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Research report

Chronic alcohol exposure induced gene expression changes in the zebrafish brain

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ABSTRACT

Chronic alcohol exposure affects the central nervous system, influences behavior, and induces neuroadaptive changes in vertebrate species including our own. The molecular mechanisms responsible for chronic alcohol effects have not been fully elucidated due to the complexity of alcohol's actions. Here we use zebrafish, a novel tool in alcohol research, to reveal a large number of genes that respond to chronic alcohol treatment. We demonstrate differential gene expression in response to chronic alcohol treatment using full genome DNA microarrays and find a total of 1914 genes to show a minimum of 2-fold and significant expression level change (1127 were up- and 787 were down-regulated). Approximately two-thirds of these genes had no known previous functional annotation. The results of the microarray analyses correlated well with those obtained on a selected subset of genes analyzed by quantitative real-time RT-PCR. Analyses of the differentially expressed genes with known annotations were enriched for a variety of molecular functions. Only a fraction of these known genes has been reported in the literature to be alcohol related. We conclude that the zebrafish is an excellent tool for the analysis of genes associated with alcohol's actions in vertebrates, one which may facilitate the discovery and better understanding of the mechanisms of alcohol abuse.

1. Introduction

Chronic alcohol exposure induces neuroadaptive changes leading to tolerance, adaptation, and alcohol seeking in humans and other non-human animals [19,13,26,51]. Modification of gene expression may underlie the altered brain function resulting from chronic alcohol exposure [56,45,50,22,39,16] and has been observed in cultured neurons [8] and in the brain of the rat [9,49,70], the mouse [2,3,70] and humans (postmortem brain tissue from alcoholics, e.g. [41,46,63,43]).

Zebrafish has also been utilized in alcohol research (e.g. [29,10,23]). The first DNA microarray analysis of the effects of alcohol has been published using zebrafish [39]. However, this latter study employed a complex mixed alcohol dosing regimen that involved repeated short exposure to alcohol and repeated withdrawal from the substance followed by a long term withdrawal before the gene expression analysis. Briefly, the effect of continuous chronic alcohol exposure has not been analyzed at the gene expression level. Nevertheless, significant behavioral adaptation to the substance in zebrafish has been demonstrated after such chronic exposure using a social behavior paradigm [28]. The goal of the current study is to investigate the gene expression changes that accompany chronic alcohol exposure using a genome wide DNA microarray system in zebrafish.

The rationale for conducting this work in zebrafish is several fold. Most importantly, this small and prolific vertebrate offers unprecedented efficiency for high throughput mutagenesis and drug screens, a major advantage because the complexity of alcohol's actions requires global analyses. Our work is the first to attempt comprehensive gene expression profiling of the effects of chronic alcohol exposure alone. With this analysis we hope to identify individual molecular targets, cluster of targets, and/or key biochemical interactions associated with functional changes induced by chronic alcohol in the brain. These putative targets then may serve as candidate genes in future follow-up forward and reverse genetic studies or drug screens using zebrafish.

2. Materials and methods

2.1. Zebrafish: the rationale for its use and the methods of its maintenance

Although rodents have been successfully utilized in alcohol research, a recent upsurge of alcohol studies with zebrafish suggests that this species also has some utility in this endeavor. First, analysis of zebrafish responses to alcohol may reveal evolutionarily conserved mechanisms common to vertebrates. Second, zebrafish

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may allow perhaps the most efficient discovery of novel mechanisms associated with alcohol exposure among vertebrate laboratory organisms. Alcohol administration in zebrafish is simple: the subject is immersed in the alcohol solution and its blood–brain alcohol reaches steady levels within 40 min [20,28] allowing precise control of the duration and dose of alcohol exposure. This dosing method may be superior to drug self-administration paradigms or passive alcohol exposure methods employed with mammalian laboratory species. For example, passive alcohol administration paradigms including alcohol vapor and/or invasive injection methods induce potential stress or anxiety whereas active alcohol administration paradigms (self-administration) depend upon motivational characteristics as well as homeostatic processes associated with fluid and/or food intake (see e.g. [47]). It is also important to note that zebrafish as a model of functional changes in behavior induced by alcohol has not only face validity (e.g. [29–31,20,21]) but also construct validity [4,44,59,39]. Last, this small (4 cm long) fish is housed in large numbers in small tanks (a shoaling fish), and its prolific nature lends it to both forward and reverse genetic studies that require testing of a large number of subjects.

Adult long fin zebrafish (6-month old) bred and raised in our vivarium (University of Toronto Mississauga, Mississauga, ON, Canada) were randomly selected for this experiment. The fish originated from founders that were purchased from a local pet store (Big Al's Aquarium Warehouse, Mississauga, ON, Canada) and were of the second filial generation bred in our facility. The rationale for the choice of this "pet store" variety fish was as follows. Genetically well defined strains of zebrafish may enhance reproducibility within and across laboratories. Nevertheless, these strains having been bred for several generations and having lost numerous alleles (due to inbreeding) are expected to possess idiosyncratic features. A genetically heterogeneous population, such as the long fin wild type (these fish originate from a Singapore breeding facility where the effective population size approaches tens of thousands of fish) may be closer to what one may consider the "prototypical" zebrafish and we felt such a population would be a better starting point for the first comprehensive DNA microarray-based gene expression analysis [28]. All fish were raised and housed in a standard manner as described previously [28]. In all experiments approximately 50–50% males and females were included in each treatment group.

2.2. Alcohol treatment

Our treatment employed for the chronic alcohol group was a continuous exposure to alcohol without repeated and/or prolonged withdrawal from the substance. We choose this simple chronic paradigm to avoid the potential complications arising from mixing alcohol adaptation related processes with withdrawal induced or sensitization induced mechanisms [11]. Although in the human clinic chronic alcohol exposure almost certainly has several components associated not only with adaptation to the substance but also with multiple withdrawal episodes, we wanted to focus on the mechanisms of adaptation alone, a reductionist approach we feel will allow us to disentangle the complexities of alcohol effects. Another reason why we decided to employ this continuous alcohol exposure dosing regimen is that we utilized the exact same procedure in our prior, behavioral, studies [28] and this way the behavioral and gene expression results will be comparable. Two groups of fish were compared: chronic alcohol exposed and chronic freshwater exposed. Fish were randomly assigned to these groups and sample sizes were 30 for both the chronic freshwater (control), and the chronic 0.50% alcohol exposed fish. All fish were maintained under identical conditions in the same vivarium room as described before [28]. The chronic alcohol concentration of 0.50% was achieved in fish of both the behavioral and gene expression studies by increasing the alcohol concentration in the holding tank by 0.125% in a stepwise manner once every 4 days as described before [28]. Once the 0.50% concentration was reached, it was maintained for an additional 9 days (total of 21 days with continuous alcohol exposure). The alcohol dose was chosen based upon previous studies with zebrafish (for references see [28] and so that the brain alcohol levels would approximate what is observed in the human clinic [58,67]). Increased mortality or morbidity was not observed in any group. To ascertain consistent and continuous alcohol exposure, the alcohol concentration of the holding tanks was monitored using the AM1 Alcohol Analyzer (Analox Instruments, London, UK) and the water (or alcohol solution) of exposure tanks was changed daily as described before [28].

2.3. Analysis of alcohol levels in the brain

In order to properly interpret the alcohol induced gene expression changes we have analyzed the amount of alcohol in the brain of our fish as described before [28]. Briefly, a small subset of fish exposed to the 0.5% EtOH concentration or the freshwater (0.0% EtOH concentration) were analyzed: immediately after the alcohol treatment period, fish were decapitated, their brains were quickly removed and homogenized. The homogenate (whole brain extract) was analyzed using the AM1 Alcohol Analyser (Analox Instruments, London, UK). The instrument works on the principle that alcohol-oxygen oxidoreductase (AOD) catalyzes the enzymatic oxidation of ethyl alcohol to acetaldehyde and the oxygen consumption by the enzyme reduces the oxygen content of the solution, which is quantified by an oxygen sensitive sensor. The instrument has been successfully utilized to measure small alcohol amounts from liquid solution or homogenized solid tissue samples and is expected to be one of the most precise methods (see [28] and references therein).

2.4. Total RNA preparation

At end of the chronic treatment period zebrafish were killed by decapitation and the heads were placed on dry-ice. The brain of the fish was removed and immediately frozen in liquid nitrogen and was subsequently stored at −80 °C until RNA extraction. Brain samples were homogenized in TRIzol reagent (100 mg/ml) and total RNA was extracted according to the manufacturer's protocol (Invitrogen Corporation, Carlsbad, CA). In order to minimize between-animal variability, samples were pooled for fish kept in the same aquarium ($n = 10$ per aquarium). We had 3 such aquaria for alcohol treated and 3 for control fish. That is, a total of 6 RNA sample pools were prepared and separately analyzed (i.e. 6 DNA microarray chips were used). The total RNA was purified using the RNeasy Mini Kit (QIAGEN Inc., Mississauga, CA). The RNA concentrations were determined; the A_{260}/A_{280} and A_{260}/A_{230} ratios were calculated as indices of protein and volatile compound contamination, respectively, using a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Inc., Wilmington, DE, USA). The integrity of the total RNA was determined by electrophoresis on a 1.5% denatured agarose formaldehyde gel stained with ethidium bromide. Total RNA was then used for microarray analysis and quantitative RT-PCR experiments.

2.5. DNA microarrays, cDNA probe synthesis, hybridization and scanning

The NimbleGen Zv7 Zebrafish Gene Expression 385K microarray used in the study contained 37,157 probe sets with up to 12 probes of 60mer oligonucleotides per gene. The arrays represented at least 24,000 genes plus additional ESTs from multiple zebrafish tissues. The design of the array relied on gene and EST information from several sources; Ensembl 46 (August 2007, Zv7), RefSeq (September 2007), TIGR (Release 14.0), UniGene (Build 54), Vega 27, and ZGC (August 2007).

10 μg total RNA of each pooled sample was converted to double-stranded cDNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). The synthesized cDNAs were then incubated with 1 μl of 4 mg/ml RNase A at 37 °C for 10 min, precipitated and the pellet dissolved with 20 μl of VWR water. Each cDNA sample was quantified and verified to meet the following requirements: (concentration ≥ 100 ng/μl; $A_{260}/A_{280} \geq 1.8$, $A_{260}/A_{230} \geq 1.8$). 1 μg cDNA of each sample was synthesized as probe and labeled with Cy3-conjugated random nonamers (TriLink Biotechnologies, San Diego, CA) and hybridized to a NimbleGen Zv7 Zebrafish Gene Expression 385K microarray following the protocol by Roche NimbleGen, Inc. (Madison, USA). The microarrays were incubated on the NimbleGen Hybridization System 4 (Roche NimbleGen) for 16 h at 42 °C. The pools of RNA (see above) from alcohol treated and control zebrafish brains were used to generate cDNA probes, which were then hybridized to microarrays separately. The gene expression level and folds changes (alcohol treated vs. control) were calculated from three separate samples.

The hybridized slides were washed at 10× wash buffer I, II and III (Roche NimbleGen), dried by nitrogen gas at room temperature, and scanned with an Axon GenePix Pro 4200A microarray scanner at 5 μm resolution, 532 nm wavelength (Molecular Devices, Sunnyvale, CA) associated software GenePix Pro software (Axon, Union city, CA, USA).

2.6. Global gene expression analysis

The scanned images of the arrays were quantified using NimbleScan software (Roche NimbleGen). The expression data were normalized by quantile normalization across replicate on arrays as described previously [6]. The gene expression values were generated by RMA (Robust Multichip Average) analysis [36]. Subsequent microarray data analysis was performed using ArrayStar software (DNASTAR, Inc., Madison, USA). Average ratios of expression values of chronic alcohol treated vs. control fish were calculated from three experiments. Genes were considered differentially expressed when the level of expression change in brain of the alcohol treated fish was at least 2-fold (up-regulation or down-regulation) as compared to the control fish brains and the difference between the alcohol treated and control groups was significant ($p < 0.05$, moderated t-test, in which the false discovery rate was controlled by the Benjamini Hochberg correction method).

2.7. Functional classification of differentially expressed genes

The UniGene ID numbers of genes that showed significant differential expression were uploaded into the Database for Annotation, Visualization and Integrated Discovery [35,18] (http://david.abcc.ncifcrf.gov) to obtain DAVID gene ID conversion and functional annotation. The genes were classified into functional groups using 'GO TERM Molecular Function at all levels'.

2.8. Real-time RT-PCR analysis

Real-time RT-PCR is often employed as a follow-up validation procedure subsequent to DNA microarray analysis. While real-time PCR and the microarray analysis may be associated with different technical subtleties and issues, the former is considered more quantitative and more precise. Ten genes were selected as targets from different functional groups and fold change ranges for follow-up investigation. β-Actin was used as the control gene for normalization. Primers were designed online with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/). The primer