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Ethanol Exposure in Rats and its Effect on Genetic Markers

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Chapter 1 Background

Aims and Objectives

Alcoholism is believed to affect an estimated 18 million people each year. In 1993, according to the Institute for Health Policy, it was responsible for more deaths and disabilities in the United States than any other cause (Institute for Health Policy, 1993). The number of people abusing alcohol is three times the number of people abusing all other substances combined (Institute for Health Policy, 2001). In 1991-1992, 5.6 million people abused alcohol and an additional 8.2 million were alcohol dependent. This compares to 9.7 million and 8.9 million, respectively, in 2001-2002, representing a 35% increase for alcohol abuse.

The social and economic burdens of alcoholism are substantial as well. An estimated \$276 billion per year (Institute for Health Policy, 2001) is spent to combat the influence of alcoholism in this country alone; this is more than cancer, heart disease, and diabetes, combined. At least two major factors contribute to these costs. First, alcoholism has affects not only the adult abuser, but also family and friends of the abuser, and innocent bystanders who are injured in alcohol-related accidents. Second, the problem of alcoholism can begin very early in one's life and extend for decades. Fetal exposures are capable of predisposing individuals toward this behavior by causing devastating and permanent effects on one's health and well-being, damage to the central nervous system, liver, and heart, as well as physical malformations. Alcohol abuse can cause deficits in learning and memory, reduced ability to

retrieve verbal and nonverbal information, compromised attention, and diminished function in visuospatial tasks (Brown and Tapert, 2004). Alcohol abuse also increases the risk of violent injury. Unfortunately this is a growing problem in young America. An estimated 10 million Americans 12-20-years-old have had at least one drink in the past month and 2/3 of them were binge drinkers. In 2003, nearly one-half of 8th graders consumed alcohol at least once per month and the vast majority of high school seniors (77%) report using alcohol. Nearly 2/3 of high school seniors self-report that they have been drunk and 1/3 claim to have binged in the last two weeks. Alcohol has been associated with the three leading causes of death and injury among adolescents: suicides, homicides, and motor vehicle crashes. (CDC, 1990) In 1999, the total economic burden of underage drinking alone was greater than \$58 billion per year (Levy and Stewart, 1999).

A full understanding of the effects of alcohol on the developing nervous system and other organ systems, and knowing when and where that central or internal organ damage has occurred after a certain period of abuse will allow us to identify at risk individuals and allow for appropriate and efficient interventional strategies. The goal of the present study was to try and identify which peripheral biomarkers indicate when early evidence for central nervous system damage has occurred. We performed this using a well-established rat model of drinking with the intention of identifying genes in peripheral blood whose expression patterns are strongly correlated with

damage to the brain but separate from changes produced by damage to the heart or liver.

The model employed chronic daily drinking beginning at an earlier adolescence or young adulthood. Ultimately, wish to test the most promising genes in human subjects currently undergoing treatment for alcohol abuse.

Literature Review

The consequences of ethanol exposure during developmental periods can be very severe. It is a potent toxin to many different cell types and organs, including the brain. The most vulnerable time is during development, it spans from the third week of gestation to the third decade after birth. The central nervous system reacts to pre- and neonatal exposure to ethanol in a different way than it does to postnatal exposure. It affects proliferating neuronal precursors (Luo and Miller 1998) and the CNS can respond to this trauma by altering the numbers of cells being generated. These effects cannot occur after birth because, by then, most of neuronal generation is complete. The postnatal animal can only respond by modifying inter-neuronal interactions, or by cell death of mature neurons. Also, in other organs, such as the liver, cell death occurs. (Luo and Miller 1998) It is useful to diagnose when such central or internal organ damage has occurred in adult humans, who began abusing alcohol either as an adolescent or mature adult

Studies have been done that demonstrate the effects of adolescent ethanol exposure. In a 4-day period of multiple ethanol intubations (resulting in exposures of 9-10 g/kg/day), rats exhibit substantially more ethanol-induced damage to brain regions including the frontal cortex than similarly treated adults (Crews et al., 2000).

Rats exposed chronically to ethanol over 20-days including much of adolescence exhibit a larger impairment in working memory than adults

exposed to ethanol for 20 days. Animals either during adolescence or adulthood were exposed to ethanol in a binge-pattern. Twenty days after the final ethanol exposure when all subjects were adults, their spatial working memory was examined in the absence and presence of an acute ethanol challenge. The subjects were tested using an eight-arm radial maze where they were trained to perform a spatial working memory task. At the beginning of these training sessions, subjects retrieved food rewards on four of the eight arms. After a time period, they were allowed to retrieve food from the remaining four arms. It was found that animals treated with ethanol during adolescence exhibited larger working memory impairments during an ethanol challenge (1.5 g/kg intraperitoneally). The findings indicate that binge pattern exposure to ethanol during adolescence enhances responsiveness to the memory-impairing effects of ethanol in adulthood. (White et al., 2000)

Also, exposure to ethanol vapor for 5 or 10 days has recently been reported to alter parietal and hippocampal EEG activity in adolescent rats (Slawewski et al., 2001). Also, in another study the opportunity to consume alcohol voluntarily during adolescence was found to increase later aggressive behavior in male golden hamsters (Ferris et al., 1998; Shtiegman et al., 1997).

Adolescents with alcohol use disorders have been reported to have smaller hippocampal volumes than comparison subjects, with these hippocampal volumes correlating positively with onset age and negatively with duration of the use disorder (De Bellis et al., 2000). As many as 30,000 synapses are lost per second during the pubertal/adolescent period; this is

believed to transform the brain into one that is leaner and presumably more efficient. It is often during periods of such rapid developmental change that the brain is especially vulnerable to perturbation---including ethanol sensitivity. This leads us to the importance of developing tools for rapid and early intervention and definitive diagnosis when problems are suspect. Using peripheral biomarkers that are correlated with central or internal organ damage can be crucial in assessing the damage that has been done.

The cerebellum can be used as a central reference tissue for the effects of ethanol. The behavioral manifestations of acute and chronic ethanol use and abuse include both motor and cognitive disturbances. The motor symptoms are similar to those associated with cerebellar dysfunction; ataxia, dysmetria, and decomposition of movement. This is a result from the direct action of ethanol on cerebellar motor circuits that transfer information between the cerebellum and sensorimotor regions of the cerebral cortex. The neural system responsible for the cognitive symptoms of ethanol abuse is more difficult to pinpoint; the profound deficits in attention, working memory, verbal fluency and reasoning, can be linked to the hippocampus and association cortex.

(Middleton and Strick 1994).

A study done by Lieber and DeCarli, showed that chronic daily ethanol consumption in adult rats alters neurotrophin expression in brain. There is a significant impact on the expression of various neurotrophins in the brain in a time- and region-dependent manner. In that study, adult (3-to 4-month-old) male Long-Evans rats were fed ethanol daily for 8 or 24 weeks,

and divided into weight-matched pairs. One animal from each pair was fed ad libitum a high-carbohydrate, liquid diet containing 6.7% (v/v) ethanol (Lieber and DeCarli, 1986). The other rat was pair-fed a control diet (with the same nutrients and calories) but with no ethanol. The five structures dissected from each brain included three cortical areas (parietal and entorhinal cortices and the hippocampus) and two segments of the basal forebrain (the basal and septal nuclei). An enzyme-linked immunosorbant assay (ELISA) was used to quantify the levels of each protein of interest. The results of these studies clearly demonstrated an effect of time and region on the levels of neurotrophin or neurotrophin receptor levels in the brain. (Lieber and DeCarli, 1986)

Another study demonstrated that repeated episodic ethanol consumption in adult rats alters neurotrophin expression in brain. Three groups of mature (3 1/2-month-old) male Long-Evans rats were used. One group was exposed to ethanol episodically, fed a liquid ethanol-containing diet ad libitum three consecutive days per week for 6, 12, 18, or 24 weeks. On the other 4 days each week, the rats were fed a liquid control diet ad libitum that was matched for caloric and nutritional content. A second group of rats was weight-matched with the Et-fed rats, fed Ct for 3 days that was defined by the amount of food consumed by the paired Et-fed rats. The other 4 days, food was provided ad libitum. The third group of rats was fed chow and water ad libitum throughout the study. These rats were used as controls for the Ct-fed rats and to assure that malnutrition and aging were not confounding factors. The results showed that the repeated episodic treatments appeared to be

equally effective at altering neurotrophins. Consequently, there is a clear interaction of the brain region and the duration of exposure on the levels of protein. These results are similar to those in the chronic daily paradigm, even though the animals had access to ethanol for less than half as many days.

There were two brain regions fairly consistent alterations at most of the time points: the parietal cortex showed trends for increased expression of NGF, BDNF and NT-3, while the basal forebrain showed decreases for NGF and NT-3 (but not BDNF). (Mooney and Miller 2004)

Chapter 2 Method and Materials

In this study, twelve pairs of rats were divided into two groups of young adults and adolescents (n=6 pairs/group/age/gender). One rat in each pair was fed a liquid diet containing 6.7% ethanol for a total of 3 weeks and the other animal in each pair received a liquid diet without alcohol, equal in calories and quantity to that which the ethanol animal consumed. These rats are called “pair-fed” controls. During the feeding period, samples of venous blood were obtained and assayed for blood ethanol concentrations. After three weeks, these animals were euthanized and blood and brain tissues (specifically the hippocampus and the cerebellum) were dissected for analysis of gene expression patterns. We extracted the RNA from both the blood and brain samples. The RNA was purified from these samples using the RNeasy kit from Qiagen, amplified and labeled it using the standard Affymetrix protocol. The labeled RNA was then sent for microarray analysis and it was these data that we analyzed. The reason for using RNA is that it tells us what genes are expressed or changed across brain and blood samples as a result of ethanol consumption.

Microarrays use hundreds of thousands of probes to screen for genes whose expression may be altered in pathologic and experimental conditions. The advantages of using this method include the fact that only a minute amount of tissue is needed and the high probability of unanticipated results. The disadvantages include the fact that it is costly, we depart from the classical scientific method of hypothesis-driven experiments, it requires validation and also it requires interpretation. For our studies, we used the

GeneChip Rat Gene 1.0 ST Array. Each of the 27,342 genes is represented on the array by approximately 26 probes spread across the full length of the gene, which allows for a complete and accurate picture of gene expression. “Gene-level” analysis of multiple probes on different exons is summarized into an expression value representing all transcripts from the same gene. (Gene Chip Arrays)

Three groups of data were collected from the baseline, the pair-fed and the ethanol treated animals. To analyze the data, we used Gene Spring GX (Agilent) software. We did a three-way analysis of variance (ANOVA) test (Diet x Tissue x Pair) so we could study the effect of ethanol consumption across samples in a pair-wise fashion. By conducting this, we were able to filter for the strongest gene expression changes.

At the midpoint of the study, blood was obtained for Blood Ethanol Concentration (BEC) measurements. At the conclusion of the study, additional samples of peripheral blood were obtained to monitor BEC levels, and for analysis of gene expression differences between ethanol consuming and pair-fed control rats.

Chapter 3 Results

We first looked at the consumption of the rats, adolescents versus adults. Examination of the consumption data in the figure below revealed a number of striking observations. First, as seen in Figure 1, adolescent rats (both males and females) consumed significantly more liquid ethanol diet (mL/kg body weight) than their adult counterparts. Moreover, within the adult rats, females were observed to consume more than their male counterparts throughout the duration of the treatment period. Beyond the consumption data, we also observed that the BEC levels produced were also correspondingly higher in adolescent rats than in adult rats. This is shown in Figure 2. Interestingly, however, while adolescent male and female rats consumed highly similar amounts of ethanol (Figure 1), the BEC levels were considerably higher in female adolescent rats than male adolescent rats (220.3 mg/dL vs 151.3 mg/dL).

Figure 1 Consumption of Adolescents and Adults (Male and Female)

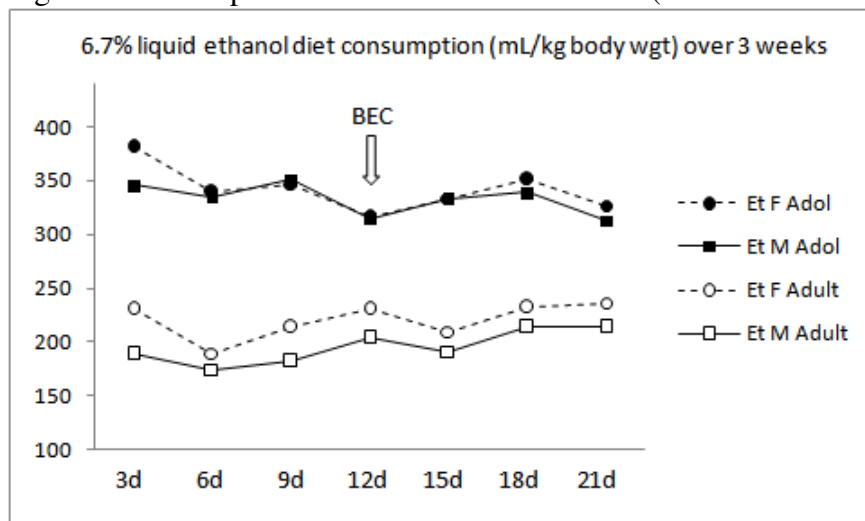
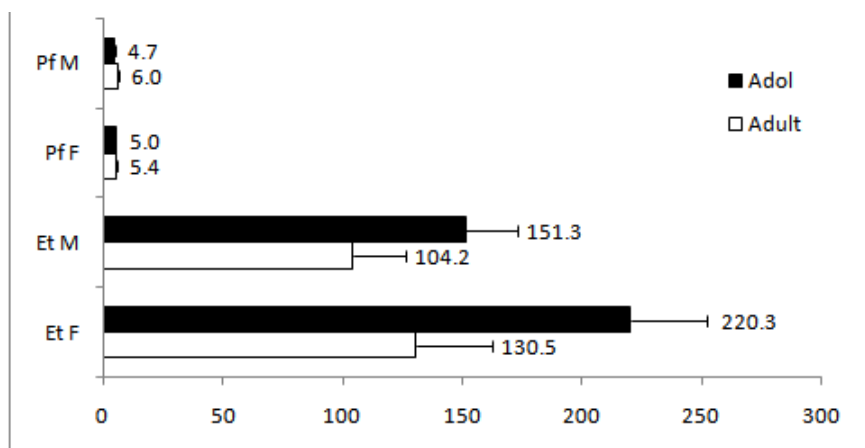
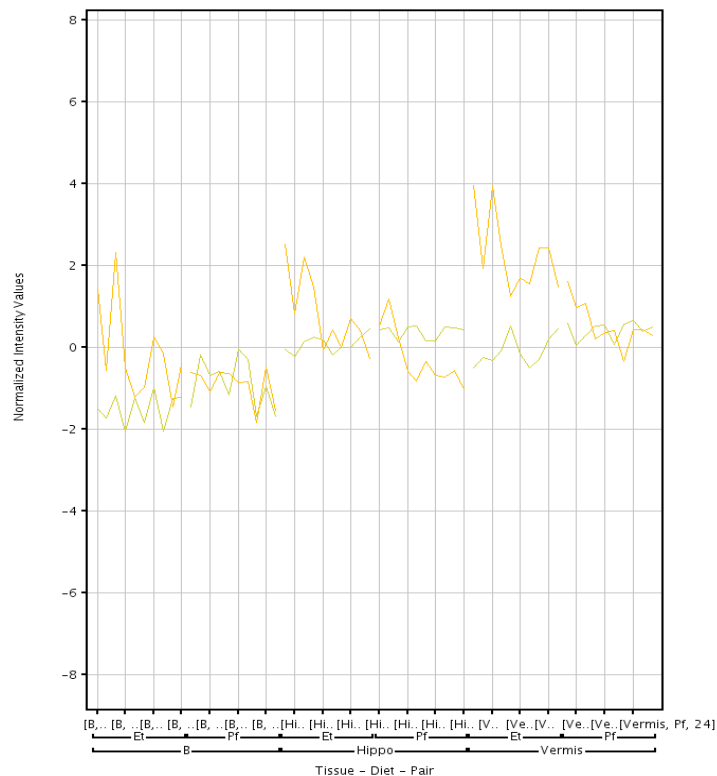


Figure 2 Blood Ethanol concentrations (mg/dL) at midpoint of 3 week study



We found two genes that showed highly significant changes in all tissues, the Fos gene and the Hfrlike gene. The following chart shows these two genes after a fold change using the analysis software program.

Figure 3 1.2 Fold Change of Microarray Data



Using the microarray data which we combined with data from our baseline, we were able to identify a distinct set of peripheral blood biomarkers which may be able to predict functional genomic changes in the brain that have occurred as a consequence of this mode of ethanol exposure. In the first stage of analysis, we used RMA normalization of the data from a set of 102 Rat ST Gene Arrays (3 tissues x 24 ethanol or pair fed adolescent rats + 3 tissues x 10 P29 rats). These data were analyzed in GeneSpring GX 9 (Agilent) using two complementary statistical approaches. First, we

performed a 3 way ANOVA (diet x tissue x pair) to identify genes with strong pairwise differences in expression according to the diet that the animals consumed. The significance thresholds were adjusted using the Holm-Bonferroni step down procedure. Next, in order to allow for greater power to detect genes with strong changes in expression due to the ethanol consumption, we used a Fisher's exact test to examine the strength of association between consumed dose and BEC levels and expression level. The P values from this test were also corrected using a Holm-Bonferroni step down procedure. Inspection of the data in the table below indicates the presence of a set of 14 genes whose expression levels differ significantly between ethanol-fed and pair-fed animals and are also robustly associated with the amount of ethanol dose that the ethanol-fed animals consumed throughout adolescence.

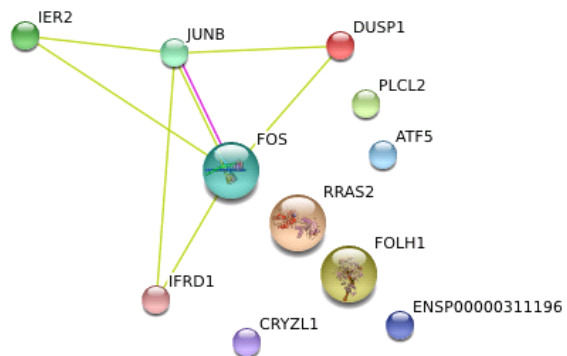
Figure 4 Table of 14 genes with significant expression changes

| Gene ID | Mean Pairwise Fold Change | | | 3 way ANOVA | Assoc to EtOH | Symbol | Gene |
|----------|---------------------------|------|--------|-------------|---------------|--------|---|
| | Blood | Hipp | Vermis | Corrected P | Fisher's P | | |
| 10721480 | 2.09 | 1.13 | 1.15 | 0.00063 | 0.00007 | Atf5 | Activating transcription factor 5 |
| 10886031 | 1.59 | 1.84 | 3.11 | 4.54E-06 | 4.11E-09 | c-fos | FBJ murine osteosarcoma viral oncogene homolog |
| 10940689 | 1.57 | 1.14 | 1.23 | 0.00582 | 2.62E-10 | lfrd1 | Interferon-related developmental regulator 1 |
| 10795548 | 1.49 | 1.15 | 1.17 | 0.00582 | 0.00011 | Khc | Kinesin family member 5B |
| 10926181 | 1.46 | 1.18 | 1.14 | 0.04630 | 0.00444 | Plc2 | Phospholipase C-like 2 |
| 10825113 | 1.31 | 1.13 | 1.37 | 0.04630 | 0.04856 | Ckip1 | Similar to TNF intracellular domain-interacting protein |
| 10723523 | 1.25 | 1.17 | 1.13 | 0.04630 | 0.04024 | Folh1 | Folate hydrolase |
| 10732652 | 1.16 | 1.89 | 2 | 0.04630 | 0.00511 | Dusp1 | Dual specificity phosphatase 1 |
| 10806585 | 1.15 | 1.57 | 1.87 | 0.00063 | 3.83E-06 | Junb | Jun-B oncogene |
| 10806685 | 1.15 | 1.4 | 1.45 | 0.00582 | 0.00034 | Ier2 | Immediate early response 2 |
| 10727717 | 1.1 | 3.03 | 3.64 | 0.00582 | 0.00008 | Nxf | Neuronal PAS domain protein 4 |
| 10753172 | 0.9 | 0.9 | 0.87 | 0.04630 | 0.02162 | Donson | Downstream neighbor of SON |
| 10743357 | 0.84 | 0.81 | 0.93 | 0.00582 | 0.00697 | Rras2 | Related RAS viral (r-ras) oncogene homolog 2 |
| 10797009 | 0.68 | 0.92 | 0.72 | 0.00582 | 0.01912 | -- | ENSRNOT00000044597, ENSRNOT00000061528 |

We then did a biological cluster analysis using the STRING database which indicated the existence of strong relationships between a set of the

genes in the table presented in Figure 4, especially JunB, Ier2, c-fos, Ifrd1, and Dusp1 (see Figure 5 below).

Figure 5 Biological Cluster Analyses



Chapter 5 Discussion

After completion of our first set of analyses, we found two genes that showed highly significant changes in all tissues, the Fos gene and the Hfrlike gene. They filtered through a 1.2 fold change, which is a statistical procedure that tells us to what extent these results are reproducible. Despite their significance, we have concluded that these two genes are not strong candidates for being specific biomarkers of ethanol consumption when we look at what is known about them. The Fos gene is known to fluctuate with exposure to other substances such as cocaine, and amphetamine, and the increase we detected in the brain and blood of ethanol consuming animals may merely reflect increases in cellular activity. We are looking for a gene expression change that is specific to ethanol.

The results of the biological cluster analysis using the STRING database indicated the existence of strong relationships between a set of the genes, especially JunB, Ier2, c-fos, Ifrd1, and Dusp1. Indeed, this is perhaps not surprising given that several of these genes are transcription factors or immediate early genes. Transcription is a process where RNA is synthesized under the direction of DNA. Messenger RNA carries a genetic message from the DNA to the protein-synthesizing machinery of the cell. So although DNA holds all the genetic information, it is selective in what is expressed through RNA. These genes are believed to be part of this process. So it is not surprising that there is a strong relationship between them. We can conclude that although it is significant findings, it is not interesting.

The consumption of the rats, adolescents versus adults, was also noted in this study. We found some interesting observations. First, adolescent rats (both males and females) consumed significantly more liquid ethanol diet (mL/kg body weight) than their adult counterparts. This can be a result of adolescents being more likely to consume more than adults.

Moreover, within the adult rats, females were observed to consume more than their male counterparts throughout the duration of the treatment period. The BEC levels produced were also correspondingly higher in adolescent rats than in adult rats. This might be due to the presence of alcohol dehydrogenase, the enzyme that breaks down ethanol which can be toxic to the body. A greater amount of this enzyme will cause a greater BEC level. It seems that more of this enzyme is present or expressed in the adolescent. It may be that with increased age, the gene that is responsible for this enzyme is not expressed as much.

Interestingly, yet, while adolescent male and female rats consumed highly similar amounts of ethanol, the BEC levels were considerably higher in female adolescent rats than male adolescent rats (220.3 mg/dL vs 151.3 mg/dL). These gender differences seem to be very interesting and validate previous findings. We attribute these differences in BEC produced after consumption of identical doses of ethanol to the well established gender differences in gastric levels of alcohol dehydrogenase in male and female rats. There is higher activity of alcohol dehydrogenase enzyme in females leading to greater BEC levels.

While it is tempting to speculate about the functional implications of these findings at this point, these observations need to be validated and compared with those seen in adults on the same diet. Comparing this data to an animal model that has undergone binge drinking ethanol might also give us some interesting results. Hopefully, with this data combined to what we already have, we will obtain stronger evidence for a specific genetic marker that will show a reproducible change in expression in the blood of animals consuming ethanol which will also be occurring in the brains of these animals. However, in justification of our findings, there are reports of changes in some of these same genes in response to ethanol consumption. Ultimately, we will be able to develop peripheral screening tools for alcohol abuse which will be very useful and relevant for today's world. Greater analysis and further studies will allow this to occur.

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Written Capstone Summary

Examining the effect of ethanol on blood and brain biomarkers

Background

Alcoholism is believed to affect an estimated 18 million people each year. In 1993, according to the Institute for Health Policy, it was responsible for more deaths and disabilities in the United States than any other cause (Institute for Health Policy, 1993). The number of people abusing alcohol is three times the number of people abusing all other substances combined.

Alcoholism is responsible for more deaths and disabilities in the United States than any other cause. Alcoholism has direct effects on the adult abuser, family and friends of the abuser, and innocent bystanders. An estimated \$276 billion per year (Institute for Health Policy, 2001) is what is spent to confront this impact; this is more than cancer, heart disease, and diabetes, combined.

Perhaps most troubling is that alcoholism can begin during adolescence and that even fetal exposures can predispose individuals toward this behavior by causing devastating and permanent effects on one's health and well-being, CNS, liver, and heart damage, as well as physical malformations.

A full understanding of the effects of alcohol on the developing nervous system and other organ systems, and knowing when and where central or internal organ damage has occurred after a certain period of abuse

will allow us to identify at risk individuals and allow for appropriate and efficient interventional strategies.

Methods

In this study, the means of going about investigating this problem involves an animal model of chronic alcohol abuse. In the first studies, male and female adolescent rats were used. One rat in each pair was fed a liquid diet containing 6.7% ethanol for a total of 3 weeks and the other animal in each pair received a liquid diet without alcohol, equal in calories and quantity to that which the ethanol animal consumed. These rats are called “pair-fed” controls. During the feeding period, samples of venous blood were obtained and assayed for blood ethanol concentration. After three weeks, these animals were euthanized and blood and brain tissues were dissected for analysis of gene expression patterns. We extracted the RNA from both the blood and brain samples. This RNA was then transcribed into labeled DNA for the gene chips. This was sent for microarray analysis and it was these data that we analyzed. The reason for using RNA is that it tells us what genes are expressed or changed across brain and blood samples as a result of ethanol consumption.

Microarrays use hundreds of thousands of probes to screen for genes whose expression may be altered in pathologic and experimental conditions. The advantages of using this method include the fact that only a minute amount of tissue is needed and the high probability of unanticipated results.

The disadvantages include the fact that it is costly, we depart from the classical scientific method of hypothesis-driven experiments, it requires validation and also it requires interpretation. For our studies, we used the GeneChip Rat Gene 1.0 ST Array. Each of the 27,342 genes is represented on the array by approximately 26 probes spread across the full length of the gene, which allows for a complete and accurate picture of gene expression. “Gene-level” analysis of multiple probes on different exons is summarized into an expression value representing all transcripts from the same gene.

Three groups of data were collected; the baseline, the pair-fed and the ethanol treated animals. To analyze the data, we used Gene Spring GX (Agilent) software program. We did a three-way analysis of variance (ANOVA) test (Diet x Tissue x Pair) so we could study the effect of a ethanol consumption across samples in a pair-wise fashion. By conducting this, we were able to filter for the strongest gene expression change.

Results

After completion of our first set of analyses, we found two genes that showed highly significant changes in all tissues, the Fos gene and the Hfrlike gene. A biological cluster analysis also revealed strong relationships between a set of the genes, especially JunB, Ier2, c-fos, Ifrd1, and Dusp1. We also found different consumption rates between the adolescent and adult groups, and males and females.

Discussion

Despite their significance, we have concluded that the Fos and Hfr like genes are not strong candidates for being specific biomarkers of ethanol consumption when we look at what is known about them. The Fos gene is known to fluctuate with exposure to other substances such as cocaine, and amphetamine, and the increase we detected in the brain and blood of ethanol consuming animals may merely reflect increases in cellular activity. We are looking for a gene expression change that is specific to ethanol.

The strong relationships between a set of the genes, especially JunB, Ier2, c-fos, Ifrd1, and Dusp1 can also be dismissed. Indeed, this is perhaps not surprising given that several of these genes are transcription factors or immediate early genes. So it is not surprising that there is a strong relationship between them. We can conclude that although it is significant findings, it is not interesting.

Adolescent rats (both males and females) consumed significantly more liquid ethanol diet (mL/kg body weight) than their adult counterparts. This can be a result of adolescents being more likely to consume more than adults. Also, the presence of alcohol dehydrogenase, the enzyme that breaks down ethanol which can be toxic to the body can cause a greater BEC level. It seems that more of this enzyme is present or expressed in the adolescent. It may be that with increased age, the gene that is responsible for this enzyme is not expressed as much.

We attribute the differences in BEC between males and females produced after consumption of identical doses of ethanol to the well established gender differences in gastric levels of alcohol dehydrogenase in male and female rats. There is higher activity of alcohol dehydrogenase enzyme in females leading to greater BEC levels.

While it is tempting to speculate about the functional implications of these findings at this point, these observations need to be validated and compared with those seen in adults on the same diet. We need to obtain stronger evidence for a specific genetic marker that will show a reproducible change in expression in the blood of animals consuming ethanol which will also be occurring in the brains of these animals. However, in justification of our findings, there are reports of changes in some of these same genes in response to ethanol consumption. Ultimately, we will be able to develop peripheral screening tools for alcohol abuse which will be very useful and relevant for today's world. Greater analysis and further studies will allow this to occur.