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CREB-mediated alterations in the transcriptome of the amygdala following cocaine conditioned reward and extinction

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Abstract

The neuronal circuitry underlying stress- and drug-induced reinstatement of cocaine seeking has been relatively well-characterized; however, less is known regarding the long-term molecular changes following cocaine administration that may promote future reinstatement. The transcription factor cAMP response element binding protein (CREB) is necessary for stress- but not cocaine-induced reinstatement of conditioned reward, suggesting that different molecular mechanisms may underlie these two types of reinstatement. To explore the relationship between this transcription factor and reinstatement, we utilized conditioned place preference (CPP) to examine alterations in gene expression in the amygdala, a neural substrate critically involved in stress-induced reinstatement, following the development of cocaine reward and subsequent extinction. Our findings demonstrate that the amygdala transcriptome was altered by CREB-deficiency more than by previous cocaine experience, with an over-representation of genes involved in the immune response. However, a subset of genes involved in stress and immune response demonstrated a drug×genotype interaction, indicating that cocaine produces different long-term alterations in gene expression depending on the presence or absence of CREB. This profile of gene expression in the context of addiction enhances our understanding of the long-term molecular changes that occur throughout the addiction cycle and identifies novel genes and pathways that might lead to the creation of better therapeutic agents.

Keywords

gene expression; CREB; conditioned place preference; immune response; stress

Introduction

Drug addiction is a psychiatric disorder characterized by a transition from recreational to compulsive drug use that continues in spite of severe negative consequences (O'Brien, 2003). Despite attempts by individuals to quit, the desire or need to resume drug taking can last for months or years (Sinha and Li, 2007). The persistence of addiction over time suggests that exposure to drugs results in long-term adaptations in the brain that likely involve alterations in transcription and genetic regulation.

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Statement of Interest:
None.

Attempts to further characterize these long-term changes have identified a variety of candidate genes that may promote and maintain addictive behaviors (Lu et al., 2003a), including ΔFosB (Ang et al., 2001; Chen et al., 1995; McClung and Nestler, 2003), brain-derived neurotrophic factor (Grimm et al., 2003; Lu et al., 2004), dynorphin (Redila and Chavkin, 2008; Shippenberg et al., 2007) and corticosterone releasing factor (Maj et al., 2003; Sarnyai et al., 2001; Zorrilla et al., 2001). Most of these changes in gene expression occur in the mesolimbic dopamine system, which includes the ventral tegmental area (VTA) and the nucleus accumbens (NAc), as well as limbic structures like the amgydala. The VTA and NAc are critically important in initial reward (Pierce and Kumaresan, 2006; Roberts et al., 1977; Wise, 2004) as well as reinstatement of drug seeking (Kalivas and McFarland, 2003; McFarland et al., 2004; McFarland and Kalivas, 2001). The amygdala, more specifically the central nucleus, has been shown to be particularly important in mediating stress-induced reinstatement (Erb et al., 2001; Leri et al., 2002; McFarland et al., 2004).

The involvement of these brain regions in reward and reinstatement have been characterized primarily using the self-administration model. However, conditioned place preference (CPP) is another model that measures drug reward in rodents and can also be used to study reinstatement elicited by various stimuli (Kreibich and Blendy, 2004; Lu et al., 2003b; Mueller et al., 2002; Parker and McDonald, 2000; Redila and Chavkin, 2008). In mice, stress-induced reinstatement of CPP is associated with an augmentation of phosphorylated CREB levels in the amygdala relative to non-stressed mice, which does not occur following cocaine-induced reinstatement (Kreibich and Blendy, 2004). Furthermore, mice lacking the α and Δ isoforms of CREB (CREBαΔ mutant mice) have a 90% deletion in CREB protein levels and do not exhibit stress-induced reinstatement but maintain robust reinstatement to a priming dose of cocaine (Kreibich and Blendy, 2004). These findings strongly suggest that different molecular mechanisms underlie the two forms of reinstatement. Thus, CREB-induced changes in the amygdala may occur following drug administration and act to promote stress-induced reinstatement.

To generate a broader inventory of genes that may underlie the prolonged persistence of addictive behaviors and to identify novel targets involved in this cycle of addiction, a variety of microarray studies have been performed (Freeman et al., 2010; Krasnova et al., 2008; McClung and Nestler, 2003; Yuferov et al., 2003; Yuferov et al., 2005). The majority of these microarrays, however, have been focused on brain areas classically involved in mediating the rewarding properties of drugs, namely the VTA and NAc, and the long-term changes that occur in the amygdala during abstinence and may promote reinstatement have been largely unexplored.

Thus, in the present study we utilized expression profiling to examine changes in gene expression following cocaine administration, which might contribute to the reinstatement of place preference. We focused our gene analysis on the amygdala of wildtype and CREBαΔ mice following cocaine conditioning and extinction but prior to reinstatement. This time-point was chosen to identify gene changes that occur during extinction and could potentially underlie subsequent reinstatement behavior elicited by a stressor, while avoiding the acute changes in gene expression that occur following stress exposure. A number of novel genes that are classically involved in stress and immune response were identified. These findings may lead to a better understanding of the long-term genetic alterations in the amygdala that act to promote reinstatement behavior.

Materials and Methods

Animals

Mice (3–6 months of age; 20–40 grams; mixed sexes) were group-housed in a 21°C humidity-controlled animal facility approved by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) and had access to food and water *ad libidum*. All experiments were performed in accordance with NIH guidelines for "Guiding principles in the care and used of animals".

Both wildtype and CREBαΔ mice were maintained as a 129SvEvTac:C57BL/6J F1 hybrid strain, obtained from crossing mice heterozygous for the CREB mutation from each parental strain. Both parental strains (129SvEvTac and C57BL/6J) had been backcrossed with vendor-supplied wild type mice for >20 generations.

Conditioned Place Preference (CPP)

Place conditioning boxes were divided into two sides (20 X 20 X 20 cm); one side consisted of a striped wall with plastic flooring, and the other side, solid grey-colored walls with a metal grid floor. The solid side was illuminated throughout the 10-day paradigm, while the striped side was dark. *Preconditioning Day.* Mice were placed on one side of the box and allowed to roam freely between both sides for 900 seconds. Time spent on each side was recorded and data was used to separate the mice into groups that had average bias on each side. *Pairing Days.* For days 2–9, mice underwent conditioning, with the cocaine group receiving cocaine (20 mg/kg, i.p.; NIDA Drug Supply, Research Triangle, NC) on one side of the box and saline on the other, and the saline group receiving saline on both sides of the apparatus. *Test Day.* Mice were given a saline injection, placed into the box, and allowed to roam freely between both sides for 900 seconds. Time spent on each side was recorded, and data was expressed as time spent on the paired side minus time spent on the unpaired side. *Extinction.* For days 11–22, mice were given saline injections on both sides of the conditioning boxes. *Extinction Test Day.* On day 23, mice were given a saline injection, placed into the box and allowed to roam freely between both sides for 900 seconds. Time spent on each side was recorded and data was analyzed using a two-way ANOVA.

Brain dissections

Mice were killed 24 hours following extinction test by cervical dislocation and brains were rapidly removed and dissected on ice. Brains were first sliced into 1 mm slices using a mouse brain matrix (Braintree Scientific, MA) and specific regions identified using coordinates from the mouse stereotaxic atlas (Amygdala, bregma: −1.2mm; Cortex, bregma: 0.15mm) (Franklin and Paxinos, 2007). Tissue was macrodissected and immediately frozen in liquid nitrogen.

Expression profiling

RNA was extracted from brain tissue by homogenization in 800 μL of Trizol and 160 μL chloroform. Samples were sedimented at 13,000 rpm for 15 minutes and the aqueous layer removed. RNA was purified using an RNeasy Mini Kit (Qiagen). RNA concentration was determined using a Nanodrop spectrometer (Nanodrop Technologies, Wilmington, DE) and quality was evaluated using a RNA NanoChip on the Bioanalyzer (Agilent). 1,000 ng of total RNA was amplified and labeled with Cy3 using the Low RNA Input Linear Amp Kit PLUS, One-Color (Agilent Technologies, CA). After purification, 1.65 μg of cRNA was fragmented and hybridized to the Whole Mouse Genome Oligo Microarray G4122A (Agilent Technologies, CA) for 17 hrs at 65°C. One animal per group was hybridized to one array tile (n=4 per group). Following hybridization, the slides were washed and scanned

with an Agilent G2565BA Microarray Scanner. Images were analyzed with Feature Extraction 9.5 (Agilent Technologies, CA). Mean foreground intensities were obtained for each spot and imported into the mathematical software package "R", which normalized the data using Limma Quantile Normalization (Smyth 2004, Bolstad et al., 2003). Complete statistical analysis was then performed in "R" using both the LIMMA (Linear Models for Microarray Data) and SAM (Significance Analysis of Microarrays) packages. Hierarchical clustering was performed on the samples using "pvclust" (Suzuki and Shimodaira, 2006). This package calculates *p*-values for hierarchical clustering via multiscale bootstrap resampling. Differentially regulated genes were determined for four comparisons: WT/Cocaine - WT/Saline, CREBαΔ/Cocaine - CREBαΔ/Saline, CREBαΔ/Cocaine - WT/Cocaine, and CREBαΔ/Saline - WT/Saline. Further analysis of the dataset to determine functional classes of these genes was completed using the UCSC mouse genome browser and EntrezGene.

Functional analysis of genes

GO (Gene ontology) biological functions were determined from gene lists in each array by NIH David (Dennis et al., 2003; Hosack et al., 2003). Significance was determined using a modified *Fisher's exact test* (EASE) score (Hosack et al., 2003).

Quantitative real-time polymerase chain reaction (QPCR)

To verify the differentially expressed genes found in the microarray, a separate cohort of mice were taken through an identical behavioral paradigm and their brain tissue obtained as described for the array, however, no amplification was required for the biological validation of gene expression changes using QPCR. RNA (500 ng) was used for cDNA synthesis using 1 microgram Oligo dT primer (Operon) and Superscript II reverse transcriptase with its accompanying reagents (Invitrogen). All QPCR reactions were run using the Stratagene MX3000 and the MXPro QPCR software. Reactions were assembled using Applied Biosystems 2X SYBR-Green master mix along with 300 nM primers (final concentration) in accordance with the manufacturer's instructions, except that the total reaction volume was scaled down to 25 μL. Cycling parameters were 95°C for 10 min and then 40 cycles of 95°C (30 s) and 60°C (1 min), followed by a melting curve analysis. All reactions were performed in triplicate and the median cycle threshold was used for analysis. Cycle threshold values were normalized to TATA Binding Protein (TBP). Two-way ANOVAs were used to confirm significance and direction of fold changes predicted by the array. Primer sequences are available upon request.

Results

Both wildtype and CREBαΔ mutant mice show development and extinction of cocaine conditioned place preference as previously demonstrated

Following conditioning, both wildtype and CREBαΔ groups developed preference for the cocaine-paired context (Two-way ANOVA: $F_{(1,28)}=33.96$, $p<0.0001$; Bonferroni *post-hoc*: *$p<0.01$ from corresponding saline-treated animals), and this preference was no longer evident following 12 days of extinction (Figure 1). Mice were sacrificed 24 hours following extinction test day but prior to reinstatement in order to identify candidate genes whose expression might be changed throughout conditioning and extinction, while avoiding the confounding effects of an acute stressor on gene expression.

CREB genotype plays a key role in the expression of genes in the amygdala

Microarray analysis was performed on the amygdala of both wildtype (WT) and CREBαΔ mutant (MT) mice exposed to the CPP paradigm. Comparisons were designed to determine

the effect on gene expression of genotype (MT/Saline - WT/Saline), drug treatment in wildtype (WT/Cocaine - WT/Saline) and mutant mice (MT/Cocaine - MT/Saline), and the interaction between drug and genotype (MT/Saline - MT/Saline VS. MT/Cocaine - WT/Cocaine) (Figure 2a). Hierarchical clustering revealed a significant impact of the CREB genotype on gene expression, with 807 genes differentially expressed in the MT/Saline - WT/Saline comparison (Figure 2b). More than half of the genes found in the MT/Cocaine - MT/Saline comparison (24 out of 47 genes), as well as a majority of genes in the MT/Cocaine - WT/Cocaine comparison (21 out of 29 genes), were identical to differentially expressed genes found in the MT/Saline - WT/Saline comparison, further supporting the observation that CREB plays a significant role in gene expression in this brain region (Figure 2b).

Clustering analysis also indicated that cocaine treatment did not have a significant effect on gene expression in the amygdala, as mice given cocaine did not cluster separately from those receiving saline, regardless of genotype. Significance Analysis of Microarrays (SAM) further supported this finding as no genes were differentially expressed in the WT/Cocaine - WT/Saline comparison (Figure 2b, see also Supplemental Table I). However, forty-seven genes were differentially expressed by SAM analysis in the MT/Cocaine - MT/Saline comparison, indicating that prior cocaine treatment in the mutants, but not the wildtype mice, influences gene transcription following extinction of cocaine preference.

Table I lists the top 10 differentially expressed genes found in each comparison (full list of genes, see Supplemental Table I). In the MT/Saline - WT/Saline comparison, there is both an upregulation and a downregulation in the expression of various genes in the mutant animals. This indicates that while CREB is a positive regulator of transcription and thus reduced levels of this protein are likely to downregulate gene expression, compensatory mechanisms may come into play to increase expression of other genes. Furthermore, CREBαΔ mutants treated with cocaine exhibited downregulated gene expression compared to wildtype mice treated with cocaine. However, many of these genes were also downregulated to a similar degree in the MT/Saline – WT/Saline comparison (Supplemental Table I), suggesting that this downregulation is simply due to the absence of CREB and is not affected by cocaine. Lastly, mutants treated with cocaine exhibited downregulated gene expression compared to mutants treated with saline, further supporting the observation that cocaine treatment alters gene expression only in mutant animals as there were no differences in the WT/Cocaine - WT/Saline comparison.

Table II lists the top 10 differentially expressed genes similar between two different comparisons: MT/Cocaine - WT/Cocaine vs. MT/Saline - WT/Saline and MT/Saline - WT/Saline vs. MT/Cocaine - MT/Saline. The first comparison demonstrates that lack of CREB causes comparable downregulation in expression of these genes regardless of drug treatment. The second comparison again highlights a more interesting change: saline-treated mutants demonstrate increased expression of certain genes compared to saline-treated wildtypes and cocaine-treated mutants. This suggests that at baseline, mutants show greater expression of these genes and that this enhanced expression is diminished following cocaine conditioning and extinction.

Analysis of gene functions in the genotype comparison: MT/Saline - WT/Saline

Gene Ontology (GO) was used to analyze patterns of functionality among the differentially expressed genes in the MT/Saline - WT/Saline comparison (Table III). The highest-scoring biologically relevant category found to be over-represented in the genotype comparison was ‘immune response’ ($p<1.19E-06$, *EASE score*), followed by ‘transcription’ as well as ‘G-protein coupled receptor binding’. Of interest, analysis of putative CRE sites in the promoters of differentially expressed genes in this comparison demonstrated that over half

did not have a CRE site in their promoter (data not shown), indicating that many of these genes are likely regulated by CREB indirectly. As there were very few genes differentially expressed in the other group comparisons, analysis of the GO biological function was limited and produced no significant results.

Verification by QPCR of genes found to be differentially expressed in the amygdala

Sixteen candidate genes from a range of functional categories were sampled from the three lists of comparisons that demonstrated changes in gene expression: MT/Saline - WT/Saline, MT/Cocaine - WT/Cocaine, and MT/Cocaine - MT/Saline (Table IV; Supplemental Table I). All four group comparisons for each of these 16 genes were consolidated to better visualize how CREB and cocaine alter patterns of gene expression (Table V). The fold-change values across all four comparisons suggested that while many of the genes demonstrated a genotype effect (typically decreased expression in the CREBαΔ mice regardless of drug experience), some of the genes demonstrated a possible drug × genotype interaction. Some of these comparisons are not represented in other tables either because their fold change was lower than 1.5 or their false discovery rate (FDR) was higher than 20%. Changes in these sixteen candidate genes were then verified by QPCR in a separate cohort of mice that underwent the same drug conditioning and extinction paradigm.

Overall, the validation rate of the microarray in a second cohort of mice, which served as the biological replicate, was over 75% (Table V: validated genes are highlighted). Genes that indicated a genotype effect and were validated as such included *Camk1* (calcium/calmodulin-dependent protein kinase I), *Crem* (cAMP responsive element modulator), *Dhrs7* (dehydrogenase/reductase member 7), *IL18* (interleukin 18), *Pecr* (peroxisomal trans-2-enoyl-CoA reductase), *Sln* (sarcolipin), and *Zfp367* (zinc finger protein 367) (Figure 3). CREBαΔ mice displayed decreased gene expression regardless of drug experience for all genes except *Crem* (data not shown), which was, and has previously been shown to be, upregulated in CREBαΔ mice (Blendy et al., 1996). Both *Dhrs7* and *Pecr* are genes that appeared to be unique to the MT/Cocaine - WT/Cocaine comparison. When QPCR was used to verify the microarray results, however, these genes were shown to be downregulated in the absence of CREB regardless of drug treatment. This makes sense in light of the comparisons in Table V, which demonstrate a -1.3-fold change for both genes in the MT/Saline - WT/Saline comparison. This difference did not appear on the MT/Saline - WT/Saline comparison list of 807 genes, which used a fold change cutoff value of 1.4 (Supplemental Table I), emphasizing the importance of analyzing the trend in all four group comparisons when attempting to understand how these genes are regulated by both CREB as well as cocaine conditioning (Table V). Genes that suggested an interaction effect and were validated as such included *Avpr1a* (arginine vasopressin receptor 1A), *IL10* (interleukin 10), *Sgk2* (serum/glucocorticoid regulated kinase 2), *Stat1* (signal transducer and activator of transcription 1), and *Tnfrsf1b* (tumor necrosis factor receptor superfamily, member 1b) (Figure 4). These genes are all involved in stress and immune response and they all demonstrate the same pattern of expression; saline-treated CREBαΔ mice exhibited increased expression of these genes relative to wildtype mice, and treatment with cocaine abolished this difference.

To determine whether this effect was specific to the amygdala, we examined a subset of these genes in an area of the cortex consisting mainly of motor and somatosensory areas, which are typically not associated with drug or stress response. The drug × genotype interaction observed for *IL10* and *Stat1* in the amygdala was no longer present in the cortex, while *Sgk2* appeared to have a subtle but significant genotype effect. We tested *IL18* as well because it is also known to be involved in stress and immune response. This gene was downregulated in the amygdala of CREBαΔ mutant mice regardless of drug experience in

contrast to the other stress and immune response genes that demonstrated a clear drug × genotype interaction (Figure 5). These data suggest that depletion of CREB elicits widespread downregulation of certain genes in the brain, however many of the drug × genotype interactions observed following our behavioral paradigm are specific to the amygdala: a brain region critically involved in the stress response. *Abcb1b* (ATP-binding cassette, sub-family B, member 1B), *Gpr21* (G protein-coupled receptor 21), *Pomc1* (pro-opiomelanocortin-alpha), and *Slc6a3* (dopamine transporter) were also tested (individual data not shown), but none of these gene changes were validated in a new cohort of mice. Results of all 16 genes that were examined in both cohorts of mice are summarized in Table V.

Discussion

The molecular mechanisms underlying the transition from initial drug use to the compulsive drug taking that characterizes an addictive state have only been partially identified. Exposure to drugs of abuse results in long-term adaptations in the brain, which may result from activation of transcription factors such as CREB and concomitant alterations in gene expression. Studies in humans and animal models indicate that stress can increase vulnerability to addiction as well as enhance susceptibility to relapse. To identify gene expression changes that may underlie stress-induced drug relapse, we focused our expression profile analysis on alterations in the amygdala that occur following extinction of cocaine CPP but prior to reinstatement. We focused our analysis on CREB and its requirement in cocaine-induced changes in gene expression since mice lacking this transcription factor do not exhibit stress-induced reinstatement of cocaine place preference. Our data demonstrate that CREB regulates a wide variety of genes in the amygdala. Approximately half of these genes do not contain CRE elements in their promoters, suggesting that CREB is influencing these changes in gene transcription both directly and indirectly.

Functional analysis of these gene expression differences revealed that genes involved in the immune response are differentially regulated by CREB in the amygdala. This suggests that genes classically involved in immune function could be changing stress-related behaviors in the CREBαΔ mice. Recent evidence has linked immune dysfunction to psychological stress in both humans and rodents (Godbout and Glaser, 2006). Furthermore, the cytokine theory of depression suggests that enhanced production of proinflammatory cytokines is associated with the pathogenesis of depression (Roque et al., 2009). For instance, the pro-inflammatory cytokine *IL18* is over-expressed in human stress disorders, including depression and panic disorder (Takeuchi et al., 1999). In rodent models, levels of *IL18* mRNA are significantly increased in subordinant rats following a social dominance paradigm, demonstrating a link between stress and cytokine gene expression in the brain (Kroes et al., 2006).

In the present study, *IL18* was significantly decreased in the CREBαΔ mice regardless of drug treatment. *IL18* is positively regulated by corticotropin releasing factor (CRF) *in vitro* (Park et al., 2005; Yang et al., 2005) and has a putative CRE element in its promoter, located at position +197 (Zhang et al., 2005). The decreased *IL18* gene expression observed in the mutant mice might be playing a role in their altered behaviors by imparting them with a resilience to stress. Indeed, previous findings have shown that the CREBαΔ mice have a blunted stress response (Conti et al., 2002) and do not exhibit stress-induced reinstatement of cocaine CPP (Kreibich and Blendy, 2004). However, future studies must be completed to examine the role of immune-related genes in the altered stress responses of CREBαΔ mice.

Other stress- and immune-related genes demonstrated a drug × genotype interaction. Among these is *Avpr1a*, a G-protein coupled receptor that binds the hormone arginine vasopressin

when it is released from the hypothalamus. *Avpr1a* has been implicated in regulating aggression, social bonding, and maternal behaviors (Goodson and Bass, 2001). Furthermore, increases in *Avpr1a* are linked to increased anxiety, while decreases in this receptor are linked to decreased anxiety (Bielsky et al., 2005; Bielsky et al., 2004). Interestingly, our data show increased baseline expression of this gene in the amygdala of CREBαΔ mice, which display increased anxiety despite their antidepressant phenotype (Graves et al., 2002; Gur et al., 2007).

Another gene of interest identified in this analysis is *IL10*, an anti-inflammatory interleukin that may play a key role in modulating depressive-like behaviors (Roque et al., 2009). *IL10*, along with other cytokines, is known to elicit phosphorylation of the transcriptional activator, *Stat1*, which also demonstrated an interaction on the microarray (Zocchia et al., 1997). *Tnfrsf1b* is the receptor for tumor necrosis factor (TNF), increased production of which has also been observed in depressed patients (Raison et al., 2006). Additionally, a recent microarray study examining expression changes in the NAc following abstinence from cocaine self-administration identified a TNF-centered network of genes, suggesting that long-term changes following cocaine administration may involve alterations in TNF signaling (Freeman et al., 2010). A subunit of the transcription factor nuclear factor-KB (NF-KB), a central mediator of the immune response, was also identified as being upregulated by chronic cocaine administration (Ang et al., 2001). Together, these studies provide further evidence linking immune-related pathways to cocaine-induced adaptations and also highlight the importance of microarray studies in identifying novel targets and pathways not classically involved in drug addiction and therefore often overlooked.

Overall, CREBαΔ mice displayed higher levels of these various immune-related genes at baseline compared to wildtype mice, but cocaine treatment abolished this difference. The drug × genotype interaction observed using QPCR is consistent with the more general observation that there were 807 genes differentially expressed in the MT/Saline - WT/Saline comparison of the microarray relative to only 29 in the MT/Cocaine - WT/Cocaine comparison. This suggests that differences in gene expression between CREBαΔ and wildtype mice diminish if the animals have undergone development and extinction of cocaine CPP. Since only the CREBαΔ mice demonstrate decreased expression of stress- and immune-related genes following cocaine, this response might be contributing to their greater stress resilience. However, it is unclear whether this altered gene expression has a direct effect on subsequent behavioral responses or whether these changes are simply a downstream readout of alterations in certain signaling pathways, possibly immune-related, that are affecting the behavior.

It was surprising to observe upregulated gene expression at baseline in animals lacking an activating transcription factor. This upregulation may be an indirect effect of CREB deletion, such as decreased transcriptional repression or increased availability of the coactivator CREB binding protein (CBP), which may promote binding to other transcription factors that directly upregulate these genes (Kamei et al., 1996; Manna and Stocco, 2007). Since this upregulation was no longer evident following cocaine CPP and extinction, the drug exposure and experience with conditioning may have blunted this increased gene expression via CREB-independent mechanisms. This idea is consistent with the microarray results, which demonstrated that forty-seven genes were downregulated in the CREBαΔ mice following cocaine.

One such CREB-independent mechanism could be driven by glucocorticoid receptors (GR). GRs are known to be involved in mediating the behavioral responses to cocaine as well as the cross-sensitization between drugs and stress (Ambroggi et al., 2009; de Jong and de Kloet, 2009; de Jong et al., 2009; Deroche-Gamonet et al., 2003; Izawa et al., 2006). These

studies indicate that decreasing GR activity using a variety of methods decreases the rewarding properties of cocaine, suggesting that under normal conditions cocaine is activating GR signaling pathways. GRs are also involved in combating inflammation by repressing key inflammatory transcription factors such as activator protein 1 (AP-1) and NF-kappaB and reducing the expression of pro-inflammatory genes (Almawi and Melemedjian, 2002; Hosoi et al., 2003; Newton and Holden, 2007; Paliogianni et al., 1993). Thus, GR activity may be increased following cocaine treatment, acting to suppress transcription of certain genes but only in the absence of CREB, which might prevent this process from occurring under normal conditions. Additional experiments must be conducted to elucidate the mechanisms underlying the upregulation of certain genes in the CREBαΔ mice and subsequent downregulation following cocaine conditioning and extinction.

The expression profiling study presented above is one of the first to investigate CREB's involvement in the long-term alterations in gene expression that take place in the amygdala following cocaine conditioning and extinction. Elucidating changes in gene expression that occur during cocaine administration as well as during subsequent abstinence may lead to a better understanding of why the amygdala is necessary for reinstatement of drug-seeking following exposure to a stressor. Identification of novel genes and pathways in this brain region could prove useful in creating therapeutic agents designed to promote abstinence in human addicts and diminish the likelihood of relapse following a stressful life event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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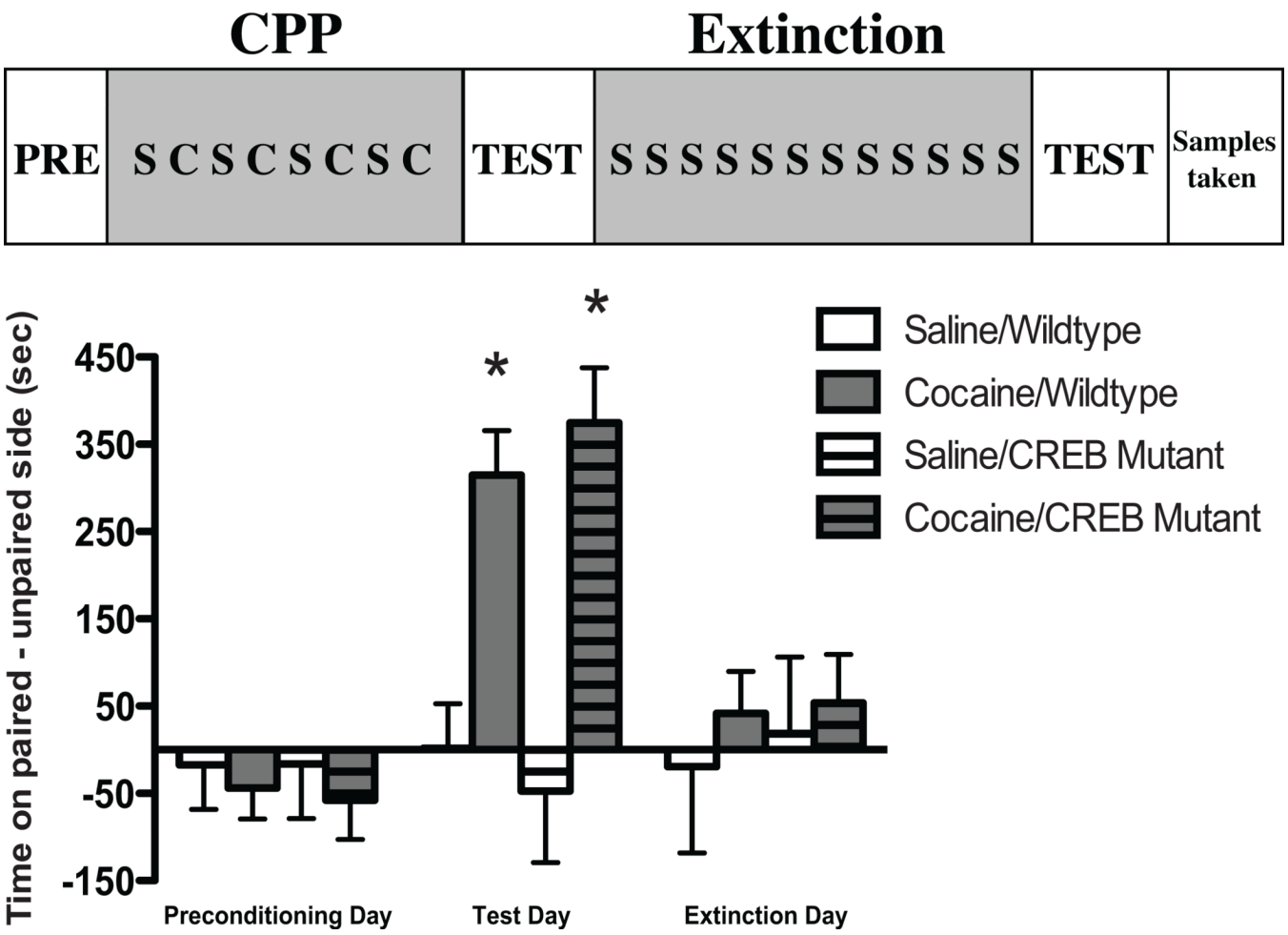

Figure 1.

Experimental timeline and behavioral data associated with microarray study. Both CREBαΔ mutant (MT) and wildtype (WT) mice were tested in the CPP paradigm to examine both reward and extinction of the reward behavior. Twenty-four hours following the extinction test day, mice were sacrificed for microarray analysis. Both wildtype and CREBαΔ mutant mice demonstrate place preference to 20 mg/kg cocaine (Significant effect of drug on test day, $F_{(1,28)}$=33.96, p<0.0001, Two-way ANOVA, *p<0.01 from corresponding saline-treated animals, Bonferroni *post-hoc*). Following extinction, wildtype and mutant mice no longer show preference for either side of the CPP chamber (not significant, Two-way ANOVA). These data are similar to results observed in the second cohort of mice used for the biological replication of the microarrays. Data is expressed as mean ± SEM. n = 8 per group.

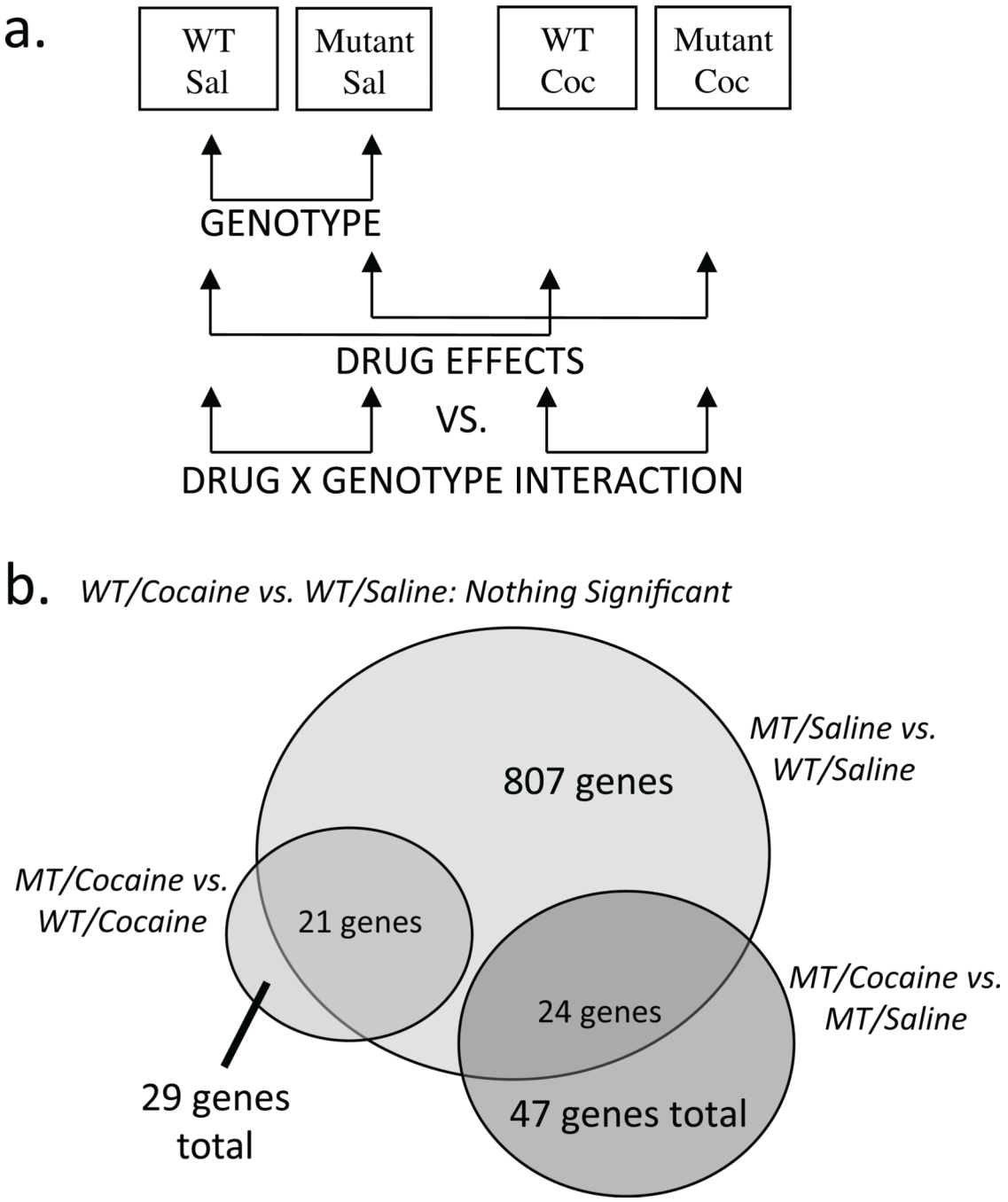

Figure 2.

Overview of results found in microarray examining the effects of the CREBαΔ genotype following extinction of cocaine place preference. a. Multiple comparisons were made between all four experimental groups with each bracket representing one comparison. b. A Venn diagram representing the number of differentially expressed genes and their overlap with other pairwise comparisons (fold change >1.4, 20% FDR as determined by SAM).

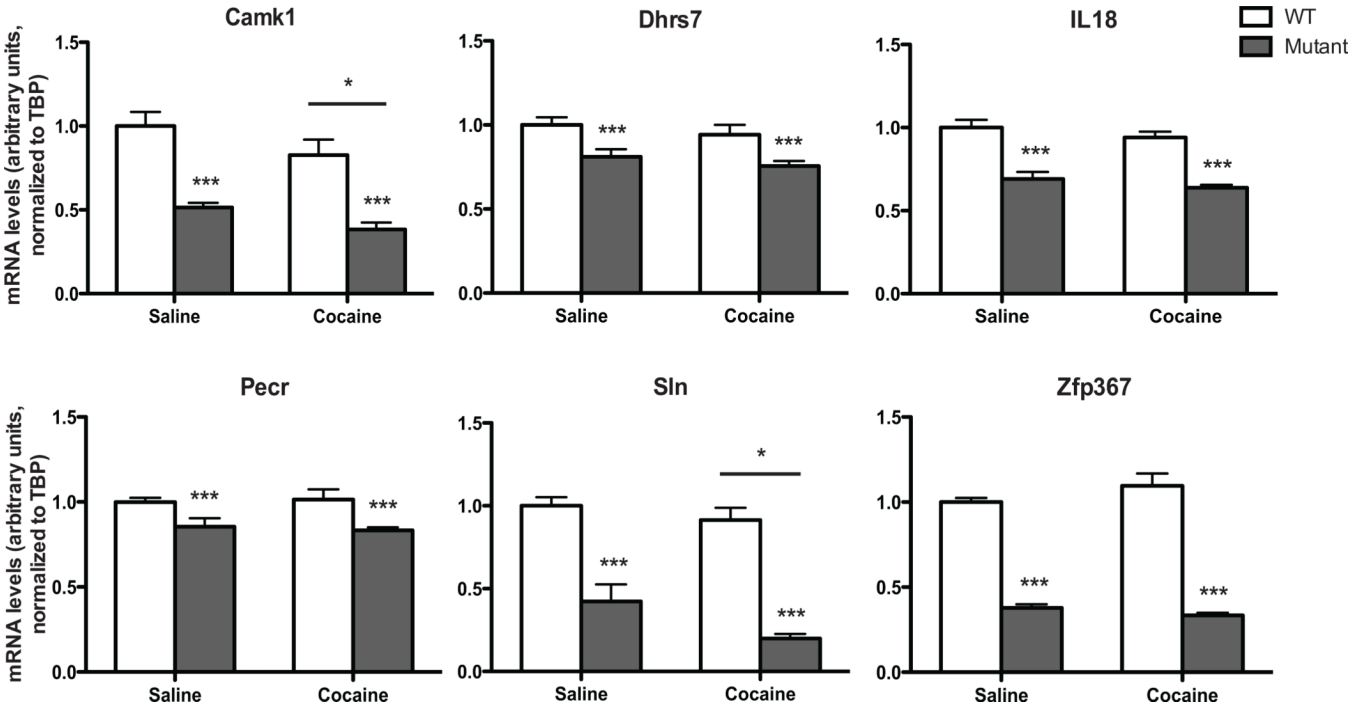

Figure 3.

Biological replication by QPCR of genes in the amygdala that demonstrated a genotype effect in the microarray. All data is expressed as mean ± SEM with n=7–8 per group. Two-way ANOVAs were used to determine statistical significance. *Camk1* (calcium/calmodulin-dependent protein kinase I): overall effect of genotype ($F_{(1,24)} = 47.44$, $p < 0.0001$) and drug ($F_{(1,24)} = 5.12$, $p < 0.05$). *Dhrs7* (dehydrogenase/reductase member 7): effect of genotype ($F_{(1,25)} = 16.63$, $p < 0.001$). *IL18* (interleukin 18): effect of genotype ($F_{(1,27)} = 67.33$, $p < 0.0001$). *Pecr* (peroxisomal trans-2-enoyl-CoA reductase): effect of genotype ($F_{(1,26)} = 13.73$, $p < 0.001$). *Sln* (sarcolipin): effect of genotype ($F_{(1,24)} = 86.51$, $p < 0.0001$) and drug ($F_{(1,25)} = 4.927$, $p < 0.05$). *Zfp367* (zinc finger protein 367): effect of genotype ($F_{(1,25)} = 302.6$, $p < 0.0001$).

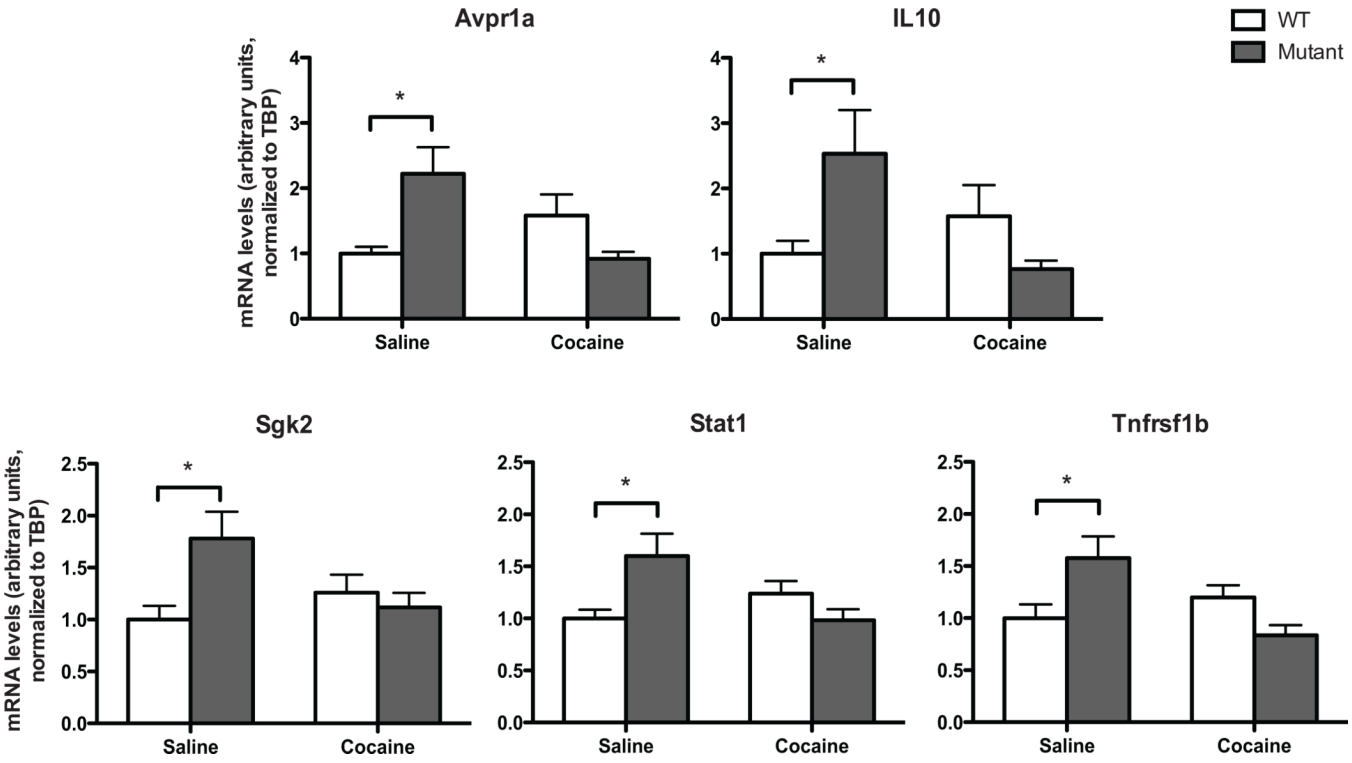

Figure 4.

Biological replication by QPCR of genes in the amygdala that demonstrated a drug × genotype interaction on the microarray. All data is expressed as mean ± SEM with n=7–8 per group. Two-way ANOVAs were used to determine statistical significance. All Bonferroni/Dunn post hoc tests revealed a significant difference between saline-treated wildtype and CREBαΔ mutant mice ($p<0.05$). *Avpr1a* (arginine vasopressin receptor 1A): drug×genotype interaction ($F_{(1,26)} = 10.71$, $p < 0.01$). *IL10* (interleukin 10): drug × genotype interaction ($F_{(1,26)} = 8.64$, $p < 0.01$). *Sgk2* (serum/glucocorticoid regulated kinase 2): drug × genotype interaction ($F_{(1,27)} = 6.184$, $p < 0.05$). *Stat1* (signal transducer and activator of transcription 1): drug × genotype interaction ($F_{(1,27)} = 8.957$, $p < 0.01$). *Tnfrsf1b* (tumor necrosis factor receptor superfamily, member 1b): drug × genotype interaction ($F_{(1,27)} = 10.04$, $p < 0.01$).

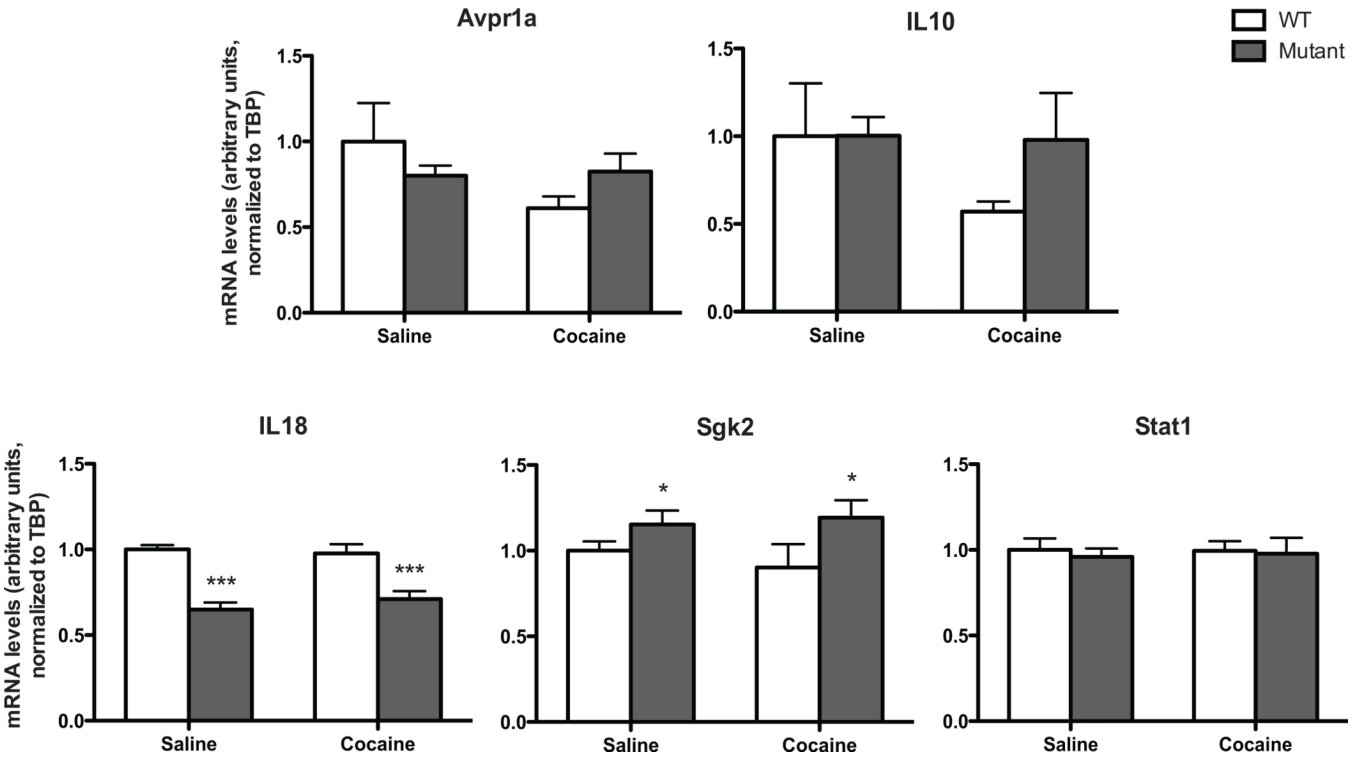

Figure 5.

QPCR of genes in the cortex to determine how patterns of gene expression in this brain region compare to those observed in the amygdala. All data is expressed as mean ± SEM with n=7–8 per group. Two-way ANOVAs were used to determine statistical significance. *IL18* (interleukin 18): effect of genotype ($F_{(1,25)} = 48.78$, $p < 0.0001$). *Sgk2* (serum/glucocorticoid regulated kinase 2): effect of genotype ($F_{(1,27)} = 5.097$, $p < 0.05$).

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Table I

List of top 10 upregulated and down regulated genes in each comparison (fold change>1.5, FDR 20%)

Upregulated				**Downregulated**			
WT/Cocaine - WT/Saline							
None found.							
MT/Saline - WT/Saline							
Accession	*Gene Symbol*	*Gene Name*	*Fold change*	*Accession*	*Gene Symbol*	*Gene Name*	*Fold change*
NM_021274	Cxcl10	chemokine (C-X-C motif) ligand 10	2.34	NM_172879	4932412H11Rik	RIKEN cDNA 4932412H11 gene	−4.13
NM_173449	4921523A10Rik	RIKEN cDNA 4921523A10 gene	2.29	NM_139219	Defb9	defensin beta 9	−3.83
NM_146296	Olfr437	olfactory receptor 437	2.27	NM_011075	Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	−3.72
NM_009490	V2r15	vomeronasal 2, receptor, 15	2.17	NM_025540	Sln	sarcolipin	−3.57
NM_013667	Slc22a2	solute carrier family 22 (organic cation transporter), member 2	2.15	NM_028434	2900092C05Rik	RIKEN cDNA 2900092C05 gene	−3.07
XM_897036	Col6a3	procollagen, type VI, alpha 3	2.01	NM_183111	4930563M21Rik	RIKEN cDNA 4930563M21 gene	−2.87
AK007727	Ppp1r12b	protein phosphatase 1, regulatory (inhibitor) subunit 12B	1.98	AK018511	Myo3a	myosin IIIA	−2.75
AK077061	D830007B15Rik	RIKEN cDNA D830007B15 gene	1.97	NM_144960	Fcamr	Fc receptor, IgA, IgM, high affinity	−2.60
NM_011338	Ccl9	chemokine (C-C motif) ligand 9	1.97	NM_010615	Kif11	kinesin family member 11	−2.53
NM_013731	Sgk2	serum/glucocorticoid regulated kinase 2	1.95	NM_019944	Hlxb9	homeobox gene HB9	−2.49
MT/Cocaine - WT/Cocaine							
Accession	*Gene Symbol*	*Gene Name*	*Fold change*	*Accession*	*Gene Symbol*	*Gene Name*	*Fold change*
NM_013498	Crem	cAMP responsive element modulator	1.83	NM_025540	Sln	sarcolipin	−3.80
				NM_175494	Zfp367	zinc finger protein 367	−2.87
				NM_138301	Trpm2	transient receptor potential cation channel, subfamily M, member 2	−2.23
				NM_025865	2310030G06Rik	RIKEN cDNA 2310030G06 gene	−1.97

Upregulated				Downregulated			
				NM_020033	Ankrd2	ankyrin repeat domain 2 (stretch responsive muscle)	−1.92
				NM_001033264	Gls2	glutaminase 2 (liver, mitochondrial)	−1.88
				NM_026972	Cd209b	CD209b antigen	−1.85
				NM_145392	Bag2	Bcl2-associated athanogene 2	−1.83
				NM_026601	Hyi	hydroxypyruvate isomerase homolog (E. coli)	−1.81
				NM_133641	Rtkn	rhotekin	−1.78
MT/Cocaine - MT/Saline							
Accession	*Gene Symbol*	*Gene Name*	*Fold change*	*Accession*	*Gene Symbol*	*Gene Name*	*Fold change*
None found.				AK047448	B930063P07Rik	RIKEN cDNA B930063P07 gene	−1.85
				XM_356184	1110001D15Rik	RIKEN cDNA 1110001D15 gene	−1.81
				XM_897036	Col6a3	procollagen, type VI, alpha 3	−1.80
				NM_026143	Mlstd2	male sterility domain containing 2	−1.76
				Y14041	Cflar	CASP8 and FADD-like apoptosis regulator	−1.74
				AK005750	1700008A07Rik	RIKEN cDNA 1700008A07 gene	−1.69
				AK028157	Mrc2	mannose receptor, C type 2	−1.68
				NM_178734	Zfp473	zinc finger protein 473	−1.66
				NM_010391	H2-Q10	histocompatibility 2, Q region locus 10	−1.65
				AK086310	Pbx3	pre B-cell leukemia transcription factor 3	−1.64

Table II

List of top 10 upregulated and down regulated genes similar between comparisons at extinction time point (fold change>1.5, FDR 20%)

Upregulated					**Downregulated**				
MT/Cocaine - WT/Cocaine compared to MT/Saline - WT/ Saline									
Accession	*Gene Symbol*	*Gene Name*	Fold change MT/Cocaine - WT/ Cocaine	Fold change MT/ Saline - WT/ Saline	*Accession*	*Gene Symbol*	*Gene Name*	*Fold change MT/Cocaine - WT/ Cocaine*	*Fold change MT/Saline - WT/Saline*
NM_013498	Crem	cAMP responsive element modulator	1.826	1.802	NM_010395	H2-T10	histocompatibility 2, T region locus 10	−1.667	−1.618
					NM_007421	Adssl1	adenylosuccinate synthetase like 1	−1.678	−1.445
					NM_001004147	AI595406	expressed sequence AI595406	−1.686	−1.645
					NM_133641	Rtkn	rhotekin	−1.783	−1.56
					NM_026601	Hyi	hydroxypyruvate isomerase homolog (E. coli)	−1.808	−1.658
					NM_145392	Bag2	Bcl2-associated athanogene 2	−1.828	−1.701
					NM_001033264	Gls2	glutaminase 2 (liver, mitochondrial)	−1.88	−1.764
					NM_025865	2310030G06Rik	RIKEN cDNA 2310030G06 gene	−1.965	−1.629
					NM_138301	Trpm2	transient receptor potential cation channel, subfamily M, member 2	−2.227	−1.901
					NM_025540	Sln	sarcolipin	−3.802	−3.571
MT/Saline - WT/Saline compared to MT/Cocaine - MT/Saline									
Accession	*Gene Symbol*	*Gene Name*	Fold change MT/Saline - WT/Saline	Fold change MT/ Cocaine - MT/ Saline	*Accession*	*Gene Symbol*	*Gene Name*	*Fold change MT/Saline - WT/Saline*	*Fold change MT/Cocaine - MT/Saline*
None found.					XM_897036	Col6a3	procollagen, type VI, alpha 3	2.007	−1.802
					AK028157	Mrc2	mannose receptor, C type 2	1.793	−1.681
					Y14041	Cflar	CASP8 and FADD-like apoptosis regulator	1.756	−1.742
					NM_026143	Mlstd2	male sterility domain containing 2	1.732	−1.757

Upregulated					Downregulated				
					XM_356184	1110001D15Rik	RIKEN cDNA 1110001D15 gene	1.713	−1.805
					NM_207260	B230218L05Rik	RIKEN cDNA B230218L05 gene	1.641	−1.441
					NM_013905	Heyl	hairy/enhancer-of-split related with YRPW motif-like	1.612	−1.458
					AK047448	B930063P07Rik	RIKEN cDNA B930063P07 gene	1.554	−1.845
					NM_013601	Msx2	homeo box, msh-like 2	1.547	−1.504
					CK791701	A030006P16Rik	RIKEN cDNA A030006P16 gene	1.534	−1.587

Table III

Functional analysis of gene changes: MT/Saline compared to WT/Saline

Category	p-value
Immune response	1.19E-06
Transcription factor complex	1.72E-04
G-protein-coupled receptor binding	7.15E-04
Transcriptional regulation	5.03E-03
Protein binding	6.97E-03
Cellular metabolism	8.24E-03

Table IV

16 genes representing a range of functional categories sampled from the 4 group comparisons on the microarray

Functional Category	Accession	GeneSymbol	GeneName	Max Fold Change (FC)
Cytokine	NM_010548	Il10	interleukin 10	1.42
	NM_008360	Il18	interleukin 18	1.61
Hormone precursor	NM_008895	Pomc1	pro-opiomelanocortin-alpha	1.73
Kinase	NM_133926	Camk1	calcium/calmodulin-dependent protein kinase I	1.59
	NM_013731	Sgk2	serum/glucocorticoid regulated kinase 2	1.95
Receptor	NM_016847	Avpr1a	arginine vasopressin receptor 1A	1.45
	NM_177383	Gpr21	G protein-coupled receptor 21	1.49
	NM_011610	Tnfrsf1b	tumor necrosis factor receptor superfamily, member 1b	1.88
Reductase	NM_025522	Dhrs7	dehydrogenase/reductase (SDR family) member 7	1.44
	NM_023523	Pecr	peroxisomal trans-2-enoyl-CoA reductase	1.47
Transcription	AK016156	Crem	cAMP responsive element modulator	1.89
	AK041599	Stat1	signal transducer and activator of transcription 1	1.71
	NM_175494	Zfp367	zinc finger protein 367	2.87
Transporter	NM_011075	Abcb1b	ATP-binding cassette, sub-family B (MDR/TAP), member 1B	3.72
	NM_010020	Slc6a3 (DAT)	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	1.94
	NM_025540	Sln	sarcolipin	3.80

Table V

Validation of the 16 genes of interest: trends were predicted based on the 4 pairwise comparisons and fold-changes of the microarray. Genes were validated in a new cohort of mice using two-way ANOVAs. Genes that were validated in the new cohort are highlighted.

Gene Symbol	FOLD CHANGE ON MICROARRAY				Microarray Prediction	Biological Replicate Validation
	WT: COC - SAL	MT: COC - SAL	COC: MT - WT	SAL: MT - WT		
Abcb1b	−1.45	2.45	−1.05	−3.72	Interaction	No change
Avpr1a	1.06	-1.45	−1.08	1.42	Interaction	Interaction
Camk1	−1.14	−1.09	−1.51	−1.59	Genotype	Genotype and drug
Crem	1.01	−1.15	1.63	1.89	Genotype	Genotype & drug; interaction
Dhrs7	−1.03	−1.12	−1.44	−1.32	Genotype	Genotype
Gpr21	−1.02	−1.49	−1.14	1.28	Interaction	Genotype
Il10	1.02	−1.31	1.06	1.42	Interaction	Interaction
Il18	1.06	−1.06	−1.61	−1.43	Genotype	Genotype
Pecr	1.09	−1.05	−1.47	−1.28	Genotype	Genotype
Pomc1	−1.05	1.07	−1.55	−1.73	Genotype	No change
Sgk2	1.09	−1.65	1.09	1.95	Interaction	Genotype; interaction
Slc6a3 (DAT)	1.19	−1.34	1.22	1.94	Interaction	No change
Sln	1.05	−1.02	−3.80	−3.57	Genotype	Genotype & drug
Stat1	−1.02	−1.71	−1.02	1.64	Interaction	Interaction
Tnfrsf1b	−1.00	−1.88	−1.14	1.64	Interaction	Interaction
Zfp367	1.11	−1.17	−2.87	−2.23	Genotype	Genotype