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Novel candidate genes for alcoholism — transcriptomic analysis of prefrontal medial cortex, hippocampus and nucleus accumbens of Warsaw alcohol-preferring and non-preferring rats

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ABSTRACT

Objective: Animal models provide opportunity to study neurobiological aspects of human alcoholism. Changes in gene expression have been implicated in mediating brain functions, including reward system and addiction. The current study aimed to identify genes that may underlie differential ethanol preference in Warsaw High Preferring (WHP) and Warsaw Low Preferring (WLP) rats.
Methods: Microarray analysis comparing gene expression in nucleus accumbens (NAc), hippocampus (HP) and medial prefrontal cortex (mPFC) was performed in male WHP and WLP rats bred for differences in ethanol preference.
Results: Differential and stable between biological repeats expression of 345, 254 and 129 transcripts in NAc, HP and mPFC was detected. Identified genes and processes included known mediators of ethanol response (*Mx2*, *Fam111a*, *Itpr1*, *Gabra4*, *Agtr1a*, LTP/LTD, renin-angiotensin signaling pathway), toxicity (*Sult1c2a*, *Ces1*, inflammatory response), as well as genes involved in regulation of important addiction-related brain systems such as dopamine, tachykinin or acetylcholine (*Gng7*, *Tac4*, *Slc5a7*).
Conclusions: The identified candidate genes may underlie differential ethanol preference in an animal model of alcoholism.
Comment: Names of genes are written in italics, while names of proteins are written in standard font. Names of human genes/proteins are written in all capital letters. Names of rodent genes/proteins are written in capital letter followed by small letters.

1. Introduction

Alcohol dependence results in various medicinal and socio-economic problems. Estimated societal costs of alcohol abuse in the USA are higher than for both tobacco and illicit drugs (http://www.drugabuse.gov/related-topics/trends-statistics). Animal models, such as genetically selected strains of rats, have been extensively used to study the neurobiological and genetic mechanisms underlying alcohol dependence (Becker, 2013; Phillips and Gubner, 2013). Only a few pairs of alcohol-preferring and alcohol-non-preferring strains of rats have been breed to this point. These pairs of strains include the University of Chile A and B (UChA/UChB) (Mardones and Segovia-Riquelme, 1983), the ALCO alcohol/non-alcohol (AA/ANA) (Eriksson, 1968), Sardinian alcohol-preferring (sP)/-non-preferring (sNP) (Lobina et al., 1997) as well as Indiana University alcohol-preferring/alcohol-non-preferring (P/NP) and high/low alcohol-drinking (HAD/LAD) (Li et al., 1979; Murphy et al., 2002).

In the present study we used Warsaw High Preferring (WHP) and Warsaw Low Preferring (WLP) rat strains that have been bred from Wistar foundation stock for differential ethanol preference and consumption (Dyr and Kostowski, 2008). WHP rats voluntarily drink

Abbreviations: WHP, Warsaw High Preferring rat strain; WLP, Warsaw Low Preferring rat strain; mPFC, prefrontal medial cortex; HP, hippocampus; NAc, nucleus accumbens; GEO, Gene Expression Omnibus database; LTP, long-term potentiation; LTD, long-term depression; AA, ALCO ethanol preferring strain; ANA, ALCO ethanol avoiding strain; sP, Sardinian alcohol-preferring strain; sNP, Sardinian alcohol-non-preferring strain; P, Indiana University alcohol-preferring strain; NP, Indiana University alcohol-non-preferring strain; HAD, Indiana University high alcohol-drinking strain; LAD, Indiana University low alcohol-drinking strain.

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excessive amounts of ethanol (over 5 g/kg/day) that induce the increase in blood ethanol concentration to 0.03–0.045 g/dl, which is in order with standard criteria for selecting pairs of strains for ethanol preference (Cicero, 1979; Murphy et al., 2002; Quintanilla et al., 2006; Sinclair et al., 1989). The WHP rats display signs of physical dependence such as exophthalmia, piloerection and tremors (Dyr and Kostowski, 2008). Similar effects to ethanol intake were reported for P rats (Kampov-Polevoy et al., 2000). WHP rats, relative to WLP, are characterized by lesser sensitivity to the sedative and hypnotic effects of high doses of ethanol (Dyr and Kostowski, 2008). This characteristic is also present in sP/sNP strains but not in P/NP strains (Colombo et al., 2000; Waller et al., 1983). Moreover, WHP, similarly to AA and P rats (Le and Kiianmaa, 1988; Lumeng and Li, 1986) develop metabolic tolerance to ethanol after its chronic consumption (Dyr and Taracha, 2012). WHP rats display increase in locomotor activity after single intraperitoneal injection of 0.5 g/kg of ethanol and this behavioral response is shared with sP rats (Agabio et al., 2001). Rok-Bujko et al. (2006) demonstrated strong reinforcing properties of ethanol for WHP line using operant oral self-administration and the same paradigm was used to confirm reinforcing properties of ethanol for most other rat lines (P, sP, AA and HAD) (Oster et al., 2006; Rodd et al., 2003; Vacca et al., 2002) and is a hallmark of alcohol preference in animals.

Brain dopaminergic system is considered crucial for regulating ethanol intake (Imperato and Di Chiara, 1986; Tupala and Tiihonen, 2004). Concentrations of dopamine (DA) and its metabolites (HVA and DOPAC) were approximately 50% lower in the striatum of naive WHP rats as compared with naive WLP rats (Dyr and Kostowski, 2004), which corresponds with findings in HAD/LAD strains (Gongwer et al., 1989; McBride et al., 1997a). It should be noted, however, that in AA/ANA and P/NP strains striatal dopamine system was not considered important for determining their difference in ethanol preference (Korpi et al., 1987; McBride et al., 1997b; Syvalahti et al., 1994).

Another neurotransmitter crucial for regulating ethanol intake is GABA (Boyle et al., 1993; Enoch et al., 2012; Kumar et al., 2009). Alcohol-drinking and alcohol-naive WHP and WLP rats displayed inter-strain differences in the density of selective GABA-A receptor ligand [-^{3}H]muscimol binding sites in the brain (Dyr et al., 2002, 1999). Significant differences in activity of brain GABAergic system were also found in most other rat strains selected for ethanol preference (Hwang et al., 1997, 1990; Saba et al., 2001; Wong et al., 1996).

Genomic studies on WHP and WLP strains are important for verifying the validity of these rats as the animal model of alcohol preference. We attempted now to identify genes whose altered expression could contribute to differences in alcohol preference between WHP and WLP rats. Expression level of protein-coding genes has been considered a major determinant of cell phenotype and function. Therefore, analyzing mRNA transcriptomic profiles of experimental groups can yield information on the correlates and/or causes of inter-strain differences. We examined the constitutive transcriptomic profiles in brain regions implicated in alcoholism that is prefrontal cortex, hippocampus and nucleus accumbens (Tabakoff and Hoffman, 2013). There are currently several transcriptomic studies relevant to ethanol preference, which were performed in these brain areas (Bell et al., 2009; Carr et al., 2007; Hashimoto et al., 2011; Kimpel et al., 2007; McBride et al., 2010; Osterndorff-Kahanek et al., 2013; Sutherland et al., 2014). However, only two of them analyzed gene expression patterns in naïve animals, with both these works done in strains iP/iNP or congenic strain derived from them — NP.P (Carr et al., 2007; Kimpel et al., 2007). Given the general low replicability of microarray studies (see: LaCroix-Fralish et al., 2011) and potentially large number of molecular mechanisms regulating ethanol preference, there is still little information on basal transcriptomic differences, which underlie differences in ethanol preference. Moreover, as WHP/WLP model of ethanol preference is currently actively studied (Acewicz et al., 2014; Dyr et al., 2014), the characterization of brain transcriptome of these strains will be crucial establishing new research direction and for interpretation of consecutive data. The results of our work suggest that selective breeding for differences in ethanol preference resulted in a large number of previously unrecognized alterations in gene expression in the brain structures that may mediate alcohol abuse.

2. Material and methods

2.1. Animals

Warsaw High Preferring (WHP, 48th generation) and Warsaw Low Preferring (WLP, 50th generation) alcohol-naive 2–3 months old (250–300 g) male rats were used. Three to four rats were kept in standard shoe-box plastic cages (57 × 37 × 20 cm) with wood chip bedding in a room with controlled temperature (22 ± 1 °C) and humidity (~60%) and a 12-h light–dark cycle (lights on at 6 a.m.). Rat chow (Labofeed H, WPiK, Kcynia, Poland) and tap water were available ad libitum. All procedures performed on the animals were conducted according to the NIH Animal Care and Use Committee Guidelines and were approved by the local ethics committee.

2.2. Sample preparation

Nine animals from each strain were decapitated at the beginning of the light phase of the light–dark cycle (between 8 a.m. and 6 p.m.), brains removed from the skull, and the prefrontal medial cortex (mPFC), hippocampus (HP), and nucleus accumbens (NAc) were isolated (see Supplementary Methods for details)). The samples were inserted into freezing vials, flash-frozen in liquid nitrogen and stored at −80 °C until analysis. Total RNA was isolated from brain samples using spin-column based RNeasy kit (Qiagen, Venlo, Netherlands). RNA isolation included DNAse-treatment step. Quantity and quality of RNA samples were estimated using Nanodrop spectrophotometer (Nanodrop, Waltham, USA) and Bioanalyzer 2100 microcapillary electrophoresis device (Agilent, Santa Clara, USA). All of the RNA samples were of high quality (RIN > 9, 260/280 ~2.1).

2.3. Microarray procedures

RNA samples were processed as followed (samples from single brain structure of both WHP and WLP were processed at the same time): 1) For each brain area and strain, equal amounts of RNA from 9 animals were pooled into 3 RNA pools, so that each of 9 RNA samples was contained in only one of the 3 RNA pools. Final amounts of RNA in each of the pools were: 100 ng for HP and mPFC and 50 ng for NAc; 2) Pooled RNA samples were amplified and labeled with cyanine 3 or 5 using Two-Color Low Input Quick Amp Kit (Agilent, Santa Clara, USA); 3) Each labeled pool from WHP strain was hybridized on a single microarray with a pool from WLP strain (labeled with complementary dye) utilizing GE Hybridization Kit (Agilent, Santa Clara, USA). Each comparison was replicated in dye swap that is in reversed cyanine configuration, to control for unequal dye fluorescence intensity. The microarrays used in the experiment were Agilent's Whole Rat Genome Microarray 4 × 44 K v2 microarrays (that contain over 43,000 50-mer oligonucleotide probes analyzing approximately 41,000 of verified and proposed genes). Scanning was performed on G2565CA Microarray Scanner (Agilent, Santa Clara, USA), and data were extracted using Agilent Feature Extraction Software (Agilent, Santa Clara, USA) on default settings (GE2_1010_Sep10). All of the procedures were performed using dedicated kits according to respective producer's protocols.

2.4. Statistics

Statistical analysis on raw data was performed using Limma package from Bioconductor software (Smyth, 2005; http://www.bioconductor.org). The analysis included background correction (method: "normexp") (Ritchie et al., 2007), within-array normalization ("loess") and between-array normalization ("Aquantile") (Smyth and Speed, 2003). It also