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Increased expression of protein kinase A inhibitor α (PKI- α) and decreased PKA-regulated genes in chronic intermittent alcohol exposure.

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Abstract

Intermittent models of alcohol exposure that mimic human patterns of alcohol consumption produce profound physiological and biochemical changes and induce rapid increases in alcohol self-administration. We used high-density oligonucleotide microarrays to investigate gene expression changes during chronic intermittent alcohol exposure in three brain regions that receive mesocorticolimbic dopaminergic projections and that are believed to be involved in alcohol's reinforcing actions: the medial prefrontal cortex, the nucleus accumbens and the amygdala. An independent replication of the experiment was used for RT-PCR validation of the microarray results. The protein kinase A inhibitor α (PKI- α , *Pkia*), a member of the endogenous PKI family implicated in reducing nuclear PKA activity, was found to be increased in all three regions tested. Conversely, we observed a down-regulation of the expression of several PKA-regulated transcripts in one or more of the brain regions studied, including the *activity and neurotransmitter-regulated early gene* (*Ania*) -1, -3, -7, -8, the transcription factors *Egr1* and *NGFI-B* (*Nr4a1*) and the neuropeptide *NPY*. Reduced expression of PKA-regulated genes in mesocorticolimbic projection areas may have motivational significance in the rapid increase in alcohol self-administration induced by intermittent alcohol exposure

Drug addiction is a chronic, relapsing disorder that results from the prolonged effects of drugs on the brain [32]. Both long-lasting maladaptive changes and conditioning contribute to continued drug use and relapse after cessation [30,65]. The positive affective state (e.g. euphoria) induced by alcohol and other drugs of abuse produces the positive reinforcement that motivates occasional use [29,30]. However, it is the relief from the negative emotional symptoms (e.g. dysphoria, anxiety, irritability, craving) induced by chronic use that is believed to be key in continued use [29,30]. The emergence of this negative reinforcement has been associated with the transition from occasional drug use to addiction [28,29].

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Behind it are believed to be functional changes in the brain reward, stress and motivation neurocircuitry that develop during dependent drug intake [25,28,30]. The elucidation of these processes will require a quantitative understanding of the many interconnected molecular networks that govern the neuronal pathways involved as well as the progression of their dysregulation during the transitions from drug-naïve to occasional use, and from occasional use to dependent use.

In the present study, we investigated gene expression changes induced by chronic intermittent alcohol exposure in three brain regions that are believed to be involved in alcohol's reinforcing actions, the medial prefrontal cortex (MPF, regions Cg1-3) [45], the nucleus accumbens (NAc) and the amygdala (AMY). Intermittent models of alcohol exposure mimic human patterns of alcohol consumption and have been shown to induce long-lasting physiological and biochemical changes [5,10,41,53,58,59]. Intermittent exposure to alcohol also induces more rapid increases in self-administration of alcohol relative to continuous exposure. In fact, work in this department showed that 4 to 6 weeks of continuous alcohol vapor are necessary to induce significant increases in operant self-administration of alcohol during withdrawal [48] while a considerably shorter time (2 weeks) of intermittent vapor is sufficient [39]. This suggests that intermittent exposure may be more efficient in producing the neuroadaptations that are believed to be responsible for the development of excessive alcohol intake [39,53].

Male Wistar rats (200 grams at the beginning of the study) n=6 per condition were exposed to alcohol vapors for two weeks with an intermittent schedule of 14 hrs exposure and 10 hrs off [39,51,53]. Daily peak blood alcohol levels (BAL) were maintained at around 200-220 mg%. For BAL determination, serum was collected biweekly by tail-bleed. Alcohol content was determined by the core facility of the TSRI Alcohol Research Center using the NAD-NADH spectrophotometric method (Sigma Biochemicals). Rats were anesthetized by CO₂ narcosis and sacrificed either at the end of the last alcohol exposure period or following 1 week of withdrawal. Two sets of control rats (n=6) were sacrificed along with the two groups of alcohol-dependent and withdrawal rats alternating between control and alcohol-treated rats. Brains were manually dissected from macroslices as previously done [3,52,53] with the assistance of the Paxinos and Watson brain atlas [45]. Briefly, a 14 gauge needle was used to collect the NAc, while the MPF and the AMY were dissected free-handedly using established anatomical landmarks [45].

Affymetrix Rat Neurobiology Arrays were used for gene expression analyses and processed as previously described [3]. Samples from each experimental group were pooled and run in duplicate. Signal intensities were scaled to a target intensity of 250 using the MAS 5.0 algorithm. A list of differentially expressed genes was generated by the consensus of two analysis strategies, the Affymetrix *Comparison Analysis Algorithm* (CAA) and t-test analysis of GeneSpring 7.2-normalized expression values. CAA utilizes a robust normalization that corrects for the sequence-dependent probe set characteristics of each probe pair in the probe set – e.g. affinity and linearity of hybridization - and compares individual probe pairs of the experimental and control conditions [2]. CAA generates a change p-value based on a Wilcoxon's Signed Rank test that is used to determine the change direction and significance for every probe set. For t-test analysis, signal intensities were

normalized across the experiment using the perGene normalization algorithm in GeneSpring 7.2. Each chip was further normalized to 1 using the perChip normalization algorithm also in GeneSpring 7.2. Based on metaanalyses of a large microarray data set that was extensively RT-PCR validated [3], the following filters were applied: genes labeled *absent* in all chips by the MAS 5.0 detection call algorithm were filtered out in both analysis strategies; genes that did not display an average expression level of at least 100 in at least one condition (after scaling to a target intensity of 250 but without any further normalization) were also excluded; a fold-change cut-off of 1.8 (equivalent to 0.85 for CAA-generated values) was then applied on each set of normalized expression values; and, lastly, a $p < 0.05$ was used to define significance of change in the t-test analysis. Results were validated by RT-PCR in individual animals from an independent replication of the experiment ($n=6$). In these animals, MPF, NAc and AMY from one side at random were collected for RNA expression, while the opposite side was used for measurements of dopamine and its metabolites. Primer design and amplicon selection for RT-PCR were done with the Beacon Designer Software (Premier Biosoft International, Palo Alto, CA). The occurrence of non-specific amplification products, such as primer-dimer formation, was checked by melt-curve analysis for each pair of primers. The iQ SYBR Green Supermix (BioRad, Hercules, CA) was used in 25 μ l reaction volume with a iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA) using 0.2 ml 96-well thin-wall PCR plates from Bio-Rad (Hercules, CA). The relative amounts of mRNA were normalized to β -actin. Sequences of primers used for RT-PCR are shown in Table 1. Dopamine and its metabolites, 3,4-dihydroxyphenyl acetic acid (DOPAC) and homovanilic acid (HVA) were measured by HPLC as previously described [23]. The ratio of DOPAC to dopamine (DOPAC/DA) and HVA levels were used as indicators of dopamine turnover [23,40,68,69].

Scatter plots of microarray expression values from the three brain regions of chronic alcohol-treated rats plotted against controls are shown in Fig. 1A. Of the 3 brain regions tested, the MPF was the most transcriptionally responsive to both alcohol dependence induced by chronic intermittent alcohol exposure and withdrawal (Fig. 1B,C).

Expression of PKI- α (Figs. 1C, 2), a member of the protein kinase A inhibitor (PKI) family of proteins that act as pseudosubstrates for PKA to inhibit PKA catalytic subunits [27] was found to be increased in all 3 brain regions tested (in the NAc, PKI- α increase only approached significance in the microarray analysis, but was found to be significantly increased by RT-PCR). PKI family members have been implicated in terminating the nuclear actions of PKA on gene expression [11]. Alcohol has been shown to induce translocation of PKA to the nucleus, cAMP response element-binding protein (CREB) phosphorylation, and cAMP response element-mediated gene transcription both *in vitro* [13,14] and *in vivo* [4]. Acutely, alcohol also reduces PKI's inhibitory activity of PKA catalytic subunits [13]. Therefore the increased PKI- α expression seen here *in vivo* may be a compensatory response to chronic alcohol exposure.

Concomitantly with PKI- α increase, various genes that are regulated by receptors that activate the cAMP-PKA pathway were found to be decreased. Among them were three members of a group of dopamine-regulated transcripts, activity and neurotransmitter-regulated early genes (Ania) [6], that were found to be decreased in the MPF and NAc (Figs.

1C, 2). In particular, as shown in Figs. 1C and 2, transcripts for Ania-1, -3, -7 and -8 were significantly decreased in the MPF and Ania-1 and -3 in the NAc (reductions of Ania-1 in the MPF and NAc and Ania-8 in the MPF only approached significance in the microarray analysis, but these transcripts were significantly reduced by RT-PCR).

Ania represent a heterogeneous group of approximately 30 transcripts originally identified by differential display as induced in the striatum by agonists of the G_S-coupled dopaminergic D₁ receptors in 6-hydroxydopamine (6-OHDA)-treated animals [6]. While the functions of most Ania remain to be defined, Ania-3 has been shown to be an inducible short splice variant of Homer1, a member of the Homer family of scaffolding proteins [8,57]. Long splice variants of Homer1 determine dendritic localization of group I mGluRs and receptor clustering, while short splice variants act as dominant negatives, producing opposite effects on mGluR distribution and function [8,26]. Ania-3 has also been shown to bind the plasma membrane Ca²⁺ ATPase (PMCA) via its PDZ domain-binding COOH-terminal tail [57]. Ania-1, -7, -8, are dramatically inducible short transcripts that lack close matches to mouse and human expressed sequences and might represent non-coding RNAs [6]. Only a subset of Ania was found to be changed in the present study, possibly reflecting previously observed differences in their regulations [6,7,38,56,57].

The expression of the immediate-early gene of the zinc finger family Egr1 (also known as NGFI-A, zif268, or Krox 24) was also reduced in the MPF and AMY (Fig. 1C, 2). Expression of Egr1 in the developing striatum has been shown to be induced by dopaminergic afferents acting on D1 receptors [24,60]. Egr1 is also induced in developed animals by D1 dopamine activation [1,24,61] and is required for behavioral sensitization to repeated cocaine [61]. NGFI-B (Nr4a1), another gene regulated by dopamine, among other stimuli, [12] was also reduced in both the MPF and NAc by chronic intermittent alcohol exposure (Fig. 2). Microarray analyses also showed decreased expression of the neuropeptide NPY in the MPF by chronic intermittent alcohol exposure (Fig. 1C, 2). NPY biosynthesis has also been shown to be under the influence of dopamine [49]. Reduced NPY biosynthesis has also been observed after repeated cocaine administration in brain areas innervated by the mesocorticolimbic dopaminergic system, including the MPF [63].

Gene expression changes observed in the present study included GABA_A genes previously shown to be transcriptionally responsive to alcohol. In agreement with previous reports, despite differences in alcohol exposure paradigms, time of sacrifice, and anatomical dissections, we observed increased GABA_A-β3 (Gabbr3) expression in the MPF and AMY (Fig. 2). The expression of the GABA_A-β3 was previously shown to be increased in the cerebral cortex of chronically alcohol-exposed rats, and is persistently elevated during withdrawal [35]. The β3 subunit was also found to be increased in the frontal cortex of human alcoholics in post-mortem samples [37]. The GABA_A subunit α1 (Gabra1) was increased in the AMY of dependent rats (Fig. 2). Previous studies did not address changes in this GABA_A subunit in the amygdala, but did show decreased α1 subunit mRNA and peptide content in whole brain samples or in the cortex [9,15,36]. Upregulation of various GABA_A α subunits – although not α1 – was seen during alcohol withdrawal in an *in vitro* setting [50]. Increased α1 subunit mRNA was also seen in the amygdala of rats selectively bred for reduced temporal lobe excitability after seizure induction with kainate [21] and it

may reflect an adaptation to increased excitability induced by the intermittent alcohol treatment in the present study. The GABA_A $\gamma 2$ (Gabrg2) subunit was also increased as previously shown during withdrawal in another intermittent alcohol exposure paradigm [10]. The mRNA for AMPA glutamate receptor subunit 2 (GluR2) gene (Gria2) was increased in the MPF (Fig. 2). Cortical induction of the AMPA GluR2 mRNA was previously seen during protracted withdrawal in another intermittent model of alcohol exposure [46]. Thus, similarly to the dopamine-regulated genes described above, changes in the expression of GABA_A and AMPA subunits at the end of alcohol exposure, appear more consistent with changes previously shown during withdrawal than with alcohol dependence.

The increased PKI- α expression and the coordinated down-regulation of genes whose expression is positively modulated by the cAMP-PKA pathway in the present study suggest changes in PKI- α as a neuroadaptive consequence of alcohol dependence. Functional changes in the cAMP-PKA pathway have been observed after chronic exposure to various drugs of abuse [54,55]. Activation of CREB-mediated transcription by acute ethanol exposure *in vivo* was recently shown using CRE-lacZ transgenic mice in various brain regions including the NAc, PFC and AMY [4]. Additionally, upregulation of the cAMP-PKA pathway has been demonstrated in alcohol preferring rats [20] and mutant mice hemizygous for CREB- α and $-\delta$ display reduced alcohol-preference [42], while mice hemizygous for Gs- α show increased consumption [64]. Together, these lines of evidence suggest that increased PKI- α expression may be integral to the adaptations of the cAMP-PKA pathway induced by alcohol dependence.

Alcohol, as well as other drugs of abuse, activates the mesolimbic dopamine system [16,22]. Conversely, alcohol withdrawal is associated with a profound decrease of the neuronal activity of mesolimbic dopaminergic neurons [17,18] and with decreased dopamine release in projection areas like the NAc [17,66]. Since these effects are reversed by alcohol administration [17,66], such hypoactivity of the mesolimbic dopaminergic system during withdrawal is believed to have motivational significance in maintaining alcohol self-administration in dependent individuals [34,67]. Measures of dopamine and dopamine turnover taken in the replicate experiment conducted concomitantly with the RT-PCR analyses did not demonstrate significant changes in the regions investigated in intermittent alcohol-exposed rats at the end of the last alcohol exposure (Table 2). Reduced expression of cAMP-PKA-sensitive genes without significantly decreased dopamine levels may indicate desensitization to the action of dopamine on the expression of cAMP-PKA-regulated genes in animals exposed to chronic intermittent alcohol. Increased expression of PKI- α may contribute to this hypothesized desensitization to the transcriptional effects of dopamine in the mesocorticolimbic projection regions studied here.

Dopamine D1 and glutamatergic NMDA receptors converge to activate the ERK pathway, which in turn regulates gene expression through the action of various transcription factors [43,62]. Our previous work showed that chronic intermittent alcohol exposure reduced the activity of the ERK pathway in various brain regions [47,53]. Additionally, high-frequency stimulation, which activates NMDA receptors, was ineffective in recruiting the ERK pathway in brain slices from rats exposed to chronic intermittent alcohol [47]. It is therefore

possible that adaptations of the ERK pathway may also be involved in the decreased expression of dopamine-regulated genes in animals exposed to chronic intermittent alcohol.

In vitro, alcohol exposure has also been shown to activate the cAMP-PKA pathway by stimulating Gs-coupled adenosine A2 receptors resulting in PKA translocation to the nucleus and CREB activation [13,14,19]. Adenosine A2 and dopamine D2 have been shown to have the potential to synergistically induce cAMP-PKA-regulated gene expression [33] as has also been shown for dopamine D1 and D2 receptors [44]. It is therefore possible that the changes in PKI- α expression seen here may be a neuroadaptive response to the recruitment of multiple alcohol-activated signaling pathways.

Addiction is a chronic, relapsing disorder that results from the prolonged effects of drugs on the brain [31]. Drug-induced adaptive changes in the brain reward and stress neurocircuitry that are believed to motivate continued drug use and the transition from occasional drug use to addiction [28,30]. In the present study we observed increased expression of the endogenous PKA inhibitor PKI- α and decreased expression of genes that are transcriptionally induced by the cAMP-PKA pathway in animals exposed to chronic intermittent alcohol. This may represent a molecular correlate of central alcohol tolerance and may have motivational significance in the rapid increase in alcohol self-administration induced by intermittent alcohol exposure [39].

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References

- [1]. Abrous DN, Desjardins S, Sorin B, Hancock D, Le Moal M, Herman JP. Changes in striatal immediate early gene expression following neonatal dopaminergic lesion and effects of intrastriatal dopaminergic transplants. *Neuroscience*. 1996; 73:145–159. [PubMed: 8783238]
- [2]. Affymetrix, Statistical Algorithms Reference Guide. 2002. http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf
- [3]. Ahmed SH, Lutjens R, van der Stap LD, Lekic D, Romano-Spica V, Morales M, Koob GF, Repunte-Canonigo V, Sanna PP. Gene expression evidence for remodeling of lateral hypothalamic circuitry in cocaine addiction. *Proc Natl Acad Sci U S A*. 2005; 102:11533–11538. [PubMed: 16076954]
- [4]. Asyyeda A, Stormb D, Diamond I. Ethanol activates cAMP response element-mediated gene expression in select regions of the mouse brain. *Brain Research*. 2006; 1106:63–71. [PubMed: 16854384]
- [5]. Becker HC, Hale RL. Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling". *Alcohol Clin Exp Res*. 1993; 17:94. [PubMed: 8452212]
- [6]. Berke JD, Paletzki RF, Aronson GJ, Hyman SE, Gerfen CR. A complex program of striatal gene expression induced by dopaminergic stimulation. *J Neurosci*. 1998; 18:5301–5310. [PubMed: 9651213]
- [7]. Berke JD, Sgambato V, Zhu PP, Lavoie B, Vincent M, Krause M, Hyman SE. Dopamine and glutamate induce distinct striatal splice forms of Ania-6, an RNA polymerase II-associated cyclin. *Neuron*. 2001; 32:277–287. [PubMed: 11683997]

- [8]. Bottai D, Guzowski JF, Schwarz MK, Kang SH, Xiao B, Lanahan A, Worley PF, Seeburg PH. Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression. *J Neurosci*. 2002; 22:167–175. [PubMed: 11756499]
- [9]. Buck KJ, Hahner L, Sikela J, Harris RA. Chronic ethanol treatment alters brain levels of gamma-aminobutyric acidA receptor subunit mRNAs: relationship to genetic differences in ethanol withdrawal seizure severity. *J Neurochem*. 1991; 57:1452–1455. [PubMed: 1654394]
- [10]. Cagetti E, Liang J, Spigelman I, Olsen RW. Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of GABAA receptors. *Mol Pharmacol*. 2003; 63:53–64. [PubMed: 12488536]
- [11]. Chen X, Dai JC, Orellana SA, Greenfield EM. Endogenous protein kinase inhibitor gamma terminates immediate-early gene expression induced by cAMP-dependent protein kinase (PKA) signaling: termination depends on PKA inactivation rather than PKA export from the nucleus. *J Biol Chem*. 2005; 280:2700–2707. [PubMed: 15557275]
- [12]. Chergui K, Svenningsson P, Nomikos GG, Gonon F, Fredholm BB, Svennson TH. Increased expression of NGFI-A mRNA in the rat striatum following burst stimulation of the medial forebrain bundle. *Eur J Neurosci*. 1997; 9:2370–2382. [PubMed: 9464931]
- [13]. Constantinescu A, Diamond I, Gordon AS. Ethanol-induced translocation of cAMP-dependent protein kinase to the nucleus. Mechanism and functional consequences. *J Biol Chem*. 1999; 274:26985–26991. [PubMed: 10480911]
- [14]. Constantinescu A, Gordon AS, Diamond I. cAMP-dependent protein kinase types I and II differentially regulate cAMP response element-mediated gene expression: implications for neuronal responses to ethanol. *J Biol Chem*. 2002; 277:18810–18816. [PubMed: 11886856]
- [15]. Devaud LL, Fritschy JM, Sieghart W, Morrow AL. Bidirectional alterations of GABA(A) receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. *J Neurochem*. 1997; 69:126–130. [PubMed: 9202302]
- [16]. Diana M, Gessa GL, Rossetti ZL. Lack of tolerance to ethanol-induced stimulation of mesolimbic dopamine system. *Alcohol Alcohol*. 1992; 27:329–333. [PubMed: 1329784]
- [17]. Diana M, Pistis M, Carboni S, Gessa GL, Rossetti ZL. Profound decrement of mesolimbic dopaminergic neuronal activity during ethanol withdrawal syndrome in rats: electrophysiological and biochemical evidence. *Proc Natl Acad Sci U S A*. 1993; 90:7966–7969. [PubMed: 8367449]
- [18]. Diana M, Pistis M, Muntoni A, Rossetti ZL, Gessa G. Marked decrease of A10 dopamine neuronal firing during ethanol withdrawal syndrome in rats. *Eur J Pharmacol*. 1992; 221:403–404. [PubMed: 1426019]
- [19]. Dohrman DP, Diamond I, Gordon AS. Ethanol causes sustained translocation of PKA C-alpha to the nucleus. *RSA meeting abstract*. 1996; 586
- [20]. Froehlich JC, Wand GS. Adenylyl cyclase signal transduction and alcohol-induced sedation. *Pharmacol Biochem Behav*. 1997; 58:1021–1030. [PubMed: 9408209]
- [21]. Gilby KL, Da Silva AG, McIntyre DC. Differential GABA(A) subunit expression following status epilepticus in seizure-prone and seizure-resistant rats: a putative mechanism for refractory drug response. *Epilepsia*. 2005; 46(Suppl 5):3–9. [PubMed: 15987246]
- [22]. Imperato A, Di Chiara G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J Pharmacol Exp Ther*. 1986; 239:219–228. [PubMed: 3761194]
- [23]. Izzo E, Sanna P, Koob G. Impairment of dopaminergic system function after chronic treatment with corticotropinreleasing factor. *Pharmacology Biochemistry and Behavior* in press. 2005
- [24]. Jung AB, Bennett JP Jr. Development of striatal dopaminergic function. II: Dopaminergic regulation of transcription of the immediate early gene zif268 and of D1 (D1a) and D2 (D2a) receptors during pre- and postnatal development. *Brain Res Dev Brain Res*. 1996; 94:121–132.
- [25]. Kalivas PW, Volkow ND. The neural basis of addiction: a pathology of motivation and choice. *Am J Psychiatry*. 2005; 162:1403–1413. [PubMed: 16055761]
- [26]. Kammermeier PJ. Surface clustering of metabotropic glutamate receptor 1 induced by long Homer proteins. *BMC Neurosci*. 2006; 7:1. [PubMed: 16393337]

- [27]. Knighton DR, Zheng JH, Ten Eyck LF, Xuong NH, Taylor SS, Sowadski JM. Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science*. 1991; 253:414–420. [PubMed: 1862343]
- [28]. Koob GF. Alcoholism: allostasis and beyond. *Alcohol Clin Exp Res*. 2003; 27:232–243. [PubMed: 12605072]
- [29]. Koob GF, Ahmed SH, Boutrel B, Chen SA, Kenny PJ, Markou A, O'Dell LE, Parsons LH, Sanna PP. Neurobiological mechanisms in the transition from drug use to drug dependence. *Neurosci Biobehav Rev*. 2004; 27:739–749. [PubMed: 15019424]
- [30]. Koob GF, Sanna PP, Bloom FE. Neuroscience of addiction. *Neuron*. 1998; 21:467–476. [PubMed: 9768834]
- [31]. Leshner AI. Addiction is a brain disease, and it matters. *Science*. 1997; 278:45–47. [PubMed: 9311924]
- [32]. Leshner AI, Koob GF. Drugs of abuse and the brain. *Proc Assoc Am Physicians*. 1999; 111:99–108. [PubMed: 10220804]
- [33]. Mailliard W, Diamond I. Recent advances in the neurobiology of alcoholism: the role of adenosine. *Pharmacology & Therapeutics*. 2004; 101:39–46. [PubMed: 14729391]
- [34]. Melis M, Spiga S, Diana M. The dopamine hypothesis of drug addiction: hypodopaminergic state. *Int Rev Neurobiol*. 2005; 63:101–154. [PubMed: 15797467]
- [35]. Mhatre M, Ticku MK. Chronic ethanol treatment upregulates the GABA receptor beta subunit expression. *Brain Res Mol Brain Res*. 1994; 23:246–252. [PubMed: 8057781]
- [36]. Mhatre MC, Ticku MK. Chronic ethanol administration alters gamma-aminobutyric acidA receptor gene expression. *Mol Pharmacol*. 1992; 42:415–422. [PubMed: 1383684]
- [37]. Mitsuyama H, Little KY, Sieghart W, Devaud LL, Morrow AL. GABA(A) receptor alpha1, alpha4, and beta3 subunit mRNA and protein expression in the frontal cortex of human alcoholics. *Alcohol Clin Exp Res*. 1998; 22:815–822. [PubMed: 9660306]
- [38]. Nairn AC, Greengard P. A novel cyclin provides a link between dopamine and RNA processing. *Neuron*. 2001; 32:174–176. [PubMed: 11683987]
- [39]. O'Dell LE, Roberts AJ, Smith RT, Koob GF. Enhanced alcohol self-administration after intermittent versus continuous alcohol vapor exposure. *Alcohol Clin Exp Res*. 2004; 28:1676–1682. [PubMed: 15547454]
- [40]. Oiwa Y, Yoshimura R, Nakai K, Itakura T. Dopaminergic neuroprotection and regeneration by neurturin assessed by using behavioral, biochemical and histochemical measurements in a model of progressive Parkinson's disease. *Brain Res*. 2002; 947:271–283. [PubMed: 12176170]
- [41]. Overstreet DH, Knapp DJ, Breese GR. Accentuated decrease in social interaction in rats subjected to repeated ethanol withdrawals. *Alcohol Clin Exp Res*. 2002; 26:1259–1268. [PubMed: 12198403]
- [42]. Pandey SC, Roy A, Zhang H, Xu T. Partial deletion of the cAMP response element-binding protein gene promotes alcohol-drinking behaviors. *J Neurosci*. 2004; 24:5022–5030. [PubMed: 15163695]
- [43]. Papadeas ST, Blake BL, Knapp DJ, Breese GR. Sustained extracellular signal-regulated kinase 1/2 phosphorylation in neonate 6-hydroxydopamine-lesioned rats after repeated D1-dopamine receptor agonist administration: implications for NMDA receptor involvement. *J Neurosci*. 2004; 24:5863–5876. [PubMed: 15229233]
- [44]. Paul ML, Graybiel AM, David JC, Robertson HA. D1-like and D2-like dopamine receptors synergistically activate rotation and c-fos expression in the dopamine-depleted striatum in a rat model of Parkinson's disease. *J Neurosci*. 1992; 12:3729–3742. [PubMed: 1357113]
- [45]. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. 2nd. Academic Press; San Diego: 1998.
- [46]. Rimondini R, Arlinde C, Sommer W, Heilig M. Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *Faseb J*. 2002; 16:27–35. [PubMed: 11772933]
- [47]. Roberto M, Nelson TE, Ur CL, Brunelli M, Sanna PP, Gruol DL. The transient depression of hippocampal CA1 LTP induced by chronic intermittent ethanol exposure is associated with an

- inhibition of the MAP kinase pathway. *Eur J Neurosci.* 2003; 17:1646–1654. [PubMed: 12752382]
- [48]. Roberts AJ, Heyser CJ, Cole M, Griffin P, Koob GF. Excessive ethanol drinking following a history of dependence: animal model of allostasis. *Neuropsychopharmacology.* 2000; 22:581–594. [PubMed: 10788758]
- [49]. Salin P, Kerkerian L, Nieoullon A. Expression of neuropeptide Y immunoreactivity in the rat nucleus accumbens is under the influence of the dopaminergic mesencephalic pathway. *Exp Brain Res.* 1990; 81:363–371. [PubMed: 1975787]
- [50]. Sanna E, Mostallino MC, Busonero F, Talani G, Tranquilli S, Mameli M, Spiga S, Follesa P, Biggio G. Changes in GABA(A) receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. *J Neurosci.* 2003; 23:11711–11724. [PubMed: 14684873]
- [51]. Sanna PP, Folsom DP, Barizo MJ, Hirsch MD, Melia KR, Maciejewski LD, Bloom FE. Chronic ethanol intake decreases vasopressin mRNA content in the rat hypothalamus: a PCR study. *Brain Res Mol Brain Res.* 1993; 19:241–245. [PubMed: 8412569]
- [52]. Sanna PP, King AR, van der Stap LD, Repunte-Canonigo V. Gene profiling of laser-microdissected brain regions and sub-regions. *Brain Res Brain Res Protoc.* 2005; 15:66–74. [PubMed: 15946894]
- [53]. Sanna PP, Simpson C, Lutjens R, Koob G. ERK regulation in chronic ethanol exposure and withdrawal. *Brain Res.* 2002; 948:186–191. [PubMed: 12383974]
- [54]. Self DW, Genova LM, Hope BT, Barnhart WJ, Spencer JJ, Nestler EJ. Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J Neurosci.* 1998; 18:1848–1859. [PubMed: 9465009]
- [55]. Self DW, McClenahan AW, Beitner-Johnson D, Terwilliger RZ, Nestler EJ. Biochemical adaptations in the mesolimbic dopamine system in response to heroin self-administration. *Synapse.* 1995; 21:312–318. [PubMed: 8869161]
- [56]. Sgambato V, Minassian R, Nairn AC, Hyman SE. Regulation of ania-6 splice variants by distinct signaling pathways in striatal neurons. *J Neurochem.* 2003; 86:153–164. [PubMed: 12807435]
- [57]. Sgambato-Faure V, Xiong Y, Berke JD, Hyman SE, Strehler EE. The Homer-1 protein Ania-3 interacts with the plasma membrane calcium pump. *Biochem Biophys Res Commun.* 2006; 343:630–637. [PubMed: 16554037]
- [58]. Slawecki CJ, Betancourt M, Cole M, Ehlers CL. Periadolescent alcohol exposure has lasting effects on adult neurophysiological function in rats. *Brain Res Dev Brain Res.* 2001; 128:63–72.
- [59]. Slawecki CJ, Somes C, Ehlers CL. Effects of chronic ethanol exposure on neurophysiological responses to corticotropin-releasing factor and neuropeptide Y. *Alcohol Alcohol.* 1999; 34:289–299. [PubMed: 10414603]
- [60]. Snyder-Keller A, Chandra R, Lin Y, Mitchell ES. Basal EGR-1 (zif268, NGFI-A, Krox-24) expression in developing striatal patches: role of dopamine and glutamate. *Brain Res.* 2002; 958:297–304. [PubMed: 12470865]
- [61]. Valjent E, Aubier B, Corbille AG, Brami-Cherrier K, Caboche J, Topilko P, Girault JA, Herve D. Plasticity-associated gene Krox24/Zif268 is required for long-lasting behavioral effects of cocaine. *J Neurosci.* 2006; 26:4956–4960. [PubMed: 16672671]
- [62]. Valjent E, Corbille AG, Bertran-Gonzalez J, Herve D, Girault JA. Inhibition of ERK pathway or protein synthesis during reexposure to drugs of abuse erases previously learned place preference. *Proc Natl Acad Sci U S A.* 2006
- [63]. Wahlestedt C, Karoum F, Jaskiw G, Wyatt RJ, Larhammar D, Ekman R, Reis DJ. Cocaine-induced reduction of brain neuropeptide Y synthesis dependent on medial prefrontal cortex. *Proc Natl Acad Sci U S A.* 1991; 88:2078–2082. [PubMed: 2006146]
- [64]. Wand G, Levine M, Zweifel L, Schwindinger W, Abel T. The cAMP-protein kinase A signal transduction pathway modulates ethanol consumption and sedative effects of ethanol. *J Neurosci.* 2001; 21:5297–5303. [PubMed: 11438605]
- [65]. Weiss F. Neurobiology of craving, conditioned reward and relapse. *Curr Opin Pharmacol.* 2005; 5:9–19. [PubMed: 15661620]

- [66]. Weiss F, Parsons LH, Schulteis G, Hyytia P, Lorang MT, Bloom FE, Koob GF. Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. *J Neurosci.* 1996; 16:3474–3485. [PubMed: 8627380]
- [67]. Weiss F, Porrino LJ. Behavioral neurobiology of alcohol addiction: recent advances and challenges. *J Neurosci.* 2002; 22:3332–3337. [PubMed: 11978808]
- [68]. Zigmond MJ, Hastings TG, Perez RG. Increased dopamine turnover after partial loss of dopaminergic neurons: compensation or toxicity? *Parkinsonism Relat Disord.* 2002; 8:389–393. [PubMed: 12217625]
- [69]. Zigmond MJ, Stricker EM. Parkinson's disease: studies with an animal model. *Life Sci.* 1984; 35:5–18. [PubMed: 6146085]

Fig. 1. Gene expression analysis in chronic intermittent alcohol exposure

A) Scatter plots of microarray expression values from the three brain regions of chronic alcohol-treated rats at the end of the exposure paradigm and after 1 week withdrawal plotted against controls. The high linear correlation seen in the present results is indicative of limited differential expression between the two conditions as expected in the brain, in which gene expression changes are typically of limited magnitude and involve a limited number of transcripts. Differentially expressed probesets are shown in blue (present or marginal probesets with expression levels >100 in at least one condition are displayed). **B)** Numbers of genes found to be changed in the three regions studied in chronic alcohol-treated rats at the end of the exposure paradigm and after 1 week withdrawal. **C)** Hierarchical clustering of genes specifically associated with chronic intermittent alcohol exposure (A) versus controls (C) in the brain regions studied. Genes are colored according to their expression values normalized to 1 as shown in the intensity scale on the right that represents expression levels on a continuous scale from yellow (overexpression) to black (underexpression). Yellow-black midtones indicates average expression.

Fig. 2. Increased expression of the endogenous PKA inhibitor α (PKI- α) and decreased expression of PKA-regulated genes in chronic intermittent alcohol exposure

The scatterplots demonstrate RT-PCR results from a replicate of the experiment that was used for validation of the microarray analyses (n=6). PKI- α , a member of the protein kinase A inhibitor family that is believed to terminate nuclear PKA activity, was found to be increased in all three regions tested. Conversely, several genes whose expression is positively modulated by PKA were downregulated in animals exposed to chronic intermittent alcohol in the 3 brain regions investigated. Among them were several Ania transcripts, the transcription factors Egr1 and NGFI-B (Nr4a1), and the neuropeptide NPY. The units on the Y-axis represent the ratios of the mRNA levels of the genes to β -actin. *p<0.05, **p<0.01, ***p<0.001.

Table 1

Oligonucleotide primers used for RT-PCR.

Target transcripts	Sense Primer	Anti-sense Primer
Ania-1	CAGTGGCTAAGAGTAGTTGTTG	CTGTGAACCATGTATGTGTCTG
Ania-3	TGAATCCTCCGCCCTACC	ACAGAGACTATCAACAAAGAGC
Ania-7	TGGGATGAGTAAGACCTAACC	TAACTGCTCTTAGATGGATTCC
Ania-8	TCTGACCTTGTGATCCATAGC	GAACGCAAACGACCAACAC
Egr1	GGACAGGAGGGAAGAAATGG	CAGGGACGCTAAGTGAAAGG
Gabrg2	CACGGGAGGATACACATTCG	ACAAGATTGAACAAGCAGAAGG
NPY	TCTCATCTCATCCTGTGAAACC	CAACGACAACAAGGGAAATGG
Gria2	CGTTGCTGGAGTATTCTACATC	ATTCTTTGCCACCTTCATTTCG
Gabra1	ACCGACTGTCAAGAATAGCC	GAGGACTGAACAACAGAATACG
Gabrb3	ACAAGAAGACGCACCTACG	CTATGGCATTACATCGGTTAG
PKIa	AGACAGAAGGTGAAGATGATGG	AGCAATGCCAGGAGATTTCG
Nr4a1	AATACAGAAAGGAAGAGGTCGG	TGGAAGGAGAGCGGAAGAG
<i>Reference mRNA</i>		
β -actin	agattactgccctggctcct	cagtgaggccaggatagagc

Table 2

Tissue levels of dopamine and its metabolites after chronic intermittent alcohol exposure.

<i>Brain Regions</i>	<i>Treatment</i>	<i>Dopamine</i>	<i>HVA</i>	<i>DOPAC/DA</i>
MPF	<i>alcohol</i> <i>control</i>	1.29 (± 0.03)	0.075 (± 0.01)	0.075 (± 0.01)
		0.98 (± 0.07) $F_{1,5}=1.611, p=0.26$	0.059 (± 0.02) $F_{1,5}=0.292, p=0.61$	0.059 (± 0.02) $F_{1,5}=0.292, p=0.61$
NAc	<i>alcohol</i> <i>control</i>	45.97 (± 2.98)	0.11 (± 0.01)	0.11 (± 0.01)
		50.56 (± 6.33) $F_{1,5}=0.258, p=0.63$	0.10 (± 0.01) $F_{1,5}=1.910, p=0.23$	0.10 (± 0.01) $F_{1,5}=1.910, p=0.23$
AMY	<i>alcohol</i> <i>control</i>	8.56 (± 1.66)	0.037 (± 0.01)	0.037 (± 0.01)
		7.96 (± 1.68) $F_{1,5}=0.564, p=0.49$	0.026 (± 0.01) $F_{1,5}=0.412, p=0.55$	0.026 (± 0.01) $F_{1,5}=0.412, p=0.55$

Levels of dopamine, HVA (ng/mg protein) and DOPAC/DA in the MPF, NAc and AMY at the end of the chronic intermittent alcohol exposure paradigm in the animals used for RT-PCR validation of the microarray analyses (n=5-6). No significant changes between control and experimental groups were revealed by two way ANOVA, as shown in the table.