Protracted abstinence from distinct drugs of abuse shows regulation of a common gene network

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ABSTRACT

Addiction is a chronic brain disorder. Prolonged abstinence from drugs of abuse involves dysphoria, high stress responsiveness and craving. The neurobiology of drug abstinence, however, is poorly understood. We previously identified a unique set of hundred mu-opioid receptor-dependent genes in the extended amygdala, a key site for hedonic and stress processing in the brain. Here we examined these candidate genes either immediately after chronic morphine, nicotine, Δ9-tetrahydrocannabinol or alcohol, or following 4 weeks of abstinence. Regulation patterns strongly differed among chronic groups. In contrast, gene regulations strikingly converged in the abstinent groups and revealed unforeseen common adaptations within a novel huntingtin-centered molecular network previously unreported in addiction research. This study demonstrates that, regardless the drug, a specific set of transcriptional regulations develops in the abstinent brain, which possibly contributes to the negative affect characterizing protracted abstinence. This transcriptional signature may represent a hallmark of drug abstinence and a unitary adaptive molecular mechanism in substance abuse disorders.

Keywords Abstinence, addiction, extended amygdala, gene expression, medium spiny neurons, mu-opioid receptor.

INTRODUCTION

Drug abuse is a complex relapsing brain disease (Everitt & Robbins 2005; Baler & Volkow 2006; Koob & Le Moal 2008). For an addicted individual, remaining drug-free for extended periods of time represents a major challenge (O’Brien 1997). Chronic exposure to drugs of abuse causes a broad spectrum of neural adaptations within reward pathways and associated neural circuits (Nestler 2001; Koob 2008). Drug-induced plasticity involves enduring modifications of neuronal connectivity, cell signaling and synaptic plasticity, which are orchestrated by transcriptional reprogramming (Rhodes & Crabbe 2005; McClung & Nestler 2008). These brain adaptations persist long after drug effects and acute withdrawal have dissipated, and contribute to relapse (Shalev, Grimm & Shaham 2002), so that full recovery from addiction is compromised (O’Brien 2008). Protracted abstinence from chronic drug use is characterized by lowered mood and a negative emotional state, whose molecular neurobiology has received little attention. Increased anxiety and stress responsiveness, as well as altered reward processing, have been reported in the clinic and in animal models (Koob & Le Moal 2008; Smith & Aston-Jones 2008; Stephens & Duka 2008). We recently demonstrated that morphine-abstinent mice develop low sociability and despair-like behavior. These modifications are not detectable 1 week after termination of the chronic drug exposure, but become remarkably strong after 4 weeks (Goeldner et al. 2011). Hence, persistent alterations develop and incubate within neural circuits that drive emotion-related behaviors. Pharmacological manipulations and cellular imaging have identified the extended amygdala (EA) (de Olmos & Heimer 1999) as a key brain site for such dysfunctions, and have implicated noradrenergic and corticotropin releasing...
factor (CRF) systems (Koob 2008; Smith & Aston-Jones 2008). Central EA, a cellular continuum formed by the central nucleus of the amygdala and the bed nucleus of the stria terminals (Alheid & Heimer 1988; Cassell, Freedman & Shi 1999), plays a major role in drug abuse and stress-induced drug seeking (Shaham et al. 2003; Waraczynski 2006; Koob 2009). Here, we focused on this particular brain structure to examine molecular adaptations associated with chronic drug exposure and drug abstinence.

Many neurotransmitter systems are involved in effects and adaptations to drugs of abuse, and regulate activity of the EA. The mu-opioid receptor is essential to mediate rewarding properties of most drugs of abuse, including drugs acting at non-opioid receptors (Contet, Kieffer & Befort 2004). As recreational drug use gradually evolves toward drug abuse, mu receptors are activated continually, a phenomenon that likely represents a first-line event in the development of addiction (Contet et al. 2004; Le Merrer et al. 2009). In a previous study (Befort et al. 2008), we used an open-ended approach to identify genes whose expression is specifically regulated upon excessive mu receptor activation in the EA. We identified a set of about a hundred genes, referred here as mu-opioid receptor-dependent genes, which represents a unique collection of promising candidates potentially involved in drug dependence and craving. In the present study, we tested whether these genes are regulated in response to chronic non-opioid drugs of abuse whose rewarding properties are strongly depending on the mu-opioid receptor, namely Δ9-tetrahydrocannabinol (THC), nicotine and alcohol. Importantly, we also examined whether regulations persist after protracted abstinence from these substances. We detected unforeseen molecular dysfunctions within the EA, and discovered a unique transcriptional signature of protracted abstinence.

The notion that most drugs of abuse, despite their distinct molecular targets, produce rewarding effects by jeopardizing dopaminergic mesolimbic circuits is largely accepted. However, the question of whether any unitary mechanism underlies the onset of addictive behaviors remains open. Here we provide first evidence that, at the molecular level, long-term adaptations occur whichever the drug, and may characterize drug abstinence.

**MATERIALS AND METHODS**

**Chronic drug treatments**

For all experiments, we used C57BL6/J male mice aged 8–12 weeks (Charles River, Lyon, France). Chronic morphine treatment was performed as described previously (Befort et al. 2008) with escalating doses of morphine (20–100 mg/kg, i.p.) over 6 days. Chronic nicotine treatment was performed using minipumps (Model 2001, Alzet®, Cupertino, CA, USA) as described previously (Berrendero, Kieffer & Maldonado 2002) with an average delivery of 25 μg/kg/day nicotine hydrogen tartrate salt over 6 days. Chronic THC treatment was performed as described previously (Castane et al. 2003) with administration of THC (20 mg/kg, i.p.) over 6 days. Chronic ethanol treatment was performed using alcohol vapor chambers with a delivery rate of ~76 ml/h for 72 hours (La Jolla Alcohol Research Inc., La Jolla, CA, USA) (Kang et al. 2004; Sanchis-Segura et al. 2007). After all chronic treatments, mice were either killed 20 minutes following the last morning drug administration (morphine and THC) or at the end of the treatment (nicotine, alcohol), or the animals were left 4 weeks free from drug. For nicotine-treated group, mice were withdrawn from their minipump under brief anesthesia and left 4 weeks free from drug (see Supporting Information Appendix S1).

**Drug dependence and withdrawal**

Independent groups of animals were used to evaluate physical dependence following chronic drug treatments (for details, see Supporting Information Appendix S1).

**Real-time quantitative polymerase chain reaction (pPCR) analysis**

For each drug, four independent chronic treatments were performed and tissue samples were dissected immediately at the end of the treatment (chronic condition, two experiments) or 4 weeks after (abstinent condition, two experiments). As previously described (Befort et al. 2008; Contet et al. 2011), mice were killed by cervical dislocation; brains were removed and were placed into a matrix with 1 mm division (ASI Instruments Inc., Warren, MI, USA) for slicing. Bilateral punches were then taken (Paxinos & Franklin 2001) from the bed nucleus of stria terminals and central nucleus of the amygdala, and pooled to form central EA samples. EA samples from 12 to 18 mice were pooled for each condition and a cDNA supermix was synthesized from total RNA with anchored-oligo-dT primer and random hexamer using Superscript II (see details in Supporting Information Appendix S1). For each treatment, real-time pPCR was performed on two cDNA supermix preparations and in triplicate on either a MyIQ (Bio-Rad, Marnes-la-Coquette, France) or a LC480 (Roche) detection system using IQ SYBR Green supermix (Bio-Rad), starting with 0.5 or 0.25 μl cDNA in a 25 or 12.5 μl final volume, respectively. Gene-specific primers were designed (see Supporting Information Appendix S1 and Supporting Information Table S1 for primer sequences). Relative
expression ratios were normalized to the level of rplp0 as the reference gene, and the $2^{-\Delta\Delta Ct}$ method was applied to evaluate differential expression level (see Supporting Information Appendix S1) (Livak & Schmittgen 2001).

Statistics
As described previously (Contet et al. 2011), quantitative pPCR (qPCR) data were transformed prior to statistical analysis to obtain a symmetrical distribution centered on 0 using the following formula: if $x < 1$, $y = 1 - 1/x$; if $x > 1$, $y = x - 1$ (x: qPCR data; y: transformed data). A one-sample t-test was then performed to assess their statistical significance. Calculated $P$ values indicated probability for a regulation to differ from 0.

Clustering analysis
Unsupervised clustering analysis was performed on the median of transformed qPCR data for all of the 97 genes of our collection using complete linkage with correlation distance (Pearson correlation) for genes and experimental conditions (Cluster 3.0 and Treeview software) (Eisen et al. 1998; de Hoon et al. 2004).

Principal component analysis (PCA)
A standard PCA was used to reduce the dimensionality of our data set (transformed expression data for all genes) and allow better visualization and identification of meaningful underlying variables (see Supporting Information Appendix S1) (Jolliffe 2004).

Ingenuity pathway analysis (IPA)
We performed an IPA (Ingenity® Systems, Redwood City, CA, USA; http://www.ingenuity.com) to identify signaling pathways underlying converging gene expression patterns within each cluster. Networks were selected based on their ingenuity score and the regional brain expression of their node. Additionally, we performed a bibliographic screen to validate relationships detected by the IPA and to connect unclassified genes to identified networks. The validity of IPA results was tested using a permutation analysis (see Supporting Information Appendix S1).

RESULTS
Mice were exposed to a chronic morphine, nicotine, THC and alcohol regimen. For each drug condition, one group of saline and drug-injected animals was used to confirm that dependence had established at behavioral level (Supporting Information Fig. S1), and two other independent groups were examined for gene expression either immediately at the end of the chronic drug regimen (chronic), or 4 weeks later (abstinent) (Goeldner et al. 2011). We quantified expression levels of 97 mu-dependent genes (Befort et al. 2008) by qPCR (Supporting Information Tables S1 and S2) in microdissected EA samples (Supporting Information Appendix S1) as described previously (Contet et al. 2011).

qPCR data for the four drugs and at the two time points revealed a widely diverse set of gene expression patterns, as illustrated in Fig. 1 (see also Supporting Information Table S2). Bok and Ramp3 are examples of genes that were regulated in chronic conditions only, but their expression normalized after 4 weeks. Cbhn1 and Crh displayed detectable regulation after protracted abstinence, whether or not a regulation was observed immediately after the chronic treatment. This finding is particularly relevant for Crh, encoding a well-known stress mediator, whose blockade reduces drug-seeking behaviors specifically in drug-dependent animals (Koob 2008; Smith & Aston-Jones 2008). Finally, a number of genes showed drug-specific regulations, such as Slc17a and Cck.

We then analyzed regulations of the 97 genes overall for each drug treatment using a PCA (Fig. 2a; Table 1). In chronic conditions, morphine and nicotine-induced regulations shared a positive correlation with a first principal component (PC1) accounting for 47.2% of variance in expression data. Ethanol-induced regulations opposed nicotine-induced regulations, and loaded negatively on PC1. THC- and morphine-induced regulations correlated negatively with PC2 (26.6% variance). The orthogonal position of THC, respective to nicotine and ethanol, indicates highly dissimilar regulation of gene expression for this drug. In sharp contrast, PCA from abstinent groups revealed very close positions for all drug-induced regulations that loaded highly and positively on main component PC1 (57.0% variance). These data suggest that, immediately after chronic treatment, each drug produces distinct sets of genetic regulations. However, these regulations converge toward a remarkable degree of similarity 4 weeks posttreatment.

In order to identify groups of genes sharing similar regulation profiles across the four drugs, we performed a clustering analysis of all gene expression data in either chronic or abstinent condition (Fig. 2b, details in Supplementary Information Fig. S2). In the chronic condition, cluster analysis organized expression data in eight main clusters. Notably, cluster C2 showed gene down-regulation that was specific to morphine and nicotine treatments, and opposed up-regulation after ethanol. The C4 cluster gathered genes with down-regulated expression after morphine and THC. Clusters C7 and C8 grouped genes whose expression was up-regulated after chronic morphine, and not by other drugs. In the abstinent condition, cluster analysis highlighted six novel main clusters. Cluster A5 was most remarkable. In this
cluster, genes displayed highly similar patterns of expression across drug treatments, with a strikingly homogenous down-regulation for the subcluster A5b. This latter finding indicates that protracted abstinence results in a set of long-term regulations, some of which are comparable regardless of the drug treatment. Collectively, therefore, PCA and clustering analysis reveal the existence of a common transcriptional signature of protracted abstinence in the EA circuitry.

We next examined whether regulation patterns may reflect modifications within shared molecular networks. We performed an IPA for the 97 genes. IPA describes

Figure 1 Quantitative polymerase chain reaction analysis for all the drugs in chronic and abstinet conditions reveals a wide diversity of gene expression patterns. This figure shows examples of genes whose expression was regulated (a) in chronic conditions only (b) after protracted abstinence, whether or not a regulation was observed immediately after chronic treatment (c) in a drug-specific manner. The asterisk indicates significant regulations (see Supporting Information Table S2)
functional relationships between gene products based on interactions described in the literature. Four networks recruited most genes: Creb/ERK, NFkB complex, glutamate/corticoid and huntingtin (HTT) pathways (Table 2 and Fig. 3a). Using the same PCA as described earlier, we analyzed regulations of each individual gene across drug treatments. This analysis revealed that genes belonging to a same network occupy close positions in the 2-D space defined by PC1 and PC2 (Supporting Information Fig. S3 and Table S3). To assess relative weights of each network in contributing to variance in expression data, we plotted the median coordinates of genes belonging to the same network (Fig. 3b, Table 3 and Supporting Information Table S3). In chronic conditions, three networks correlating with PC1 (glutamate/corticoid, Creb/ERK and HTT) contributed to data variance. In abstinent conditions, the HTT network correlated highly and negatively with PC1, and accounted for most of the variance in gene expression. Therefore, this network represents the likely main contributor to the transcriptional signature of protracted abstinence. Remarkably, eight out of the nine genes in cluster A5b belong to this network. Therefore, both IPA and hierarchical clustering independently identify the same gene network.

Table 1 Component loadings of drug-induced regulations for each drug condition across the 97 genes.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
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<tbody>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Morphine</td>
<td>0.53</td>
<td>-0.55</td>
<td>0.65</td>
<td>0.00</td>
</tr>
<tr>
<td>Nicotine</td>
<td>0.88</td>
<td>-0.01</td>
<td>-0.29</td>
<td>-0.39</td>
</tr>
<tr>
<td>THC</td>
<td>-0.24</td>
<td>-0.87</td>
<td>-0.43</td>
<td>0.05</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-0.89</td>
<td>-0.10</td>
<td>0.22</td>
<td>-0.40</td>
</tr>
<tr>
<td>% Variance explained</td>
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<td>26.58%</td>
<td>18.49%</td>
<td>7.74%</td>
</tr>
<tr>
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<tr>
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<td>-0.47</td>
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<tr>
<td>Nicotine</td>
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<td>0.15</td>
<td>0.21</td>
<td>0.51</td>
</tr>
<tr>
<td>THC</td>
<td>0.74</td>
<td>-0.29</td>
<td>-0.61</td>
<td>0.01</td>
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<tr>
<td>Ethanol</td>
<td>0.72</td>
<td>0.61</td>
<td>-0.03</td>
<td>-0.32</td>
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<tr>
<td>% Variance explained</td>
<td>57.05%</td>
<td>17.71%</td>
<td>14.46%</td>
<td>10.78%</td>
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</table>

Component loadings (variable space) over 0.7, considered most significant, are displayed in bold. Such high loadings are observed on PC1 and PC2 only. Percent of total variance explained by each component is shown at the bottom for each condition. Data for PC1 and PC2 are plotted in Fig. 2a.

THC = Δ9-tetrahydrocannabinol.
This study was focused on a collection of candidate mu-opioid receptor-dependent genes potentially involved in drug dependence and craving. We examined transcriptional regulations of these genes within the EA in response to chronic exposure to four distinct drugs of abuse. Our data show highly divergent gene expression profiles across drugs at the end of the chronic regimen.

Table 2 Distribution of mu-opioid receptor-dependent genes across four main signaling networks and cellular expression.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>HTT</th>
<th>Glu-Cort</th>
<th>Creb/ERK</th>
<th>NFkB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>Adora2a</td>
<td>Slc17a6</td>
<td>Camk2a</td>
<td>Gal</td>
</tr>
<tr>
<td></td>
<td>Arpp21</td>
<td>Ramp3</td>
<td>Crh</td>
<td>Fxsg7</td>
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<td></td>
<td>Bcl11b</td>
<td>Cbmx1</td>
<td>Gabrg2</td>
<td>Agg4</td>
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<tr>
<td></td>
<td>Cnr1</td>
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<td></td>
<td>Drd1a</td>
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<td></td>
<td>Fam40b</td>
<td></td>
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<td></td>
<td>Foxp1</td>
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<td></td>
<td>Gpr88</td>
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<tr>
<td></td>
<td>Hpc1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Neurons and astrocytes</td>
<td>Fam107a</td>
<td>Ntng1</td>
<td></td>
<td>Csdc2</td>
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<tr>
<td></td>
<td>Gent2</td>
<td>Hsd11b1</td>
<td></td>
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<tr>
<td>Neurons and oligodendrocytes</td>
<td>St8sia3</td>
<td>Tnem163</td>
<td>Plcb4</td>
<td>Serpin1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sdc4</td>
<td>Serpin1a</td>
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<td>Glap</td>
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<td>Btd11</td>
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<td>Astrocytes</td>
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<td>Cdk2</td>
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<tr>
<td>Oligodendrocytes</td>
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<tr>
<td>All cell types</td>
<td>Nrx4a1</td>
<td>Sgk1</td>
<td>Ctbhp</td>
<td>Nrn1</td>
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<tr>
<td></td>
<td>Pde10a</td>
<td>Prkcd</td>
<td>Dusp6</td>
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<td></td>
<td></td>
<td>Plek1g1</td>
<td>Eg1</td>
<td>Gng11</td>
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<td></td>
<td>Lrin7c</td>
<td>Eg3</td>
<td>Dhrc24</td>
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<td></td>
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<td>Inadl</td>
<td>Hnml</td>
<td>Cck</td>
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<td></td>
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<td>Homer1</td>
<td>Jnb</td>
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<td>Grm5</td>
<td>Lpl</td>
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<td></td>
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<td>Dl16a</td>
<td>Pp3r1</td>
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<td>Cr3ab</td>
<td>Syt9</td>
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<td>Bok</td>
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In the abstinent condition, and regardless of the drug of abuse, most variability of gene expression was the result of genes belonging to the huntingtin (HTT)-centered network, a novel signaling pathway in drug abuse research. The three other pathways were related previously to several aspects of addictive behaviors, including stress responses (glutamate/corticoids), neuronal signaling (Creb/ERK) and inflammation (NFkB). Expression of most genes in the HTT-centered network is enriched in neurons (Cahoy et al., 2008), and all genes (at the exception of St8sia3, Fam40b and Fam107a) belonging to the HTT network have been shown expressed in striatal GABAergic medium spiny neurons. In contrast, more than half of genes belonging to the NFkB pathway display specific enriched expression in glial cells, consistent with a role for this network in inflammatory processes (Ouimet, Hemmings & Greengard 1989; Gerfen 1992; Surmeier, Song & Yan 1996; Blain et al. 1999; Marsicano & Lutz 1999; Mercer et al. 2000; Paterlini et al. 2000; St-Hilaire et al. 2003, 2005; Blain et al. 2004; Tamaura et al. 2005; Kuhn et al. 2006; Ciruela et al. 2006; Ito et al. 2006; Burre, Zimmermann & Volknandt 2007; Kuhn et al. 2007; Menmiti et al. 2007; Yau et al. 2007; Arlotta et al. 2008; Artero-Castro et al. 2009; Chen et al. 2009; Fernandez et al. 2009; Goudreault et al. 2009; Healy & O’Connor 2009; Massart et al. 2009; Ramia & Kreydyjeych 2009). Thirty-two genes (Sept8, Ak5, Ankk1, Atrx, Bcn1, Cdk136, Cntn5, Gpr125, Gpr6, Gpr55, Igf1, Lmo7, Mctp1, Nrx3, Opalin, Pdub, Pike, Pllp, Pr18, Rem2, Rph5a, Sertad4, Shox2, Sla5, Sncr1, Tnnc1, Tnnc2, Tnnc5, Tnnc7, Tnnc15, Tnnc17, Tnnc18, Tnnc19, Tnnc20, Tnnc21, Tnnc22, Tnnc23) could not be included in any molecular network. Pathways were identified using ingenuity pathway analysis, combined with a review of literature.

**DISCUSSION**

This study was focused on a collection of candidate mu-opioid receptor-dependent genes potentially involved in drug dependence and craving. We examined transcriptional regulations of these genes within the EA in response to chronic exposure to four distinct drugs of abuse. Our data show highly divergent gene expression profiles across drugs at the end of the chronic regimen. Transcriptional regulations, however, converge toward a strikingly similar pattern after a 4-week drug-free period, revealing regulation of a common molecular network.

IPA using the set of 97 mu-receptor-dependent genes led to identify a combination of four molecular networks. The Creb/ERK and glutamate/corticoid networks have been largely implicated in responses to drugs of abuse (Nestler 2005; Kauer & Malenka 2007; Briand & Blendy 2009; Kalivas 2009; Uhart & Wand 2009). Acute and
chronic drug exposure both produce transcriptional regulations in these molecular pathways at the level of hypothalamus, striatum and EA (Ahmed et al. 2005; McBride et al. 2010; Piechota et al. 2010). The third molecular network involves NFkB, a transcription factor regulating the development of dependence to opiates and alcohol (Capasso 2001; Okvist et al. 2007; Rehni et al. 2008) and addiction-induced plasticity (Russo et al. 2009). Most importantly, our study reveals an unforeseen HTT-centered molecular network, which appears to be the main contributor to transcriptional modifications observed in the abstinent condition. In contrast to glutamate/corticoids, and the Creb/ERK and NFkB pathways, the HTT-centered pathway (Table 2 and Fig. 3a) has not been described earlier in addiction research. Several genes from this pathway encode G protein-coupled receptors and associated proteins involved in cell signaling (Adora2a, Cnr1, Drd1, Gpr88, Pde10a, Arpp21, Fam40b, Hpca). Among these, Drd1a, Cnr1 and Adora2a encode receptors notoriously involved in addictive behaviors (see, for example, Maldonado, Valverde & Berrendero 2006; Brown & Short 2008; Le Foll et al. 2009), and Arpp21 is proposed to modulate psychostimulant effects (Caporaso et al. 2000). Other cell-signaling genes of this network have received little attention in the context of drug abuse. Of particular interest is Gpr88, encoding an orphan G protein-coupled receptor strongly and specifically expressed in limbic areas (Becker et al. 2008; Massart et al. 2009). Gpr88 was recently proposed as a promising target to treat schizophrenia (Logue

Figure 3. A huntingtin (HTT)-centered network highly contributes to the variance of gene expression after protracted abstinence. (a) The HTT-centered network. Eight out of nine genes clustered in A5b (Fig. 1b) belong to this network (highlighted in blue). Dashed line = indirect relationship; solid line = direct relationship. (b) Principal component analysis (subject space); median and standard error of coordinates from genes belonging to the four main signaling pathways were plotted in the 2-D space defined by PC1 and PC2, as in Fig. 1a, for each condition. The HTT-centered network explained most of the variance in gene regulation.
Table 3 Median component loadings of drug-induced regulations for each of the four molecular networks identified by ingenuity pathway analysis across the four drug treatments.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HTT</td>
<td>0.51 ± 0.11</td>
<td>-0.93 ± 0.13</td>
<td>0.37 ± 0.13</td>
<td>0.21 ± 0.11</td>
</tr>
<tr>
<td>Glutamate/corticoids</td>
<td>-0.76 ± 0.21</td>
<td>0 ± 0.29</td>
<td>-0.43 ± 0.17</td>
<td>0.17 ± 0.16</td>
</tr>
<tr>
<td>Creb/ERK</td>
<td>0.55 ± 0.20</td>
<td>-0.23 ± 0.23</td>
<td>0.38 ± 0.38</td>
<td>0.09 ± 0.19</td>
</tr>
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<td>Nfkb</td>
<td>-0.06 ± 0.09</td>
<td>0.83 ± 0.16</td>
<td>0.06 ± 0.11</td>
<td>0.07 ± 0.10</td>
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<tr>
<td>% Variance explained</td>
<td>47.19%</td>
<td>26.58%</td>
<td>18.49%</td>
<td>7.74%</td>
</tr>
<tr>
<td>Abstinent</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTT</td>
<td>-1.61 ± 0.16</td>
<td>0.44 ± 0.25</td>
<td>-0.29 ± 0.31</td>
<td>0.42 ± 0.15</td>
</tr>
<tr>
<td>Glutamate/corticoids</td>
<td>0.40 ± 0.18</td>
<td>0.30 ± 0.17</td>
<td>0.13 ± 0.14</td>
<td>-0.40 ± 0.11</td>
</tr>
<tr>
<td>Creb/ERK</td>
<td>-0.84 ± 0.20</td>
<td>-0.57 ± 0.19</td>
<td>0.23 ± 0.15</td>
<td>-0.22 ± 0.19</td>
</tr>
<tr>
<td>Nfkb</td>
<td>0.63 ± 0.19</td>
<td>-0.57 ± 0.16</td>
<td>-0.25 ± 0.15</td>
<td>-0.07 ± 0.13</td>
</tr>
<tr>
<td>% Variance explained</td>
<td>57.05%</td>
<td>17.71%</td>
<td>14.46%</td>
<td>10.78%</td>
</tr>
</tbody>
</table>

Component loadings (subject space) over 0.7 (absolute value), considered as most significant, are displayed in bold. Such high loadings are observed on PC1 and PC2 only. Percent of total variance explained by each component is shown at the bottom for each condition. Data for PC1 and PC2 are plotted in Fig. 3b. HTT = huntingtin.

et al. 2009), and our data extend the therapeutic potential of GPR88 to other areas of psychiatric disorders. The HTT-centered network also includes Foxp1, Nr4a1 and Bcl11b, three genes encoding transcription factors that were previously involved in dopamine-related neuroadaptations (Tamura et al. 2004; Gilbert et al. 2006; Arlotta et al. 2008). Altogether, these genes represent potential key players of drug-induced genetic reprogramming within the EA during protracted abstinence.

A majority of genes from the HTT-centered pathway are documented as expressed in striatal medium spiny neurons (Table 2). Similar medium-sized GABAergic spiny neurons are present in the EA, which integrate complex converging information from cortical glutamatergic innervation and hindbrain monoaminergic (noradrenaline, dopamine, serotonin) and peptidergic (CRF, galanin, opioids) afferences (Hokfelt et al. 1999; Hasue & Shammah-Lagnado 2002; McDonald 2003; Poulin et al. 2006; Koob 2008; Smith & Aston-Jones 2008). Long-term modifications within these neurons most likely represent critical events in the development of drug craving and relapse (Koob and Le Moal, 2008; Smith & Aston-Jones 2008). Our data show that most genes from the HTT-centered pathway are down-regulated in the abstinent condition. This observation suggests that altered function of medium spiny neurons has developed in EA. Future functional studies of both these neurons and candidate genes will determine whether deficient signaling within EA indeed underlies negative emotional aspects of protracted abstinence, and will identify key molecular actors within the HTT-centered pathway. Notably, regulations of HTT-centered pathway genes may also occur in alternative brain circuits, including ventral and dorsal striatum (Kalivas & Hu 2006), and influence other aspects of drug dependence and abstinence.

Whether addiction could be considered a unitary disorder is debated (Nestler 2005). All drugs of abuse, regardless of their target receptor, produce their acute rewarding effects through converging modulation of the reward circuitry in midbrain and ventral forebrain. Further, chronic exposure to most drugs of abuse triggers circuit-level adaptations, which all lead to modified aminergic function and hyperactive CRF systems. At the molecular level, the transcription factors ΔFosB (Nestler 2005) and Creb (Briand & Blendy 2009) have been identified as general regulators in drug dependence. Drug abstinence has been little studied at the transcriptome level (Spijker et al. 2004; Kuntz-Melcavage et al. 2009), and the identification of gene regulation patterns, which pertain or develop after prolonged abstinence from distinct drugs of abuse, remains an open research field. This study demonstrates that four drugs of abuse produce common transcriptional changes. The regulation pattern is undetectable immediately after chronic treatment, emerges after a 4-week abstinence period and reveals a newly described HTT-centered network. This molecular signature may represent another unitary mechanism, which characterizes drug abstinence and may underlie emotional alterations associated to addictive disorders.

Ultimately, these genes will be useful in the clinic. Single nucleotide polymorphism (SNP) association studies have linked variants in two genes from the identified network, Drd1 and Cnr1, with drug dependence (Zuo et al. 2007; Batel et al. 2008; Huang et al. 2008). Moreover, meta-analysis of genome-wide association studies

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revealed associations among Fam107, Foxp1, Drd1, Bcl11 and Arpp21 loci with major mood disorders (Mitchell et al. 1992; Hosoki et al. 2009; McMahon et al. 2010; Secolin et al. 2010). Future studies considering the entire HTT-centered gene network may set the bases for novel research on addiction susceptibility genes, and provide novel target opportunities for the treatment of substance abuse.

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Authors Contribution

JLM, KB and BLK conceived and designed the experiments. JLM, KB, OG, ED and DF performed the experiments. JLM, KB, DF, OG, DD and JAJB analyzed the data. JLM, KB, JAJB and BLK wrote the article. BLK is the principal investigator. All authors have critically reviewed content and approved final version submitted for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Chronic treatment by each drug induces physical dependence. Mice were injected with morphine and THC after a habituation period, received nicotine via osmotic minipumps or inhaled alcohol in vapor chambers.

Figure S2 Hierarchical clustering analysis of data from chronic and abstinent groups was performed to compare the four drug treatments (Cluster 3 and Treeview software). In abstinent condition, all the drugs produced down-regulation of genes in subcluster A5b. Clusters: chronic (C1–C8); abstinent (A1–A6). E: ethanol; M: morphine; N: nicotine; T: THC. Light to dark red: low to high up-regulation, light to dark blue: low to high down-regulation. Critical correlation nodes are indicated for each condition.

Figure S3 Principal component analysis (subject space) shows how genes belonging to the same signaling network cluster in the same area of the 2D space defined by PC1 and PC2 (see supporting Information Appendix S1). The component loadings of drug-induced regulations are presented for each gene across drug treatments. Median and standard error of coordinates from genes belonging to the four main identified networks (huntingtin, Creb/ERK, glutamate/corticoid, NFkB) are displayed in Fig. 3b.

Table S1 List of primers used for qPCR.
Table S2 Regulations of mu-opioid receptor-dependent genes in chronic and abstinent conditions are expressed as fold-change drug versus control group (median ± SEM).

Table S3 Principal component analysis: component loadings of drug-induced regulations for each individual gene across the four drug treatments (subject space). Component loadings over 0.7 (absolute value), considered as most significant, are displayed in bold. Percent of total variance explained by each component is shown at the bottom for each condition. Data for PC1 and PC2 are plotted in Fig. S3.

Appendix S1 Supplementary Methods.

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