Published in final edited form as:

Addict Biol. 2011 October ; 16(4): 615–619. doi:10.1111/j.1369-1600.2010.00304.x.

Identification of genes regulated in the mouse extended amygdala by excessive ethanol drinking associated with dependence

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Abstract

Alcoholism is characterized by a progressive loss of control over ethanol intake. The purpose of this study was to identify transcriptional changes selectively associated with excessive ethanol drinking in dependent mice, as opposed to non-dependent mice maintaining a stable voluntary consumption or mice solely undergoing forced intoxication. We measured expression levels of 106 candidate genes in the extended amygdala, a key brain structure for the development of drug addiction. Cluster analysis identified 17 and 15 genes selectively induced or repressed, respectively, under conditions of excessive drinking. These genes belong to signaling pathways involved in neurotransmission and transcriptional regulation.

Keywords

alcoholism; C57BL/6J; clustering; ethanol dependence; extended amygdala; qPCR

Alcoholism is defined as a maladaptive pattern of alcohol consumption, characterized by compulsive alcohol intake, loss of control over drinking and a negative emotional state during withdrawal (Koob 2003). The endogenous opioid system is recruited by all drugs of abuse, including alcohol, and transcriptional changes occurring downstream of mu opioid receptor activation may therefore contribute to the development of addiction (Gerrits *et al.* 2003). Our laboratory has previously identified a collection of 83 genes, whose expression is regulated in the extended amygdala in response to prolonged stimulation of the mu opioid receptor (Befort *et al.* 2008b). The present study aims to assess whether these genes, as well as 23 additional genes previously implicated in addiction (Befort *et al.* 2008a; Gerrits *et al.* 2003; Mulligan *et al.* 2006), are differentially expressed in ethanol-dependent mice, which voluntarily drink excessive amounts of ethanol. Gene expression was measured in the

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Authors contribution CC, BLK and GFK were responsible for the study concept and design. CC and OG contributed to mouse treatment, behavioral testing and tissue sampling. DF and OG conducted gene expression analysis. JAJB conducted cluster and pathway analysis. CC drafted the manuscript. BLK and GFK provided critical revision of the manuscript. All authors reviewed content and approved final version for publication.

Conflict of interests None

extended amygdala, a macrostructure of the basal forebrain that plays a critical role in the development of alcohol dependence (Koob 2003).

Male C57BL/6J mice (Jackson Laboratories, Sacramento, CA) were tested in a model of excessive drinking induced by dependence (Becker & Lopez 2004). Mice were first subjected to 2-h sessions of two-bottle choice drinking (ethanol 15% w:v vs. water), starting 3 h into the dark cycle, five days per week, until their ethanol intake stabilized. Ethanol-naïve control mice had access to two bottles of water. Half of ethanol-drinking mice were then made dependent by chronic intermittent exposure to ethanol vapor in inhalation chambers (dependent group), while the other half (non-dependent group) and ethanol-naïve controls were continuously exposed to air. Dependent mice were subjected to four cycles of ethanol intoxication (16 h) and withdrawal (8 h) before returning to their home cages for three days. Voluntary drinking sessions were then resumed for five days. This sequence of events was repeated until the ethanol intake of dependent mice increased significantly above their baseline levels and was significantly higher than the ethanol intake of non-dependent mice. Mice were finally subjected to an additional week of chronic intermittent ethanol vapor (or continuous air) exposure and sacrificed at the end of the fourth 16-h period of intoxication. An independent group of male C57BL/6J mice (Charles River, Lyon, France) was solely exposed to continuous ethanol intoxication (intoxicated group, air for control mice) for 72 h and sacrificed at the end of this period (see Fig. 1a and Supplementary Methods for details). Bilateral punches of bed nucleus of the stria terminalis (BNST) and amygdala were rapidly collected and frozen.

RNA extraction, cDNA synthesis and qPCR assays were carried out as described previously (Befort *et al.* 2008b). For each experimental condition, two independent cohorts were tested (excessive ethanol drinking induced by dependence, $n = 5-7$ mice per subgroup; chronic ethanol intoxication, $n = 12$ mice per subgroup). Within each cohort, BNST and amygdala punches from each subgroup were pooled for RNA extraction. For each RNA preparation, two independent cDNA samples were subjected to qPCR, thereby yielding 4 distinct measures of gene expression for each experimental subgroup. Real-time PCR was performed in triplicate. Sequences of primers are provided in Table S1. Relative expression ratios were normalized to the level of *rplp0* and the $2^{-\Delta\Delta Ct}$ method was applied to evaluate differential expression level (Livak & Schmittgen 2001). Ethanol-naïve mice were used as a control for dependent and non-dependent mice, while intoxicated mice were calibrated against air-exposed mice. qPCR data were transformed prior to statistical analysis to obtain a symmetrical distribution centered on 0 (corresponding to no change in gene expression). Hierarchical clustering was performed on the median of transformed qPCR data on the entire set of 106 genes, or on a subset of 44 genes (see below), using Cluster 3.0 software (de Hoon *et al.* 2004). Molecular connectivity within each cluster of interest was explored using Ingenuity Pathways Analysis (IPA, Ingenuity® Systems, www.ingenuity.com). The top-scoring direct network was selected for each cluster and biological function and canonical pathway analyses were performed on the network molecules (see Supplementary Methods for details).

Chronic intermittent exposure to ethanol vapor increased ethanol self-administration in dependent mice, while the ethanol consumption of air-exposed (non-dependent) mice remained stable over time. In the first cohort (Fig. 1b), two-way repeated-measures ANOVA revealed a main effect of time ($F_{5,60} = 30.58$, $p < 0.001$), a strong trend for an effect of treatment ($F_{1,60} = 4.46$, $p = 0.056$) and a significant interaction between the two variables ($F_{5,60} = 2.39$, $p < 0.05$). In the second cohort (Fig. 1c), the main effects of time and treatment, and interaction between both, were all significant ($F_{3,24} = 6.46$, $p < 0.01$; $F_{1,24} = 5.95$, $p < 0.05$ and $F_{3,24} = 3.01$, $p < 0.05$ respectively). After five and three rounds,

respectively, dependent mice drank more ethanol than non-dependent mice (unpaired *t*-test, $t_{12} = 2.39$, $p < 0.05$ and $t_8 = 2.84$, $p < 0.05$).

Expression levels of the 106 genes measured in extended amygdala samples from non-dependent and dependent mice (Table S2) were subjected to hierarchical clustering, which revealed two clusters gathering 44 genes induced or repressed, respectively, in dependent mice only (Fig. 2a). We examined whether these transcriptional changes also occurred in mice that were chronically exposed to ethanol vapors, but were not given the opportunity to voluntarily drink ethanol (intoxicated mice, Table S2). A novel cluster analysis was conducted on the 44-gene subset using combined data from non-dependent, dependent and intoxicated mice, and three interesting clusters emerged (Fig. 2b). In Cluster 1, gene expression was unchanged in non-dependent mice, but was up-regulated similarly in both dependent and intoxicated mice, indicating that these transcriptional changes are induced by chronic ethanol intoxication in general and are not relevant to excessive drinking (Fig. S1a). This cluster contained four genes encoding lipid binding or modifying proteins (*Apod*, *Dhcr24*, *Lpl* and *Plip*), suggesting that chronic ethanol inhalation severely alters lipid transport and metabolism. In contrast, Clusters 2 and 3 gathered genes selectively up- or down-regulated, respectively, in dependent mice, but unaffected in non-dependent and intoxicated mice, and therefore specifically associated with increased motivation to voluntarily drink ethanol (Fig. S1b and S1c). Some of these transcriptional changes corroborate published data, such as the selective up-regulation of *Crh* and *Pdyn* reflecting the recruitment of anti-reward brain systems in dependent mice (Koob 2008). Most genes, however, have not been reported earlier in the context of alcoholism (Table S3, and see Supplementary Discussion for extensive comments). Particularly striking is the coordinated up-regulation of eight genes enriched in basal ganglia medium spiny neurons (*Pdyn*, *Penk*, *Drd1a*, *Arpp21*, *Grm5*, *Gpr6*, *Bcl11b*, *Scn4b*) in ethanol-dependent mice which suggests that their protein products interact functionally to regulate ethanol intake (Fig. S2). Moreover, expression levels of four genes encoding DNA-binding proteins (*Bcl11b*, *Egr1*, *Egr3*, *Nr4a1*) were selectively altered in dependent mice, indicative of broad transcriptional reprogramming in the extended amygdala. Finally, both clusters contained several genes expressed in oligodendrocytes or astrocytes (*Opalin*, *Inadl*, *Cmtm5*, *Aqp4*, *Grm5*), which points to an unforeseen role for glial cells in ethanol dependence, possibly through the regulation of neuronal activity by potassium clearance (*Aqp4*) or glutamate gliotransmission (*Grm5*).

Pathway analysis generated two networks incorporating 9 out of 17 genes from Cluster 2 (Fig. 2c, Network “UP”) and 14 out of 15 genes from Cluster 3 (Fig. 2d, Network “DOWN”). The remarkably high scores of these two networks (32 and 38 respectively) attest to the strong interconnectivity of genes regulated in the extended amygdala during the transition from moderate to excessive ethanol drinking. The top-scoring biological functions that emerged from the functional analysis of these two networks confirmed that alteration of neurotransmission and transcriptional regulation is critical to the development of ethanol dependence (Fig. S3a and S3b). CRF signaling was identified by canonical pathway analysis of both networks (Fig. S3c and S3d), in accordance with the recruitment of brain stress systems during the development of ethanol dependence (Koob 2003; Koob 2008). A role for cdk5 signaling, which also appeared in both networks, may be an interesting hypothesis to explore in the future, as this pathway has been little investigated in the context of alcoholism. In conclusion, using a specific animal model for excessive drinking associated with dependence, the present study validated genes hypothesized to mediate enhanced ethanol self-administration in dependent subjects and also identified novel candidate genes and signaling pathways that may drive compulsive drinking in alcoholism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank Joseph Brewer, Emily Johnson and Connie Choi for their technical assistance in conducting the drinking experiments. We are grateful to Doulai Dembele for his help with cluster analysis. This work was supported by National Institutes of Health grants 2U01AA013517-06 to GFK and 1U01AA016658-01 to BLK, as well as by the European Union (GENADDICT/FP6005166).

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Figure 1. Dependence-induced increase in voluntary ethanol intake. (a) Experimental design. (b-c) Daily ethanol intake (mean $\pm$ s.e.m.) for the last week of acquisition and the last week post-vapor for (b) first cohort ($n = 7$), and (c) second cohort ($n = 5$).

Figure 2.

(a-b) Hierarchical clustering of transcriptional changes. Light to deep red/blue represents low to high up-/down-regulation, as compared with expression levels in ethanol-naïve mice (non-dependent and dependent) or air-exposed mice (intoxicated). (a) Analysis of the entire set of 106 genes, using data from non-dependent and dependent mice. (b) Analysis of the 44-gene subset, using data from non-dependent, dependent and intoxicated mice. (c-d) Top-scoring molecular networks generated by IPA within Clusters 2 (c, Network "UP") and 3 (d, Network "DOWN").