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Transcriptomic profiling of the ventral tegmental area and nucleus accumbens in rhesus macaques following long-term cocaine self-administration*

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Abstract

Background—The behavioral consequences associated with addiction are thought to arise from drug-induced neuroadaptation. The mesolimbic system plays an important initial role in this process, and while the dopaminergic system specifically has been strongly interrogated, a complete understanding of the broad transcriptomic effects associated with cocaine use remains elusive.

Methods—Using next generation sequencing approaches, we performed a comprehensive evaluation of gene expression differences in the ventral tegmental area and nucleus accumbens of rhesus macaques that had self-administered cocaine for roughly 100 days and saline-yoked controls. During self-administration, the monkeys increased daily consumption of cocaine until

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Contributors

WDY and JKR were responsible for study concept and design. NMS and JKR were responsible for collection of animal data. WDY, SVM, DBG were responsible for brain and tissue collection. DBG and EJY were responsible for data analysis. EJY and JKR drafted the manuscript. All authors critically reviewed the manuscript and approved the final version for publication.

Conflict of Interest

No conflict declared.

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almost the maximum number of injections were taken within the first 15 min of the one hour session for a total intake of 3 mg/kg/day.

Results—We confirm the centrality of dopaminergic differences in the ventral tegmental area, but in the nucleus accumbens we see the strongest evidence for an inflammatory response and large scale chromatin remodeling.

Conclusions—These findings suggest an expanded understanding of the pathology of cocaine addiction with the potential to lead to the development of alternative treatment strategies.

Keywords

mesolimbic pathway; cocaine; rhesus monkey; genetics; neuroinflammation

1. Introduction

Drug abuse is associated with a number of molecular and cellular effects on the brain including changes in neurocircuitry, gene expression, and epigenetic regulation. These changes are believed to be linked with the transition from substance use to abuse: compulsive drug seeking, loss of restraint, and negative affect (Koob and Volkow, 2010; Luscher and Malenka, 2011). The mesolimbic dopamine system, connecting the ventral tegmental area (VTA) and the nucleus accumbens (NAc), drives the salient effects of reward and has been consistently and repeatedly shown to be the primary site of action of drugs of abuse (Nestler, 2005).

There have been a number of studies in rodents and human post-mortem tissues that focus on gene expression differences associated with addiction both in the mesolimbic pathway as well as other areas of the brain (Zhou et al., 2014b). Microarray studies in rodents have identified differences in immediate-early genes (genes activated rapidly following extracellular stimulation) and dopaminergic pathways (Piechota et al., 2010; Yuferov et al., 2003; Zhang et al., 2005) in accordance with previous findings using Northern blots and immunohistochemistry (Hope et al., 1992). More recent studies using next generation sequencing technologies to study the effect of cocaine on the mouse NAc found differences from controls across multiple neurotransmitter systems including dopaminergic, cholinergic, glutamatergic, GABAergic systems as well as cadherin and Wnt signaling pathways (Eipper-Mains et al., 2013). This has been extended to non-coding RNAs with functional effects imputed using informatics approaches (Bu et al., 2012; Chen et al., 2013).

There have also been a number of microarray studies of gene expression on human cocaine abusers in the NAc (Albertson et al., 2004; Bannon et al., 2005) and midbrain dopaminergic regions (Tang et al., 2003). While confirming many of the dopaminergic findings from rodents, more widespread transcriptional changes were also identified. These findings have been suggested to reflect epigenetic reprogramming resulting from chronic drug exposure (Zhou et al., 2011). The differences observed between rodent and human expression studies may result from methodological differences in the duration of exposure to drug, acute compared to chronic usage (Zhou et al., 2014b). Indeed, transgenic mouse work has shown

that histone acetylation is important for response to chronic, but not acute, cocaine exposure (Renthal et al., 2007).

One of the challenges in developing translational animal models is to recapitulate as closely as possible the most salient features of human behavior while maintaining precise experimental control. In addition to greater genetic and neuroanatomical similarities, nonhuman primate models in particular are valuable when modeling aspects of addiction because their consumption and patterns of drug taking so closely reflect that seen in human drug addiction (Platt and Rowlett, 2012). A rhesus macaque model of self-administration provides a unique opportunity to explore the molecular mechanisms involved in the entirety of the neurochemical systems and circuitry associated with the addictive processes (Weerts et al., 2007).

In the present study, we used a cocaine self-administration procedure in rhesus macaques over three months (approximately 100 consecutive days). After this exposure period, transcriptomic analysis was performed on the NAc and the VTA using next generation RNA-seq. This allows for an unbiased and holistic view of the differences between cocaine- and saline-treated animals in these regions and provides a simultaneous assessment of the two brain regions in long-term cocaine consumption.

2. Materials and Methods

2.1 Ethics statement

Animals were maintained in accordance with the guidelines of the Committee on Animals of Harvard Medical School and the Guide for the Care and Use of Laboratory Animals (8th edition, 2011). Research protocols were approved by the Harvard Medical School Institutional Animal Care and Use Committee.

2.2 Animals

Subjects were 10, experimentally naïve, male young adult (4–7 years) rhesus macaques (*Macaca mulatta*). All animals were raised in shared conditions with identical diet and husbandry; paired animals balanced for age and weight were randomly assigned to cocaine exposure or yoked saline. All animals were of Indian ancestry and unrelated to at least kinship coefficient < 0.02. Prior to the cocaine self-administration protocol, drug exposure was limited to periodic sedation (~1 injection/quarterly) with ketamine, a commonly used veterinary sedative, for preventative healthcare, isoflurane anesthesia for surgery, and post-operative antibiotics. All monkeys were housed individually and maintained on a 12-hr lights-on/12-hr lights-off cycle (lights on at 7:00 AM) with water available continuously. Monkeys received Teklad monkey diet, supplemented with fruits and vegetables, at least 1 hour after the end of the daily session, in quantities that allowed them to gain no more than 1 kg during the 100+ days of the study. Initial weights were 6–8 kg, with no significant changes noted over the course of the experiment.

Monkeys were prepared with a chronic indwelling venous catheter (polyvinyl chloride, i.d.: 0.64 mm; o.d.: 1.35 mm) according to previously described procedures (Platt et al., 2011). Monkeys were anesthetized initially with 10–20 mg/kg i.m. of ketamine. Throughout

surgery, anesthesia was maintained by an isoflurane/oxygen mixture. Under aseptic conditions, a catheter was implanted in the internal jugular vein and passed to the level of the right atrium. The distal end of the catheter was passed subcutaneously and exited in the mid-scapular region. The external end of the catheter was fed through a fitted jacket and tether system (Lomir Biomedical, Toronto, Canada) and attached to a fluid swivel mounted to the animal's cage. The catheters were flushed daily with heparinized saline (150–200 U/mL).

2.3 Self-administration and yoked saline control

Daily drug self-administration sessions occurred in each monkey's home cage (MetalSmiths, Boston, MA). Monkeys were trained to self-administer cocaine (0.03 mg/kg/injection) under a 1-response, fixed-ratio schedule (FR 1) of i.v. drug injection. At the beginning of each session, a set of two white stimulus lights above a response lever was illuminated (Med Associates, St Albans, VT). Upon pressing the lever, the white lights were extinguished and a set of two red stimulus lights were illuminated for 1-s, coinciding with a 1-s infusion. Sessions were available for 1 h or 100 injections, whichever occurred first. This allowed for a maximum of 3 mg/kg cocaine per day, an amount producing relevant physiological effects in the rhesus macaque and corresponding to recreational doses in humans (Barnett et al., 1981). A single session occurred each day, 7 days/week, until a minimum of 100 sessions occurred for each monkey (achieved for 4 of 5 monkeys). A target cumulative dose of 300 mg/kg (i.e., 0.03 mg/kg/injection x daily number of injections x total number of sessions) was chosen a priori for terminating the study.

In order to determine the extent to which transcriptional differences were due to cocaine self-administration, a yoked design was employed for these studies. Each cocaine self-administration monkey was paired with an age- and weight-matched control monkey. The monkey's surgeries were conducted on consecutive days, and the dyad was housed in the same room, although not directly beside the matched animal. The lever apparatus was made available to the yoked monkey, and the light conditions were the same: Two white stimulus lights above the lever were illuminated at the beginning of the session. When the cocaine self-administration monkey of the dyad pressed the lever, the white lights were extinguished and the red lights were illuminated, coinciding with a 1-s infusion of drug for the cocaine animal and saline for the yoked animal. For the yoked saline monkey, pressing the lever had no programmed consequences and thus the behavior was infrequent. The yoked saline monkey was euthanized on the same day as the matched cocaine monkey, but tissue collection occurred sequentially rather than simultaneously (the order of yoked saline vs. matched cocaine was counterbalanced). Therefore, the saline control animals experienced all conditions that were experienced by the cocaine self-administration monkeys with the exception of cocaine exposure.

2.4 RNA isolation and library preparation

All monkeys were euthanized approximately 24 h after the last session. Animals are sedated with ketamine (10–15 mg/kg, IM) followed by sodium pentobarbital (>75 mg/kg, IV) to effect. Standard gross pathology is then conducted and tissues removed. No more than three hours elapsed between euthanasia and freezing of samples. Brains were manually sliced into

coronal sections with 2mm thickness from the frontal pole to the end of the midbrain. Tissues were stored at -80°C until all samples were collected. When all samples were available, two brain regions (NAc and VTA) were punched from brain blocks for use in molecular studies. The boundaries for the VTA were medial and slightly ventral to the substantia nigra compacta (SNC) and ventral and slightly lateral to the ventral medial nucleus of the hypothalamus (VMH) at the level of the oculomotor nerve (3n). The boundaries of the NAc were ventral and medial to the putamen and anterior commissure at the level of the optic chiasm and slightly dorsal to the anterior hypothalamic area (AHA). No attempt was made to dissociate shell and core subregions, opting instead for a more inclusive definition of the region of interest. All regions were identified and defined based on published literature (Paxinos et al., 2000). Samples were removed from the anterior to posterior limits for each animal and adjacent sections were combined to create a single regional sample for each animal. All samples were processed contemporaneously for RNA extraction using a standard Trizol protocol (Life Technology, Carlsbad, CA). RNA integrity was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). All the samples found to have an RNA integrity number ≥ 8 were considered adequate and sufficient for further use (Schroeder et al., 2006).

2.5 μg of total RNA from each sample was used for transcriptomic analysis. The polyA mRNA was selected using an IntegenX Apollo 324 robot and associated PrepX-PolyA kit according to manufacturer's protocols (IntegenX Inc., Pleasanton, CA). Library size and concentration was evaluated for quality using an Agilent D1K High Sensitivity DNA chip (Agilent Technologies Inc., Santa Clara, CA) followed by a SYBR qPCR assay performed using a Stratagene MxPro 3005P qPCR System. Duplicate measurements were carried out following previously established protocols (Meyer et al., 2008). Libraries were pooled, index tagged, and multiplexed 5 samples/lane, in equimolar amounts, and then denatured, clustered and sequenced on the Illumina HiSeq 2500 using a 50bp single end read protocol. Library preparation, and next generation sequencing was performed at the Biopolymers Facility, Department of Genetics, Harvard Medical School, Boston, MA.

2.5 Statistical and bioinformatic analysis

Cocaine self-administration was recorded as the number of injections per daily session. One animal (C2) had a catheter failure and only completed 84 days of cocaine self-administration; the remainder achieved 100 of cocaine self-administration. A linear-trend analysis was conducted on the number of injections/session across the 84 days for all monkeys and 100 days for the subset. Daily sessions were parsed into 4 bins of 15 min each and the number of injections that occurred in each bin was analyzed in order to assess within-session patterns of consumption. The injections/bin data were averaged for the first week (sessions 1–7), a mid-point (sessions 47–53), and final week (sessions 93–100). Sessions were grouped to minimize stochastic variability across individual days. An extra sum of squares F test was used following linear regression to detect a non-zero slope. The timing of self administration was analyzed with 2-way repeated measures ANOVA with Bonferroni correction for multiple comparisons.

Initial transcriptomic analysis was processed through DNAnexus (DNAnexus Inc., Mountain View, CA). All reads were initially aligned to the rhesus genome (MGSC Merged 1.0/rheMac2) and annotated using RefSeq annotations. Sample read depth was normalized using total count normalization and differentially expressed genes were identified using a Poisson log-linear model as implemented in the 'PoissonSeq' package for R (Li et al., 2012). This methodology calculates a single gene significance value (p-value) as well as a false discovery rate corrected significance value (q-value).

A reanalysis of the data was performed following the publication of a new rhesus macaque genome (Mmul8.0.1) in early 2016. This analysis used the TopHat2 (Trapnell et al., 2012) suite of tools including 'bowtie2' for alignment, 'cufflinks' for transcript identification (again using the new RefSeq annotation), 'cuffdiff' to identify differentially expressed transcripts across conditions, and 'cummeRbund' for visualization. While this newer reference genome did improve the percent of mapped reads (roughly 10% greater mapping to the new genome) and marginally affect the significance values of the differential expression analysis, it did not change either the specific, or general, conclusions described herein. Heatmaps were generated using k-means clustering based on the Jensen-Shannon distance to group genes of similar expression profiles.

Data were analyzed using QIAGEN's IngenuityR Pathway Analysis (IPAR, QIAGEN Redwood City, www.qiagen.com/ingenuity). IPA uses an extensive knowledge base to recapitulate biological networks and to associate biological function with genes in a regulated ontology. Overrepresented categories within the differentially expressed genes are identified using a right-tailed Fisher's exact test with Benjamini-Hochberg correction for multiple testing. A q-value of 0.05 for these categories was used for further analysis.

3. Results

3.1 Cocaine self-administration

While there is some variability across animals (Supplemental Figure 1¹), when cocaine self-administration was averaged across animals, there was evidence of a gradual increase in self-administration over sessions (Figure 1A,1B). Linear trend analysis revealed a significant fit of these data (84 days (n=5) [F(1,82)=18.23, p<0.001]; 100 days (n=4) [F(1,98)= 25.06, p<0.001]) with a positive slope, although the relationship of session and number of injections/hour was relatively weak ($r^2 = 0.18$ or 0.20 respectively). A relatively robust increase in self-administration was seen when the session was separated into 15-min bins (Figure 1C). In general, more self-administration was observed in the first 15 minutes than the other bins, with the mean number of injections/session increasing significantly over sessions for the first bin (1–7 vs. 47–53 [t(48)=6.307, p<0.001]; 1–7 vs. 93–100: [t(48)=8.601, p<0.001]) but not the other bins. In fact, there is some evidence for a decrease in self-administration from later bins, coincident with the increase in injections earlier in the session. Although a possible reason for the lower numbers in the latter bins could have been the animals finishing the sessions early (i.e., completing all 100 injections prior to the end of the 60-min period), this was not observed. Further analyses of the time required to complete

¹Supplementary material can be found by accessing the online version of this paper at <http://dx.doi.org> and by entering doi:...

the sessions, either by self-administering all 100 injections or by “timing out” after 60 minutes, over the same 1-week time intervals revealed no significant effects (data not shown) with all sessions ending at or near the 60-min mark. While there is some inter-individual variability with regard to timing of injections, it is not significant and represents a much lesser effect than intra-individual across days. Generally, as animals progress there is a modest increase in total amount of cocaine self-administered across the entirety of the session with a more substantial shift to rapid consumption immediately upon availability.

3.2 mRNA expression profiling

At the end of the behavioral study, animals were sacrificed and brains were removed and dissected, keeping target regions intact. The VTA and NAc were isolated for subsequent transcriptomic analysis using standard single-end RNA-seq technologies as described above. An average of 30.5 million reads (1,350 Mbases) were sequenced from each sample (Supplemental Table 1,2). Of these, an average of 54% (ranging from 4.5 million reads to 35.8 million reads/sample) were successfully mapped to the original, rheMac2, rhesus genome. A subsequent realignment to the newly released Mmul8.0.1 genome raised the average mapping to 63%. The RefSeq annotation of the rhesus genome was used for subsequent analyses though Ensembl annotations did not produce significantly different results.

We used a false discovery rate (FDR, $q < 0.05$) that was conservative (Storey and Tibshirani, 2003). The number of genes identified in this manner, both before and after correction for multiple testing, was roughly equivalent to a similar study focused on human postmortem hippocampus following chronic cocaine exposure (80 genes differentially expressed at $q < 0.05$) (Zhou et al., 2011). Overall a smaller number of genes were found to be differentially expressed ($q < 0.05$) in the VTA (53; Table 1) compared to the NAc (328; Table 2). While there were more genes upregulated than downregulated in the VTA were more similar (66% and 34% respectively, Figure 2A), in the NAc this trend was much more exaggerated (96% to 4%, Figure 2B). The relative magnitude of differences in gene expression overall were also greater in the NAc compared to the VTA. Moreover, while genes showing effects in the VTA largely were associated with nervous system development and function ($p < 0.0001$), this pattern was entirely absent in the NAc. Instead, the most enriched categories of genes were those associated with inflammatory response ($p < 0.001$) notably including macrophage recruitment ($p < 0.0005$) and activation ($p < 0.005$) and leukocyte apoptosis ($p < 0.0005$).

Using these lists of dysregulated genes, further analysis focusing on the identification of common or shared pathways was undertaken using Ingenuity Pathway Analysis. In the VTA, the most notable differences were found in pathways associated with dopaminergic signaling (Figure 3A). This pathway, as well as others that highly overlap (including catecholamine biosynthesis and serotonin signaling), is significantly enriched for differentially expressed genes. In the NAc, many of the enriched pathways were associated with immune response, broadly or specifically, and cellular responses to stress (Figure 3B). These findings were broad-based and robust to methodologies for assigning differential expression.

3.3 Expression differences in the VTA

The most notable differences in the VTA (Figure 4A) of animals exposed to cocaine were found in the dopaminergic pathway. Both tyrosine hydroxylase (TH), responsible for converting L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), and dopa decarboxylase (DDC), responsible for the conversion of L-DOPA to dopamine, were down-regulated ($q < 0.05$). And while there is also a robust down-regulation of the dopamine transporter (SLC6A3; $q < 0.01$), there were no significant differences in expression of any of the dopamine receptors. These findings confirm previous studies in post-mortem human cocaine abusers (Bannon et al., 2014; Bannon et al., 2015; Little et al., 1998; Zhou et al., 2014a) as well as in rats following extended cocaine exposure (Cerruti et al., 1994; Letchworth et al., 1997; Letchworth et al., 1999).

In rhesus macaques exposed to cocaine, the transcription factors FOXA1 and FOXA2 were both down regulated ($q < 0.05$ and $q < 0.01$ respectively). These genes have previously been shown to specify midbrain dopaminergic fate (Ferri et al., 2007) and are direct regulators not only of DDC and TH in mature dopaminergic neurons, but also of engrailed 1 and 2 (EN1, EN2) and the nuclear receptor NR4A2 (also known as NURR1) in immature neurons. FOXA2 down regulation has likewise been observed in the midbrain of human cocaine abusers (Bannon et al., 2014; Bannon et al., 2015). In addition to developmental roles in specifying dopaminergic neuronal fate, FOXA1 and FOXA2 have also been demonstrated to regulate midbrain dopaminergic function and survival in the adult brain (Kittappa et al., 2007).

3.4 Expression differences in the NAc

Dominating the differences observed in the NAc (Figure 4B) were genes associated with immune response and inflammation. This included numerous cytokines (IL1B, CXCL1, CXCL8 (IL8), CXCL10, CCL20, CCL23) and receptors (CXCR2, CCR1). We also see significant differences in expression of several toll-like receptors (TLR3, TLR6, TLR10). These pathways have previously been associated with a neuroprotective response to stress modulated as well by EIF2AK2, also known as the dsRNA responsive protein kinase PKR (Hsu et al., 2004). This gene was also differentially expressed in the NAc of cocaine administering animals ($q < 0.05$). This finding extends to genes downstream of PKR that also show significant differences in cocaine-exposed animals (EIF2 signaling; Figure 3B). These results show strong evidence reflective of an inflammatory response coinciding with cellular stress and neuronal death.

A large number of other genes of diverse function were also upregulated. This included a large number of genes that were not detected in the saline-yoked animals, but that were detected at extremely low, but non-zero, levels in the cocaine animals. This, perhaps, can be explained in part through the effects of chromatin remodeling. H3 and H4 were both differentially expressed ($q < 0.005$ and $q < 0.05$ respectively). It is hypothesized that a large amount of the upregulated genes observed here, particularly those that have near absent expression levels in saline control animals, are the result of “leaky” expression resulting from epigenetic changes and resulting chromatin remodeling. It is notable that at least one gene, KRT23, unrelated to brain function that was upregulated ($q < 0.05$) has previously

been demonstrated to be highly inducible through histone hyperacetylation (Zhang et al., 2001). While this does not preclude a functional relevance for many of these specific expression changes, it may represent a symptom rather than an underlying causal factor.

4. Discussion

Under conditions of limited access (1 hr/day) and long-term exposure (approximately 100 consecutive days), we observed a general increase over time in cocaine self-administration in monkeys. However, while statistically significant, this escalation was modest in size. While there was a limit on the total amount of cocaine that was available for the animals to consume (so as to not lead to overdose), this did not fully account for the magnitude effect. Rather, what was observed was a relatively robust increase in the rate at which cocaine was consumed early in the daily sessions. In later sessions not only do animals consume, on average, more cocaine (roughly 30% increase over the 100 days), but their consumption increases more than 80% in the first 15 minutes while decreasing 15% in the remaining 45 minutes.

The increase in cocaine taking across sessions has some similarities to the "escalation" phenomenon documented in rats self-administering cocaine for prolonged periods (Edwards and Koob, 2013). Specifically, animals increased their consumption over time and this consumption began to occur more heavily earlier in the session. Although our findings may reflect a similar escalation effect, there were notable differences. First, the increase in self-administration was observed with relatively short (1 hr) session lengths and manifested primarily as an increase in the number of sessions in which the maximum amount of cocaine was taken (escalation studies in rats typically do not limit the number of infusions available in a session). Second, the increase in cocaine taking did not "ramp up" over the first week of self-administration as seen with rats (Edwards and Koob, 2013), rather the increase was much more gradual over time. Regardless, an increase in the amount of cocaine consumed with increasing experience is a hallmark of human cocaine addiction that can be modeled in nonhuman subjects and it may be the case that the differences in magnitude and pattern of escalation across species merely result from as-yet fully defined parametric variables.

It is also important to note that prior studies on escalation of cocaine taking using rhesus macaques as subjects generally did not observe consistent increases in self-administration over time with either marked individual differences in the extent to which escalation occurred or no change in self-administration across sessions (Czoty et al., 2007; Henry and Howell, 2009; Kirkland Henry et al., 2009). There are many differences among the studies (e.g., schedule of reinforcement, number of sessions) and it is unclear at present which factor is the primary determinant of the extent to which escalation occurs in nonhuman primates. Nevertheless, modelling this important feature of human addictive behavior suggests an important avenue into translationally relevant interventions.

Developing an animal model that faithfully recapitulates the human disease state is important because of our increased levels of experimental control. In addition to a much greater understanding of the exact nature of exposure to drug, we have a much more precise and meaningful opportunity to look at specifically associated molecular effects. The

neuroanatomy, genetics, and physiology of the brain of a rhesus macaque are highly similar to a human, but the specifics of drug exposure are better defined and tissue collection post-mortem is much more rapid. This allows us to compare the findings here in the animal model to post-mortem human studies and be confident of the direct causal relationships between drug use and brain molecular differences.

The initial focus of these studies was on the mesolimbic system, specifically the ventral tegmental area and the nucleus accumbens. This has been the traditional focus for addiction studies and it allows for direct comparisons with previous literature. Differences observed in gene expression in the ventral tegmental area have generally centered on the dopaminergic system. Indeed, in this work we also see a down-regulation of genes associated with dopaminergic neuron development and function, including the genes traditionally associated with dopamine synthesis (DDH and TH) and transport (SLC6A3), consistent with findings from human cocaine abusers. Previous studies have observed decreases in the dopamine transporter in post-mortem studies from human cocaine abusers (Bannon et al., 2014; Bannon et al., 2015; Little et al., 1998; Zhou et al., 2014a) as well as in rats following extended cocaine exposure (Cerruti et al., 1994; Letchworth et al., 1997; Letchworth et al., 1999; Zhang et al., 2012). The parallels between these studies and previous human studies extend to a reduced expression of tyrosine hydroxylase in the VTA (Bannon et al., 2014; Bannon et al., 2015), although rat studies have been more ambiguous (Freeman et al., 2000; Rodriguez-Espinosa and Fernandez-Espejo, 2015; Vrana et al., 1993).

We also see corresponding differences in the upstream regulators of these genes, notably FOXA2, that are similar to human post-mortem findings (Bannon et al., 2014; Bannon et al., 2015). FOXA1 and FOXA2 have previously been demonstrated to regulate midbrain dopaminergic development and function (Ferri et al., 2007; Kittappa et al., 2007; Lin et al., 2009; Metzakopian et al., 2015; Metzakopian et al., 2012; Stott et al., 2013). While there does seem to be a developmental role for FOXA1 and FOXA2 (Hallonet et al., 2002; Mavromatakis et al., 2011; Nakatani et al., 2010), it is their role in maintaining dopaminergic identity in mature neurons that is likely more relevant to studies of cocaine effects. Perhaps most notable beyond this general down-regulation of dopaminergic tone, is the absence of evidence of any other major coordinated effects. While it certainly remains that individual differences in gene expression detected may be important, the primary finding of this study emphasizes the central role of changes in the dopamine system in the VTA. While other effects cannot be ruled out, this and other studies place dopaminergic dysregulation centrally.

The NAc in the present study is characterized mainly by differences associated with an activation of microglia akin to that seen in brain injury and chronic disease. This activation has been suggested to be important in the overall neurobiology of addiction (Crews et al., 2011). TLR-mediated pathways have previously been demonstrated to have proinflammatory neuroprotective effects on astrocytes (Bsibsi et al., 2006). Moreover, this immune response also has been previously demonstrated to be involved in the remyelination process (Glezer et al., 2006). Consistent with these observations, a number of post-mortem human studies have reported myelin dysregulation in the NAc (Albertson et al., 2004; Bannon et al., 2005), although we see little evidence for demyelination here, possibly as a

result of the timing of drug exposure. Although this work does not address whether the activation of innate immunity is solely a response to insult or if these changes are driving the addiction process, it does demonstrate an important, region specific, role for immune signaling that further builds upon an existing neuroimmune hypothesis of addiction.

Coupled with the immune activation, was a widespread up-regulation of a diverse array of genes in the NAc, including a significant subset that show no expression in unexposed animals and are not generally expressed in brain tissue. This is hypothesized to be attributable to cocaine-mediated chromatin remodeling, accounting for both the diversity of genes as well as the overrepresentation of upregulation compared to downregulation. A substantial literature exists identifying epigenomic changes in the NAc following long-term cocaine exposure specifically and addiction generally (Nestler, 2014; Sadri-Vakili, 2014). These epigenetic changes have been shown to correlate with drug-induced behavioral change and neuronal plasticity (Schmidt et al., 2013). In essence, chromatin is thought to be moving from a “closed” state in which transcription is tightly repressed, to an “open” state where more transcriptional activity is occurring. Cocaine mediated chromatin remodeling in the NAc has been previously demonstrated (Feng et al., 2014; Kumar et al., 2005; Maze et al., 2011) and the role of chromatin remodeling in gene expression is under increasing scrutiny (Bell et al., 2011; Mirabella et al., 2016). In particular, the role of chromatin reorganization in macrophage response is increasingly being recognized with open chromatin and high gene expression consistent with activation (Glass and Natoli, 2016; Schmidt et al., 2016). The indirect evidence here for epigenetic change not only includes this broad upregulation of gene expression but also differences in expression of multiple genes encoding histones.

Epigenetic changes have previously been suggested to correlate with the transition from use to abuse (Schmidt et al., 2013). It is perhaps notable, then, that the differences we observe here seem to be much more pronounced in the NAc and less so in the VTA, further bolstering the concept that while the VTA is associated with the initial and acute action of the drug, it is the NAc that is associated with longer-term neuro-adaptations (Koya et al., 2009). The picture emerging here is consistent with hypotheses that suggest dopaminergic changes in presynaptic neurons from the VTA lead to, or act in concert with, epigenetic remodeling and immune activation post-synaptically in the NAc. This model supports a central mechanism for developing addiction, independent of the specific mechanisms of action of drugs of abuse.

It is important to note, however, that this study is not exhaustive. While environmental variables, notably including cocaine administration, are controlled in these studies, sample sizes are necessarily small; together, these factors limit the effect size that it is possible to detect. While some inter-individual variability does exist in this study it is relatively minor and it is difficult to draw meaningful conclusions. The findings presented here do not exhaustively represent all the differences that occur with cocaine exposure, but rather a subset, likely of the largest effect sizes. It is meaningful that many of the key findings here are supported by, and supportive of, previous studies, particularly humans both with regards to behaviors associated with cocaine self-administration as well as gene expression responses.

Another limitation of the present study is the level of structural and cellular detail interrogated. In the NAc, differences exist between the core and shell (Brauer et al., 2000; Heimer et al., 1997; Martin and Cork, 2014; Meredith et al., 1996) that extend to circuitry and behavior (Baliki et al., 2013; Meredith et al., 2008; Saddoris et al., 2013), but these regions have been conflated in an effort to prioritize consistency between animals. Nor, have the cell-type distributions of the regions been explored. From the data, microglial activation is inferred in the NAc, but further studies that dissociate neuronal and glial effects are needed for confirmation. It is perhaps important to note, however, that the conflation of cell types or regions have the effect of reducing signal, effectively making the present study conservative.

In conclusion, this work demonstrates the power of an unbiased approach to transcriptomic profiling in a nonhuman primate model of long-term cocaine self-administration. Not only is this a meaningful and translationally-relevant model of human addiction, but it also affords added control of environmental variables and the ability to parse cause and effect in relationships between drug exposure and differences in gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Patterns of cocaine self-administration in rhesus macaques model humans
- Gene expression differences are observed in the mesolimbic system after exposure
- Changes in the ventral tegmental area are associated with the dopaminergic system
- Changes in the nucleus accumbens reflect neuroinflammation and chromatin remodeling

Figure 1.

Grouped data is shown for 5 rhesus monkeys self-administering cocaine. Average number of injections/session are shown over the course of the 100 days ($n=4$, A) or 84 days ($n=5$, B). A significant ($p < 0.05$) linear escalation is observed. (C) Timing of self-administered injections (grouped in 15 minute bins) are shown for the first and last weeks of study and the midpoint week. The preponderance of injections in each case occur in the first 15 minute block. It is during this time block that a significant change ($p < 0.05$) is observed over the course of the study.

Figure 2. Volcano plots comparing the normalized expression of genes in long-term cocaine self-administering animals to yoked saline controls in the ventral tegmental area (A) and nucleus accumbens (B).

Figure 3.

Canonical pathway enrichment analysis of genes differentially expressed in the VTA (A) and NAc (B) of cocaine-self-administering animals. Significance values (dashed lines; $p < 0.05$, $-\log(p\text{-value}) > 1.3$) are determined through IPA default methodologies. The ratio of genes differentially expressed to the total number found in the pathway is also shown.

Figure 4. Heatmap showing the effects of long-term cocaine self-administration on gene expression in the ventral tegmental area (A) and nucleus accumbens (B). Higher expression levels are indicated in red and lower expression levels in yellow. Cocaine and saline animals are grouped together.

Genes differentially expressed ($q < 0.05$) in the ventral tegmental area of rhesus macaques with long term cocaine self-administration compared to yoked controls.

Table 1

P-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0000	0.0000	1.918	CRYM	crystallin, mu
0.0000	0.0000	-1.761	C1QL1	complement component 1, q subcomponent-like 1
0.0000	0.0000	-2.349	CHRNA2	cholinergic receptor, nicotinic, alpha 2 (neuronal)
0.0000	0.0157	1.387	DIRAS3	DIRAS family, GTP-binding RAS-like 3
0.0000	0.0157	-1.704	FOXA2	forkhead box A2
0.0000	0.0200	1.436	CBLN4	cerebellin 4 precursor
0.0000	0.0200	-2.934	SLC6A3	solute carrier family 6 (neurotransmitter transporter), member 3
0.0000	0.0200	-2.099	NKX6-1	NK6 homeobox 1
0.0000	0.0200	1.109	TPD52L1	tumor protein D52-like 1
0.0000	0.0200	-1.343	CA8	carbonic anhydrase VIII
0.0000	0.0221	4.748	PTH2	parathyroid hormone 2
0.0001	0.0221	-1.144	SLC17A8	solute carrier family 17 (vesicular glutamate transporter), member 8
0.0001	0.0221	1.247	LRRC23	leucine rich repeat containing 23
0.0001	0.0221	1.225	DNAH12	dynein, axonemal, heavy chain 12
0.0001	0.0221	1.28	ARX	aristaless related homeobox
0.0001	0.0221	1.158	DYDC2	DPY30 domain containing 2
0.0001	0.0221	2.229	SLC18A3	solute carrier family 18 (vesicular acetylcholine transporter), member 3
0.0001	0.0221	1.157	DNAH5	dynein, axonemal, heavy chain 5
0.0001	0.0221	2.599	TLL1	tollid-like 1
0.0002	0.0221	1.537	CALCB	calcitonin-related polypeptide beta
0.0002	0.0221	-1.437	TH	tyrosine hydroxylase
0.0002	0.0221	-1.231	FOXA1	forkhead box A1
0.0003	0.0221	1.468	CXorf30	chromosome X open reading frame 30
0.0003	0.0221	-1.423	ASAH2	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2
0.0003	0.0221	1.367	AKAP14	A kinase (PRKA) anchor protein 14
0.0003	0.0221	1.426	AK9	adenylate kinase 9

P-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0003	0.0221	1.242	CCDC60	coiled-coil domain containing 60
0.0003	0.0221	1.318	VSIG8	V-set and immunoglobulin domain containing 8
0.0004	0.0221	1.483	NAALADL1	N-acetylated alpha-linked acidic dipeptidase-like 1
0.0004	0.0221	-1.574	DDC	dopa decarboxylase (aromatic L-amino acid decarboxylase)
0.0004	0.0221	1.169	IQGAP2	IQ motif containing GTPase activating protein 2
0.0004	0.0221	1.151	KRT18	keratin 18
0.0004	0.0221	1.998	SNRPD2	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa
0.0004	0.0222	1.171	AGR3	anterior gradient 3
0.0004	0.0222	-1.219	EBF3	early B-cell factor 3
0.0005	0.0227	1.358	PPP1R32	protein phosphatase 1, regulatory subunit 32
0.0005	0.0227	1.23	CL1orf70	chromosome 11 open reading frame 70
0.0005	0.0227	1.101	MYB	v-myb avian myeloblastosis viral oncogene homolog
0.0006	0.0230	1.334	CTXN3	cortixin 3
0.0006	0.0230	-1.376	FOXO2	forkhead box D2
0.0006	0.0231	-1.294	DMRTA2	DMRT-like family A2
0.0006	0.0232	-1.303	MYOC	myocilin, trabecular meshwork inducible glucocorticoid response
0.0006	0.0236	-1.498	TWIST1	twist family bHLH transcription factor 1
0.0007	0.0238	1.723	CDH9	cadherin 9, type 2 (T1-cadherin)
0.0008	0.0256	1.556	GPC3	glypican 3
0.0009	0.0270	1.389	TRHDE	thyrotropin-releasing hormone degrading enzyme
0.0010	0.0288	1.103	MARCH10	membrane-associated ring finger (C3HC4) 10, E3 ubiquitin protein ligase
0.0011	0.0293	1.18	ANKFN1	ankyrin-repeat and fibronectin type III domain containing 1
0.0012	0.0297	1.236	KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2
0.0012	0.0297	1.107	LMO1	LIM domain only 1 (rhombotin 1)
0.0012	0.0297	-1.635	T	T, brachyury homolog (mouse)
0.0024	0.0398	1.456	OSBPL3	oxysterol binding protein-like 3
0.0024	0.0404	-1.332	TK1	thymidine kinase 1, soluble

* Supplementary material can be found by accessing the online version of this paper at <http://dx.doi.org> and by entering doi:....

Table 2

Genes differentially expressed ($q < 0.05$) in the nucleus accumbens of rhesus macaques with long term cocaine self-administration compared to yoked controls.

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0000	0.0000	1.249	ATP2B4	ATPase, Ca++ transporting, plasma membrane 4
0.0000	0.0000	7.353	LECT2	leukocyte cell-derived chemotaxin 2
0.0000	0.0016	1.271	C15orf26	chromosome 15 open reading frame 26
0.0000	0.0016	2.637	C3orf70	chromosome 3 open reading frame 70
0.0000	0.0016	1.604	C6orf57	chromosome 6 open reading frame 57
0.0000	0.0016	3.098	FAM129C	family with sequence similarity 129, member C
0.0000	0.0016	2.161	FAM76A	family with sequence similarity 76, member A
0.0000	0.0016	2.24	H3F3A/H3F3B	H3 histone, family 3A
0.0000	0.0016	4.335	HBM	hemoglobin, mu
0.0000	0.0016	3.716	LILRA4	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 4
0.0000	0.0016	4.799	RAG2	recombination activating gene 2
0.0000	0.0016	5.25	RPL21	ribosomal protein L21
0.0000	0.0016	2.271	RPL36	ribosomal protein L36
0.0000	0.0016	2.255	SFRP4	secreted frizzled-related protein 4
0.0000	0.0033	1.194	STX17	syntaxin 17
0.0000	0.0035	3.561	REP15	RAB15 effector protein
0.0000	0.0035	1.849	RPL36A	ribosomal protein L36a
0.0000	0.0035	1.994	SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4
0.0000	0.0035	5.53	SLPI	secretory leukocyte peptidase inhibitor
0.0000	0.0035	1.94	ZBED4	zinc finger, BED-type containing 4
0.0000	0.0037	4.155	RPL37	ribosomal protein L37
0.0000	0.0039	4.218	ADI6	adipogenin
0.0000	0.0039	2.311	RAB22A	RAB22A, member RAS oncogene family
0.0000	0.0041	2.808	ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit G
0.0000	0.0041	1.709	MYC	v-myc avian myelocytomatosis viral oncogene homolog
0.0000	0.0041	1.523	PCDHBI	protocadherin beta 1

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0000	0.0041	2.413	SPA17	sperm autoantigenic protein 17
0.0000	0.0041	3.033	UTS2	urotensin 2
0.0000	0.0042	3.482	SLC22A8	solute carrier family 22 (organic anion transporter), member 8
0.0000	0.0044	5.432	CLDN16	claudin 16
0.0000	0.0044	2.153	ELK4	ELK4, ETS-domain protein (SRF accessory protein 1)
0.0000	0.0046	2.133	IRF6	interferon regulatory factor 6
0.0000	0.0046	1.564	WNT4	wingless-type MMTV integration site family, member 4
0.0000	0.0047	4.107	DEFA5	defensin, alpha 5, Paneth cell-specific
0.0000	0.0047	2.192	TLR10	toll-like receptor 10
0.0000	0.0055	2.139	FMN1	formin 1
0.0000	0.0055	2.322	RPL31	ribosomal protein L31
0.0000	0.0055	3.147	RPLP1	ribosomal protein, large, P1
0.0000	0.0062	2.71	DEFB119	defensin, beta 119
0.0000	0.0062	3.177	RPL10L	ribosomal protein L10-like
0.0000	0.0065	3.804	DSG4	desmoglein 4
0.0001	0.0070	2.32	GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type
0.0001	0.0070	1.908	MAP1LC3B2	microtubule-associated protein 1 light chain 3 beta 2
0.0001	0.0070	3.009	MYCT1	myc target 1
0.0001	0.0070	4.163	OR1M1	olfactory receptor, family 1, subfamily M, member 1
0.0000	0.0070	4.609	OR8B8	olfactory receptor, family 8, subfamily B, member 8
0.0000	0.0070	2.166	RPL17	ribosomal protein L17
0.0001	0.0070	2.342	RPS27A	ribosomal protein S27a
0.0001	0.0070	1.961	TMEM45B	transmembrane protein 45B
0.0001	0.0075	2.919	CYP3A43	cytochrome P450, family 3, subfamily A, polypeptide 43
0.0001	0.0075	3.645	DEFB121	defensin, beta 121
0.0001	0.0075	1.608	KIAA1958	KIAA1958
0.0001	0.0075	2.026	LDHAL6B	lactate dehydrogenase A-like 6B
0.0001	0.0075	1.152	UQCRH	ubiquinol-cytochrome c reductase hinge protein
0.0001	0.0075	1.453	FAM196B	family with sequence similarity 196, member B

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0001	0.0076	3.561	RPL12	ribosomal protein L12
0.0001	0.0079	1.74	GABRB2	gamma-aminobutyric acid (GABA) A receptor, beta 2
0.0001	0.0079	2.94	YDJC	YdjC homolog (bacterial)
0.0001	0.0081	1.383	CLN8	ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)
0.0001	0.0081	1.764	PSD3	pleckstrin and Sec7 domain containing 3
0.0001	0.0081	1.374	TRAPPC3L	trafficking protein particle complex 3-like
0.0001	0.0081	1.531	ZMAT3	zinc finger, matrin-type 3
0.0001	0.0082	1.748	PGAP1	post-GPI attachment to proteins 1
0.0001	0.0087	3.735	CLEC9A	C-type lectin domain family 9, member A
0.0001	0.0087	1.236	CTU1	cytosolic thiouridylase subunit 1
0.0001	0.0087	3.723	CXCL8	chemokine (C-X-C motif) ligand 8
0.0001	0.0087	2.625	KBTBD13	kelch repeat and BTB (POZ) domain containing 13
0.0001	0.0087	3.448	LCN8	lipocalin 8
0.0001	0.0087	3.668	NPSR1	neuropeptide S receptor 1
0.0001	0.0087	1.316	TMEM156	transmembrane protein 156
0.0001	0.0088	2.365	HMG2	high mobility group nucleosomal binding domain 2
0.0001	0.0089	1.726	CATSPER3	cation channel, sperm associated 3
0.0001	0.0089	1.985	ELSPBP1	epididymal sperm binding protein 1
0.0001	0.0089	1.728	PCP2	Purkinje cell protein 2
0.0001	0.0089	3.16	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15
0.0001	0.0089	1.888	ZNF483	zinc finger protein 483
0.0001	0.0090	2.422	USP6	ubiquitin specific peptidase 6
0.0001	0.0090	1.369	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
0.0001	0.0092	2.676	C10orf82	chromosome 10 open reading frame 82
0.0002	0.0094	1.507	HMX1	H6 family homeobox 1
0.0002	0.0100	4.809	IRF2BP2	interferon regulatory factor 2 binding protein 2
0.0002	0.0100	2.439	RC3H1	ring finger and CCH-type domains 1
0.0002	0.0103	1.355	NDUFA1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa
0.0002	0.0105	2.368	CLRN1	clarin 1

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0002	0.0105	3.849	CTLA4	cytotoxic T-lymphocyte-associated protein 4
0.0002	0.0105	2.812	CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
0.0002	0.0105	2.021	GPR160	G protein-coupled receptor 160
0.0002	0.0105	2.831	GTSF1	gametocyte specific factor 1
0.0002	0.0105	2.122	LOC102723532/OR4N4	olfactory receptor, family 4, subfamily N, member 4
0.0002	0.0105	1.618	MALAT1	metastasis associated lung adenocarcinoma transcript 1 (non-protein coding)
0.0002	0.0105	2.169	MCHR2	melanin-concentrating hormone receptor 2
0.0002	0.0105	2.383	PPP3R2	protein phosphatase 3, regulatory subunit B, beta
0.0002	0.0105	3.612	PRR30	proline rich 30
0.0002	0.0105	2.024	PTPN20B	protein tyrosine phosphatase, non-receptor type 20B
0.0002	0.0105	3.008	SLC5A8	solute carrier family 5 (sodium/monocarboxylate cotransporter), member 8
0.0002	0.0105	3.126	SSMEM1	serine-rich single-pass membrane protein 1
0.0002	0.0108	2.933	CD28	CD28 molecule
0.0002	0.0108	3.91	FLG2	flaggrin family member 2
0.0002	0.0108	3.442	IL2RA	interleukin 2 receptor, alpha
0.0002	0.0108	1.794	LDHB	lactate dehydrogenase B
0.0002	0.0108	4.586	OR10G2	olfactory receptor, family 10, subfamily G, member 2
0.0003	0.0111	1.206	AQP4	aquaporin 4
0.0003	0.0111	1.499	CAMK1D	calcium/calmodulin-dependent protein kinase ID
0.0003	0.0111	1.851	CSRNP3	cysteine-serine-rich nuclear protein 3
0.0002	0.0111	4.068	DPF3	D4, zinc and double PHD fingers, family 3
0.0003	0.0111	1.121	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
0.0002	0.0111	1.716	HEATR9	HEAT repeat containing 9
0.0003	0.0111	5.028	LAMP3	lysosomal-associated membrane protein 3
0.0003	0.0111	1.241	MAPK13	mitogen-activated protein kinase 13
0.0003	0.0111	2.144	NAA11	N(alpha)-acetyltransferase 11, NatA catalytic subunit
0.0003	0.0111	2.652	OR4D5	olfactory receptor, family 4, subfamily D, member 5
0.0003	0.0111	2.904	OR4M1	olfactory receptor, family 4, subfamily M, member 1
0.0003	0.0111	4.603	SIGLEC12	sialic acid binding Ig-like lectin 12 (gene/pseudogene)

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0003	0.0111	1.359	SLC1A2	solute carrier family 1 (glial high affinity glutamate transporter), member 2
0.0003	0.0111	2.832	TMA7	translation machinery associated 7 homolog (<i>S. cerevisiae</i>)
0.0003	0.0117	2.123	TMEM211	transmembrane protein 211
0.0003	0.0120	1.175	B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1
0.0003	0.0120	1.968	CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5
0.0003	0.0120	1.982	GLRA1	glycine receptor, alpha 1
0.0003	0.0120	-1.739	HBQ1	hemoglobin, theta 1
0.0003	0.0120	1.956	NDUFV2	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24kDa
0.0003	0.0120	1.276	PLCXD2	phosphatidylinositol-specific phospholipase C, X domain containing 2
0.0003	0.0120	1.124	RNF152	ring finger protein 152
0.0003	0.0120	2.255	SLC19A3	solute carrier family 19 (thiamine transporter), member 3
0.0003	0.0120	1.334	SLC4A4	solute carrier family 4 (sodium bicarbonate cotransporter), member 4
0.0003	0.0121	1.201	OR1F1	olfactory receptor, family 1, subfamily F, member 1
0.0004	0.0123	2.899	CXCL10	chemokine (C-X-C motif) ligand 10
0.0004	0.0124	4.338	CLDN8	claudin 8
0.0004	0.0124	1.494	SLC24A2	solute carrier family 24 (sodium/potassium/calcium exchanger), member 2
0.0004	0.0126	1.841	RPS2	ribosomal protein S2
0.0004	0.0127	3.004	CCL23	chemokine (C-C motif) ligand 23
0.0004	0.0127	3.349	IL7	interleukin 7
0.0004	0.0128	1.574	AANAT	aralkylamine N-acetyltransferase
0.0004	0.0128	1.607	DMD	dystrophin
0.0004	0.0128	1.546	GRIN2B	glutamate receptor, ionotropic, N-methyl D-aspartate 2B
0.0004	0.0128	2.285	MRPL18	mitochondrial ribosomal protein L18
0.0004	0.0128	3.19	OR10Q1	olfactory receptor, family 10, subfamily Q, member 1
0.0004	0.0128	1.127	TMEM220	transmembrane protein 220
0.0004	0.0131	2.909	CCR4	chemokine (C-C motif) receptor 4
0.0005	0.0132	1.3	ADD3	adducin 3 (gamma)
0.0005	0.0132	2.062	ATP5J2	ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2
0.0005	0.0132	3.716	CCL20	chemokine (C-C motif) ligand 20

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0005	0.0132	2.559	CD84	CD84 molecule
0.0004	0.0132	1.436	CLCN6	chloride channel, voltage-sensitive 6
0.0004	0.0132	1.107	GIMAP2	GTPase, IMAP family member 2
0.0005	0.0132	1.28	KDM5A	lysine (K)-specific demethylase 5A
0.0004	0.0132	1.216	PPP1R16B	protein phosphatase 1, regulatory subunit 16B
0.0005	0.0132	1.788	RPL23A	ribosomal protein L23a
0.0005	0.0132	1.329	RPL41	ribosomal protein L41
0.0005	0.0132	1.295	SESN3	sestrin 3
0.0004	0.0132	3.293	SLC26A3	solute carrier family 26 (anion exchanger), member 3
0.0005	0.0132	1.391	SLC9A7	solute carrier family 9, subfamily A (NHE7, cation proton antiporter 7), member 7
0.0005	0.0132	2.003	SLC9C1	solute carrier family 9, subfamily C (Na ⁺ -transporting carboxylic acid decarboxylase), member 1
0.0005	0.0132	1.475	TAZ	tafazzin
0.0005	0.0132	2.221	TTR	transthyretin
0.0005	0.0132	2.383	WEE2	WEE1 homolog 2 (S. pombe)
0.0005	0.0133	3.245	TSP02	translocator protein 2
0.0005	0.0134	4.467	OR5L1	olfactory receptor, family 5, subfamily L, member 1
0.0005	0.0134	2.422	SLC6A2	solute carrier family 6 (neurotransmitter transporter), member 2
0.0005	0.0139	1.609	KLHL11	kelch-like family member 11
0.0006	0.0140	1.336	OR6X1	olfactory receptor, family 6, subfamily X, member 1
0.0006	0.0142	-1.685	HBA1/HBA2	hemoglobin, alpha 1
0.0006	0.0142	1.577	INSM2	insulinoma-associated 2
0.0006	0.0142	1.416	RPL24	ribosomal protein L24
0.0006	0.0149	1.341	AR	androgen receptor
0.0007	0.0157	1.547	KSR2	kinase suppressor of ras 2
0.0007	0.0159	1.474	LY6G6C	lymphocyte antigen 6 complex, locus G6C
0.0007	0.0159	1.813	SAMD8	sterile alpha motif domain containing 8
0.0007	0.0162	1.338	CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
0.0007	0.0162	2.951	MEOX2	mesenchyme homeobox 2
0.0007	0.0162	1.132	PDE10A	phosphodiesterase 10A

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0007	0.0163	2.957	COX6B2	cytochrome c oxidase subunit VIb polypeptide 2 (testis)
0.0007	0.0163	1.786	GPR128	G protein-coupled receptor 128
0.0007	0.0165	1.111	ITGAV	integrin, alpha V
0.0007	0.0165	1.719	ZNF460	zinc finger protein 460
0.0008	0.0167	1.198	TNFRSF8	tumor necrosis factor receptor superfamily, member 8
0.0008	0.0169	1.103	TTL7	tubulin tyrosine ligase-like family, member 7
0.0008	0.0172	1.607	CDKL5	cyclin-dependent kinase-like 5
0.0008	0.0172	2.582	CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5
0.0008	0.0172	1.339	MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A
0.0008	0.0172	1.953	NT5DC3	5'-nucleotidase domain containing 3
0.0008	0.0172	1.798	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa
0.0008	0.0173	1.184	CCR1	chemokine (C-C motif) receptor 1
0.0008	0.0177	3.888	PAPOLB	poly(A) polymerase beta (testis specific)
0.0009	0.0178	1.109	ADAT2	adenosine deaminase, tRNA-specific 2
0.0009	0.0178	4.485	HOXC4	homeobox C4
0.0009	0.0178	2.925	LPHN3	latrophilin 3
0.0009	0.0178	-2.549	SPAG17	sperm associated antigen 17
0.0009	0.0178	1.237	THSD7A	thrombospondin, type I, domain containing 7A
0.0009	0.0179	1.365	DDX6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
0.0009	0.0179	1.164	PIWIL2	piwi-like RNA-mediated gene silencing 2
0.0009	0.0180	1.232	DENND5B	DENN/MADD domain containing 5B
0.0009	0.0180	4.287	TAAR6	trace amine associated receptor 6
0.0009	0.0181	1.217	KIAA1324	KIAA1324
0.0009	0.0181	1.683	RYR1	ryanodine receptor 1 (skeletal)
0.0009	0.0182	1.419	TLR6	toll-like receptor 6
0.0010	0.0183	1.99	ZNF593	zinc finger protein 593
0.0010	0.0184	1.454	MIB1	mindbomb E3 ubiquitin protein ligase 1
0.0010	0.0185	1.12	SLC35F1	solute carrier family 35, member F1
0.0010	0.0187	1.885	APLN	apelin receptor

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0010	0.0187	1.319	CAV1	caveolin 1, caveolae protein, 22kDa
0.0010	0.0187	1.204	IL1B	interleukin 1, beta
0.0010	0.0187	1.228	LONRF2	LON peptidase N-terminal domain and ring finger 2
0.0010	0.0187	1.261	TAOK1	TAO kinase 1
0.0011	0.0189	1.247	EXOC6B	exocyst complex component 6B
0.0011	0.0192	-1.265	SNRPD2	small nuclear ribonucleoprotein D2 polypeptide 16.5kDa
0.0011	0.0193	2.165	MTIL	metallothionein IL (gene/pseudogene)
0.0011	0.0193	1.142	NEAT5	nuclear factor of activated T-cells 5, tonicity-responsive
0.0011	0.0193	1.145	NRIP1	nuclear receptor interacting protein 1
0.0011	0.0194	1.954	FABP12	fatty acid binding protein 12
0.0011	0.0194	1.104	GRINA	glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)
0.0011	0.0194	1.263	HIPK3	homeodomain interacting protein kinase 3
0.0012	0.0202	2.898	SCGB1D2	secretoglobulin, family 1D, member 2
0.0012	0.0206	1.984	TNFRSF9	tumor necrosis factor receptor superfamily, member 9
0.0013	0.0211	1.523	RAB11FIP4	RAB11 family interacting protein 4 (class II)
0.0014	0.0220	-1.495	ACTB	actin, beta
0.0014	0.0220	1.13	BTBD9	BTB (POZ) domain containing 9
0.0014	0.0220	1.491	PANK3	pantothenate kinase 3
0.0014	0.0220	1.991	SERF2	small EDRK-rich factor 2
0.0014	0.0222	-2.489	CGB	chorionic gonadotropin, beta polypeptide
0.0014	0.0222	1.908	LTB4R2	leukotriene B4 receptor 2
0.0015	0.0223	1.618	ACR	acrosin
0.0015	0.0223	1.109	APBP2	amyloid beta precursor protein (cytoplasmic tail) binding protein 2
0.0015	0.0223	1.83	CXCR2	chemokine (C-X-C motif) receptor 2
0.0015	0.0223	1.153	GMFB	glia maturation factor, beta
0.0015	0.0223	1.113	ZBED6	zinc finger, BED-type containing 6
0.0016	0.0224	1.703	B3GALT5	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5
0.0015	0.0224	1.484	DBNL	drebrin-like
0.0016	0.0224	2.839	OR7D4	olfactory receptor, family 7, subfamily D, member 4

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0016	0.0224	2.077	SUMO2	small ubiquitin-like modifier 2
0.0016	0.0226	1.985	P1WIL3	piwi-like RNA-mediated gene silencing 3
0.0016	0.0226	1.108	SLC38A2	solute carrier family 38, member 2
0.0016	0.0227	1.505	DTX3L	deltex 3 like, E3 ubiquitin ligase
0.0016	0.0227	2.99	MS4A15	membrane-spanning 4-domains, subfamily A, member 15
0.0017	0.0229	1.268	DRAM1	DNA-damage regulated autophagy modulator 1
0.0017	0.0232	1.234	CNOT6	CCR4-NOT transcription complex, subunit 6
0.0017	0.0232	1.606	SERPINA10	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10
0.0017	0.0235	1.652	FAT1	FAT atypical cadherin 1
0.0018	0.0239	1.606	FAM26F	family with sequence similarity 26, member F
0.0018	0.0239	1.717	LCN15	lipocalin 15
0.0018	0.0240	1.233	PRR14L	proline rich 14-like
0.0018	0.0242	1.553	TRPC4	transient receptor potential cation channel, subfamily C, member 4
0.0019	0.0249	2.268	MUC6	mucin 6, oligomeric mucus/gel-forming
0.0020	0.0251	1.177	LIN7C	lin-7 homolog C (C. elegans)
0.0020	0.0251	1.207	MGAT4B	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme B
0.0020	0.0252	1.489	HTR1F	5-hydroxytryptamine (serotonin) receptor 1F, G protein-coupled
0.0021	0.0256	2.431	SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa
0.0021	0.0256	1.136	SNX27	sorting nexin family member 27
0.0023	0.0267	1.119	DPP8	dipeptidyl-peptidase 8
0.0022	0.0267	1.613	DUSP19	dual specificity phosphatase 19
0.0022	0.0267	1.953	LRFN3	leucine rich repeat and fibronectin type III domain containing 3
0.0023	0.0267	1.166	PLXNA4	plexin A4
0.0022	0.0267	1.12	PRSS44	protease, serine, 44
0.0022	0.0267	1.967	TAS2R1	taste receptor, type 2, member 1
0.0023	0.0268	1.998	XIAP	X-linked inhibitor of apoptosis
0.0023	0.0269	1.317	C21orf91	chromosome 21 open reading frame 91
0.0023	0.0269	1.223	TMEM170B	transmembrane protein 170B
0.0024	0.0270	1.462	GUCY1A2	guanylate cyclase 1, soluble, alpha 2

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0024	0.0270	1.275	SLC5A3	solute carrier family 5 (sodium/myo-inositol cotransporter), member 3
0.0024	0.0274	1.702	KIAA2018	KIAA2018
0.0024	0.0275	1.121	PDCD1LG2	programmed cell death 1 ligand 2
0.0025	0.0278	1.378	MPV17L	MPV17 mitochondrial membrane protein-like
0.0026	0.0283	1.126	LRRRC8C	leucine rich repeat containing 8 family, member C
0.0028	0.0291	1.117	C1QTNF9	C1q and tumor necrosis factor related protein 9
0.0028	0.0291	1.102	SMOC2	SPARC related modular calcium binding 2
0.0029	0.0296	1.513	MAGT1	magnesium transporter 1
0.0029	0.0296	-1.158	OBP2A	odorant binding protein 2A
0.0029	0.0297	1.472	NRCAM	neuronal cell adhesion molecule
0.0029	0.0298	1.172	RPL35A	ribosomal protein L35a
0.0030	0.0299	1.552	DOK2	docking protein 2, 56kDa
0.0031	0.0303	1.271	ADH1A	alcohol dehydrogenase 1A (class I), alpha polypeptide
0.0032	0.0303	-1.435	NPIPA1	nuclear pore complex interacting protein family, member A1
0.0033	0.0307	1.688	PPARA	peroxisome proliferator-activated receptor alpha
0.0034	0.0312	1.197	CX3CR1	chemokine (C-X3-C motif) receptor 1
0.0034	0.0312	1.106	RALGPS2	Ral GEF with PH domain and SH3 binding motif 2
0.0035	0.0312	1.749	TMPPE	transmembrane protein with metallophosphoesterase domain
0.0035	0.0313	2.607	SIRPD	signal-regulatory protein delta
0.0035	0.0314	1.458	LCLAT1	lysocardiolipin acyltransferase 1
0.0035	0.0314	1.39	LMF1	lipase maturation factor 1
0.0035	0.0314	1.475	TCP11	t-complex 11, testis-specific
0.0037	0.0319	1.251	CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1
0.0037	0.0319	1.301	RFX3	regulatory factor X, 3 (influences HLA class II expression)
0.0037	0.0319	1.448	THSD7B	thrombospondin, type I, domain containing 7B
0.0036	0.0319	2.124	VBP1	von Hippel-Lindau binding protein 1
0.0040	0.0332	1.258	CCDC79	coiled-coil domain containing 79
0.0040	0.0332	1.72	CD5L	CD5 molecule-like
0.0040	0.0332	1.841	SLC26A2	solute carrier family 26 (anion exchanger), member 2

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0039	0.0332	1.17	TLR3	toll-like receptor 3
0.0040	0.0335	1.151	PTGFR	prostaglandin F receptor (FP)
0.0042	0.0339	2.154	SNTB2	syntrophin, beta 2 (dystrophin-associated protein A1, 59kDa, basic component 2)
0.0043	0.0343	1.237	KAT7	K(lysine) acetyltransferase 7
0.0043	0.0343	1.583	POU1F1	POU class 1 homeobox 1
0.0045	0.0355	-2.002	LRRC71	leucine rich repeat containing 71
0.0046	0.0356	1.155	OYGP1	oviductal glycoprotein 1, 120kDa
0.0046	0.0357	-1.212	C1orf158	chromosome 1 open reading frame 158
0.0046	0.0357	1.311	ENPP6	ectonucleotide pyrophosphatase/phosphodiesterase 6
0.0046	0.0357	1.251	TIPARP	TCDD-inducible poly(ADP-ribose) polymerase
0.0047	0.0358	1.334	ZNF280B	zinc finger protein 280B
0.0047	0.0359	1.255	UNC5C	unc-5 homolog C (C. elegans)
0.0048	0.0364	1.381	FNIP1	folliculin interacting protein 1
0.0049	0.0368	-1.177	RPL19	ribosomal protein L19
0.0049	0.0368	1.116	SLC5A7	solute carrier family 5 (sodium/choline cotransporter), member 7
0.0050	0.0369	1.121	MDM4	MDM4, p53 regulator
0.0050	0.0371	1.592	EXPH5	exophilin 5
0.0050	0.0373	1.73	ALG10	ALG10, alpha-1,2-glucosyltransferase
0.0052	0.0380	1.521	SNX30	sorting nexin family member 30
0.0054	0.0386	1.639	ILDR1	immunoglobulin-like domain containing receptor 1
0.0054	0.0386	1.253	RPL34	ribosomal protein L34
0.0054	0.0387	-1.959	GALNT8	polypeptide N-acetylgalactosaminyltransferase 8
0.0056	0.0390	1.231	CMTM4	CKLF-like MARVEL transmembrane domain containing 4
0.0056	0.0390	1.839	HIST1H4E	histone cluster 1, H4e
0.0056	0.0390	1.951	NSL1	NSL1, MIS12 kinetochore complex component
0.0057	0.0393	1.251	RBM12B-AS1	RBM12B antisense RNA 1
0.0058	0.0396	1.449	CACNA1E	calcium channel, voltage-dependent, R type, alpha 1E subunit
0.0059	0.0399	1.377	ITGA1	integrin, alpha 1
0.0060	0.0399	1.221	ZFP14	ZFP14 zinc finger protein

p-value	FDR (q-value)	Log Ratio	Symbol	Entrez Gene Name
0.0061	0.0403	1.343	RAB27B	RAB27B, member RAS oncogene family
0.0065	0.0417	2.172	ATXN1	ataxin 1
0.0067	0.0425	1.141	NUP155	nucleoporin 155kDa
0.0067	0.0425	1.11	SERINC5	serine incorporator 5
0.0067	0.0425	1.74	ZNF529	zinc finger protein 529
0.0070	0.0436	1.66	CCDC68	coiled-coil domain containing 68
0.0074	0.0449	1.507	MTTP	microsomal triglyceride transfer protein
0.0078	0.0459	1.81	KRT23	keratin 23 (histone deacetylase inducible)
0.0077	0.0459	1.222	ZDHC21	zinc finger, DHHC-type containing 21
0.0080	0.0465	1.452	COMMD9	COMM domain containing 9
0.0088	0.0491	1.112	ANKRD20A4	ankyrin repeat domain 20 family, member A4
0.0089	0.0496	1.201	TTC39B	tetratricopeptide repeat domain 39B

* Supplementary material can be found by accessing the online version of this paper at <http://dx.doi.org> and by entering doi:...