrates a different gene expression profile in βgal-positive neurons from sensitized cocaine-challenged rats, as compared to βgal-negative neurons from the same rats, or all neurons from saline-challenged rats

(a) mRNA levels for the immediate early genes *arc*, *fosB*, and *nr4a3* are increased in βgal-positive neurons. (b) mRNA levels for the D1-type cell marker prodynorphin (*pdyn*) are increased in βgal-positive neurons, while the D2 receptor *drd2* is decreased in βgal-positive neurons. (c) mRNA levels for the kinase that activates p38 MAPK (*map2k6*) are decreased in βgal-positive neurons, while mRNA levels for the phosphatase that inactivates p38 MAPK (*mkp1* or *dusp1*) are increased in βgal-positive neurons. n=3 for every sample. See Table 3 for p values. ***** indicates $p<0.05$ versus Neun-positive neurons from saline-injected rats.† indicates $p<0.05$ versus βgal-negative neurons from cocaine-injected rats.

Table 1

Primers and probes used for qPCR

Gene	Forward primer	Reverse primer	Probe	Efficiency
c-fos	Cagcctttcctactaccattcc	acagatctgcgcaaaagtcc	#67	2.14
fosB	Tgcagctaaatgcagaaacc	Ctcttcgagctgatccgttt	#109	1.87
arc	Gctgaagcagcagacctga	ttcactggtatgaatcactgctg	#79	1.94
nr4a3	Acgcccagagaccttgatt	Cacgtgctcagcgtctgt	#123	2.01
pdyn	Atcggccatcctatcacct	agcctgtggatgagtgacct	#129	1.99
drd2	Tctgcaccgttatcatgaagtc	Tctccatttccagctcctga	#118	2.03
adora2a	Gcagcgctagtttcgaagtc	Cggcttcagggttctgag	#78	1.98
kcnc1	Cttatcaaccggggagtacg	Aatgacagggctttctttgc	#22	2.01
map2k6	Ttggagcctatagtggagctg	ctattaactgtggcccgtatcc	#77	2.09
dusp1	agccataactttgtttttcagactc	Tggcagtgcacaaacacc	#67	1.94

Table 2

Summary of microarray and qPCR data from the acute cocaine experiment.

Gene symbol	Description	Gene ID	Microarray	Quantitative PCR
Fos	c-fos; FBJ osteosarcoma oncogene; Immediate early gene	314322	+2.7 fold	$F_{(2,9)}$=1.887, p=0.207 βgal+ *vs* βgal−: p=0.229 βgal+ *vs* NeuN+: p=0.09 βgal− *vs* NeuN+: p=0.446
FosB	fra-2; FBJ osteosarcoma oncogene B; Immediate early gene	308411	---	$F_{(2,11)}$=5.520, p<0.05 βgal+ *vs* βgal−: +6.3 fold; n=5,6; p<0.05 βgal+ *vs* NeuN+: +7.5 fold; n=5,3; p<0.05 βgal− *vs* NeuN+: p=0.938
Arc	Activity-regulated cytoskeleton-associated protein	54323	+4.6 fold	$F_{(2,8)}$=17.402, p<0.005 βgal+ *vs* βgal−: +6.6 fold; n=4,4; p<0.005 βgal+ *vs* NeuN+: +3.4 fold; n=4,3; p<0.005 βgal− *vs* NeuN+: p=0.419
Nr4a3	Nuclear receptor subfamily 4, group A, member 3; transcription factor	58853	+5.2 fold	$F_{(2,9)}$=11.456, p<0.005 βgal+ *vs* βgal−: +8.0 fold; n=4,5; p<0.005 βgal+ *vs* NeuN+: +5.1 fold; n=4,3; p<0.01 βgal− *vs* NeuN+: p=0.742
Pdyn	Prodynorphin; peptide found in D1 expressing striatal neurons	29190	+2.3 fold	$F_{(2,6)}$=70.047, p<0.001 βgal+ *vs* βgal−: +3.6 fold; n=3,3; p<0.001 βgal+ *vs* NeuN+: +3.1 fold; n=3,3; p<0.001 βgal− *vs* NeuN+: p=0.515
Adora2a	Adenosine A2a receptor; found in D2 expressing striatal neurons	25369	−3.0 fold	$F_{(2,8)}$=2.690, p=0.128 βgal+ *vs* βgal−: p=0.204 βgal+ *vs* NeuN+: p=0.051 βgal− *vs* NeuN+: p=0.342
Kcnc1	Kv4; Kv3.1; Potassium voltage gated channel, Shaw-related subfamily, member 1	25327	−2.6 fold	$F_{(2,9)}$=16.942, p<0.005 βgal+ *vs* βgal−: −2.2 fold; n=4,5; p<0.01 βgal+ *vs* NeuN+: −3.1 fold; n=4,3; p<0.001 βgal− *vs* NeuN+: −1.4 fold; p=0.031
Map2k6	Mkk6; Mitogen-activated protein kinase kinase 6; activator of p38 MAPK	114495	−5.2 fold	$F_{(2,9)}$=17.196, p<0.005 βgal+ *vs* βgal−: −7.9 fold; n=4,5; p<0.05 βgal+ *vs* NeuN+: −15.1 fold; n=4,3; p<0.001 βgal− *vs* NeuN+: −1.9 fold; p=0.013

One way ANOVA (F and p values are shown in Quantitative PCR column) was used to compare gene expression in three groups of cells: βgal-positive neurons and βgal-negative neurons from the same rats after a single injection of cocaine, and all neurons (NeuN-positive; NeuN+) from different rats after a single injection of saline. Fold-level changes are relative to control levels in NeuN+ cells. Positive fold-level changes indicate higher levels while negative fold-level changes indicate lower levels than in NeuN+ control samples.

Table 3

Summary of qPCR data from the repeated cocaine experiment.

Gene symbol	Description	Gene ID	Quantitative PCR
FosB	fra-2; FBJ osteosarcoma oncogene B; Immediate early gene	308411	$F_{(2,5)}$=62.652, p<0.001 βgal+ *vs* βgal−: +6.3 fold; n=2,3; p<0.001 βgal+ *vs* NeuN+: +10.0 fold; n=2,3; p<0.001 βgal− *vs* NeuN+: p=0.478
Arc	Activity-regulated cytoskeleton-associated protein	54323	$F_{(2,6)}$=10.374, p<0.05 βgal+ *vs* βgal−: +3.5 fold; n=3,3; p<0.01 βgal+ *vs* NeuN+: +3.0 fold; n=3,3; p<0.01 βgal− *vs* NeuN+: p=0.807
Nr4a3	Nuclear receptor subfamily 4, group A, member 3; transcription factor	58853	$F_{(2,6)}$=43.423, p<0.001 βgal+ *vs* βgal−: +6.5 fold; n=3,3; p<0.001 βgal+ *vs* NeuN+: +6.5 fold; n=3,3; p<0.001 βgal− *vs* NeuN+: p=1.000
Pdyn	Prodynorphin; peptide found in D1 expressing striatal neurons	29190	$F_{(2,6)}$=20.757, p<0.005 βgal+ *vs* βgal−: +3.8 fold; n=3,3; p<0.005 βgal+ *vs* NeuN+: +3.2 fold; n=3,3; p<0.005 βgal− *vs* NeuN+: p=0.716
Drd2	Dopamine Receptor D2	24318	$F_{(2,6)}$=14.389, p<0.01 βgal+ *vs* βgal−: −2.9 fold; n=3,3; p<0.005 βgal+ *vs* NeuN+: −2.8 fold; n=3,3; p<0.005 βgal− *vs* NeuN+: p=0.874
Adora2a	Adenosine A2a receptor; found in D2 expressing striatal neurons	25369	$F_{(2,6)}$=3.963, p=0.080 βgal+ *vs* βgal−: −2.9 fold; n=3,3; p=0.190 βgal+ *vs* NeuN+: −4.6 fold; n=3,3; p<0.05 βgal− *vs* NeuN+: p=0.230
Kcnc1	Kv4; Kv3.1; Potassium voltage gated channel, Shaw- related subfamily, member 1	25327	$F_{(2,6)}$=2.704, p=0.145 βgal+ *vs* βgal−: p=0.10 βgal+ *vs* NeuN+: p=0.09 βgal− *vs* NeuN+: p=0.941
Map2k6	Mkk6; Mitogen-activated protein kinase kinase 6; activator of p38 MAPK	114495	$F_{(2,6)}$=38.591, p<0.001 βgal+ *vs* βgal−: −8.5 fold; n=3,3; p<0.001 βgal+ *vs* NeuN+: −7.3 fold; n=3,3; p<0.001 βgal− *vs* NeuN+: p=0.239
Mkp1	Mkp1; Dual specificity phosphatase 1; dephosphorylates and inactivates p38 MAPK	114856	$F_{(2,6)}$=7.600, p<0.05 βgal+ *vs* βgal−: +1.3 fold; n=3,3; p<0.05 βgal+ *vs* NeuN+: +1.6 fold; n=3,3; p<0.01 βgal− *vs* NeuN+: p=0.219

One way ANOVA (F and p values are shown in Quantitative PCR column) was used to compare gene expression in three groups of cells: βgal-positive neurons and βgal-negative neurons from the same sensitized rats that received an injection of cocaine 90 minutes before obtaining tissue, and all neurons (NeuN-positive; NeuN+) from sensitized rats that received an injection of saline 90 minutes before obtaining tissue. Fold-level changes are relative to control levels in NeuN+ cells. Positive fold-level changes indicate higher levels while negative fold-level changes indicate lower levels than in NeuN+ control samples.