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# Measuring and Correlating Blood and Brain Gene Expression Levels: Assays, Inbred Mouse Strain Comparisons, and Applications to Human Disease Assessment

A dissertation submitted in partial satisfaction of the requirements
for the degree of Doctor of Philosophy

in

Biomedical Sciences

by

Mary Elizabeth Winn

Committee in charge:

Professor Nicholas J Schork, Chair
Professor Gene Yeo, Co-Chair
Professor Eric Courchesne
Professor Ron Kuczenski
Professor Sanford Shattil

2011

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The dissertation of Mary Elizabeth Winn is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

________________________________________
Chair

University of California, San Diego

2011

## DEDICATION

To my parents,
Dennis E. Winn II and Ann M. Winn,
to my siblings,
Jessica A. Winn and Stephen J. Winn,
and
to all who have supported me throughout this journey.

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## ABSTRACT OF THE DISSERTATION

### Measuring and Correlating Blood and Brain Gene Expression Levels: Assays, Inbred Mouse Strain Comparisons, and Applications to Human Disease Assessment

by


Mary Elizabeth Winn

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2011

Professor Nicholas J Schork, Chair

Professor Gene Yeo, Co-Chair



Microarray-based gene expression profiling is a frequently utilized tool in the search for disease-specific molecular patterns and the development of clinically relevant panels of biomarkers. Although advances in high-throughput gene expression technology make for more reliable and interpretable studies, investigations of living humans are often limited by tissue accessibility. This is especially true for neural-based illnesses, where studies rely heavily on post-mortem brain tissue. As a result, medical researchers have focused on blood, a more easily

accessible and clinically obtainable tissue. In this work I explore: 1.) the technical aspects associated with assessing peripheral whole blood gene expression via microarray; and 2.) the biological significance of blood-based gene expression patterns with respect to brain-based gene expression patterns and behavioral phenotypes in mice and humans. I describe the effects of globin reduction on blood-based gene expression in mice by comparing gene expression patterns before and after globin reduction of mouse whole blood (Chapter 2). Globin reduction was found to improve the ability to detect low abundance, biologically relevant genes. I also evaluated globin reduction in the context of human blood and two Illumina gene expression assays: (i) the IVT-based direct hybridization assay; and (ii) the WG-DASL assay (Chapter 4). As in mice, I was able to recapitulate the known benefits of globin reduction in both assays, while WG-DASL appeared to be more sensitive compared to IVT. Lastly, I characterized the correlations between blood gene expression levels and behavioral phenotypes and compared blood gene expression-trait correlations with brain gene expression-trait correlations in respect to neuropsychiatric phenotypes in mice (Chapter 3) and autism in humans (Chapter 5). In both mice and humans, blood was only able to capture a small portion of the associations identified in the brain on an individual gene level. At a pathway level, blood was able to capture a larger portion of the associated brain pathways in humans as compared to mice. I conclude blood gene expression, although it may capture a small portion of the expression patterns associated with ‘primary’ neural insults, is more likely to capture variation due to ‘secondary’ perturbations or other biological and environmental insults.

# CHAPTER 1

## Introduction and Background

## INTRODUCTION

### Translational Genomics, Genome-wide Expression Analysis, and Biomarker Discovery

Since the completion of the Human Genome Project in 2003 (International Human Genome Sequence Consortium 2004), the Phase1 and Phase 2 HapMap Projects in 2005 (International HapMap Consortium 2005) and 2007 (Frazer et al. 2007), and the pilot phase of the Encyclopedia of DNA Elements (ENCODE) project in 2007 (Birney et al. 2007), there has been an explosion in the development of resources and studies aimed at identifying the fundamental causes of complex human disease (Topol et al. 2007). In order to determine the specific genetic factors mediating disease susceptibility, researchers have utilized a variety of strategies including direct DNA sequencing, single-nucleotide polymorphism (SNP) genotyping-based genome-wide association studies, global gene expression, proteomic and metabolomic studies, and *in silico* and computational model analysis of gene and sequence function. Much of the focus of these research efforts has been to identify genetic factors contributing to disease – either as stable markers of disease susceptibility or as ‘biomarkers’ whose elevations and de-elevations are indicative of disease pathogenesis – so that the resulting insights can be ‘translated’ into viable medical practices and there by usher in an era of genetics-based “personalized medicine” (Feero et al. 2008).

Biomarker discovery thus plays a key role in the process of translating scientific breakthroughs to the bedside. As a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention, a biomarker

can be used to classify disease states, develop diagnostic and prognostic tools, or illustrate drug efficacy and toxicity (Atkinson et al. 2001). Classic biomarkers have included physiological measurements (blood pressure, cholesterol levels), imaging (x-ray), and protein molecules (human chorionic gonadotropin (hCG), C-reactive protein (CRP)). Advances in genomics and associated molecular biology and pathology technologies have served to revolutionize the field, giving rise to hundreds (if not thousands) of potential genetic and molecular markers of disease, such as SNP genotypes and gene expression profiles. However, many of these candidate biomarkers have failed to be effective when tested in clinical settings. As of August 1, 2011, the US Food and Drug Administration (FDA) had approved 81 genetic biomarkers from the hundreds that have been implicated in pharmacogenetic studies (**Table 1-1**) (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm).

Gene expression is a highly regulated process by which genomic information is converted to a functional gene product such as RNA or protein. Individual differences in gene expression that arise from genetic, epigenetic, or environmental variation are likely an underlying cause of complex human disease. Microarray-based gene expression profiling has become a frequently employed tool in the search for disease-specific molecular patterns (i.e., disease 'fingerprints') and the development of clinically relevant panels of biomarkers. Since the advent of microarray technology in the mid-1990s, over 30,000 articles have been published on microarray gene expression with over fifty percent published in the last four years (**Figure 1-1**). Investigations utilizing microarrays have yielded insights into disease classifications (Golub et al. 1999, Alizadeh et al. 2000, Dyrskjot et al. 2003),

diagnostic and prognostic gene profiles (Mirnics et al. 2000, Welsh et al. 2001, van 't Veer et al. 2002), and drug efficacy and toxicity profiles (Gunther et al. 2003).

**Neuropsychiatric Diseases, Tissue Accessibility and Blood-based Gene Expression**

Although advances in gene expression technologies probably made studies of gene expression more reliable and interpretable than they were 20 years ago, gene expression studies on living humans are still limited by tissue accessibility. This is especially true for neuropsychiatric illnesses, where it is nearly impossible to collect brain samples from living individuals. As a result, the majority of studies of neuropsychiatric diseases rely on post-mortem brain tissue. Alternatively, medical researchers have begun to focus on peripheral blood (e.g., leukoyctes, lymphocytes, lymphoblastoid cell lines (LCLs), peripheral blood mononuclear cells (PBMCs), whole blood), a more easily accessible and clinically obtainable tissue.

Aside from accessibility, the physiological characteristics of blood cells suggest peripheral blood is an ideal surrogate for primary tissue (Liew et al. 2006). Importantly, the natural variation, heritability, and processing-induced variation of blood-based gene expression have been examined (**Table 1-2**), while the advantages and disadvantages of studying blood gene expression patterns have been carefully reviewed (Fan et al. 2005, Mohr et al. 2007). Nonetheless, the biological relevance of gene expression levels in the blood to human neuropsychiatric disease remains relatively unknown, given that brain tissue and blood would be difficult to collect from the same living individual (Sullivan et al. 2006). Given the significant amount of intra- and inter- individual variation in blood gene expression, such an approach limits

interpretability and brings in to question a study's ability to identify reliable and replicable biomarkers for the diagnosis and treatment of human disease.

Blood-based gene expression correlations with neuropsychiatric conditions and behaviors are quite likely to reflect 'secondary' if not 'primary' molecular perturbations in the diseased brain, such as the presence of a tumor or an immunological insult. As a result, the secondary patterns of, or changes in, gene expression identified in the blood may be seen as biomarkers of a disease state rather than potential targets for pharmacological intervention. On the other hand, in certain neuropsychiatric states, there could be an actual over- or under- deposition of, e.g., neurohormones, into the bloodstream that reflects the primary (or one of the *many* primary) etiological defects contributing to these states. Thus, ultimately, blood-based gene expression patterns associated with certain neuropsychiatric states may indeed reflect a combination of primary and secondary effects.

**Mouse Models of Human Disease**

Unlike human subjects, blood and primary tissue samples can be easily collected from the same living mouse under highly controlled conditions. Intra- and inter-individual sources of variation introduced by gender, age, time of day, genetic variation, and environment can be studied and accommodated (Whitney et al. 2003, Radich et al. 2004, Cobb et al. 2005), while clinically acceptable and highly standardized protocols for blood collection, RNA isolation, and globin reduction can be employed to lessen technical-induced variation (Debey et al. 2004, Cobb et al. 2005, Debey et al. 2006). Mice have also been widely used to mimic and provide insight into the genetic basis of human disease since the early 1900s (Rosenthal and

Brown 2007) with an assortment of valuable online resources now available (**Table 1-3**)(Peters et al. 2007).

Studies of human disease have benefited from the vast pool of genetic resources developed and utilized in mice, such as: 1. quantitative trait locus (QTL) mapping to identify genomic locations harboring mutations that influence a relevant phenotype's expression; 2. the creation of chromosome substitution, recombinant-inbred, and congenic mouse strains to explore the impact of specific chromosomes and genetic locations on phenotype expression; 3. *in silico* mapping studies which compare known polymorphic sites across different mouse strains to phenotypic differences exhibited by those strains; 4. complex "genetical genomics" analyses mixing mouse strain crosses, QTL mapping strategies, and gene expression studies to uncover complex gene expression regulatory networks behind phenotype expression; 5. mutagenesis strategies designed to correlate specific mutations with specific phenotypes; and 6. knockout and transgenic studies which consider the effects of specific genes on the expression of a particular phenotype (Peters et al. 2007). Putting such efforts and studies into context requires understanding the genetic and phenotypic backgrounds of the strains used in these studies. In this light, characterizing correlations between blood and brain gene expression levels and gene-phenotype relationships across commonly used inbred mouse strains, although not trivial, may contribute to our understanding of previous mouse-based studies, yield compelling candidate genes for human neuropsychiatric diseases, and provide insight into the potential of blood-based gene expression patterns as biomarkers of neuropsychiatric conditions.

As noted, however, mouse models of human disease are not without their

drawbacks (i.e. genetic background) (Rivera et al. 2008). This is especially true for models of neuropsychiatric disorders and behavioral phenotypes, which are hampered not only by a variety of genetic and environmental factors but also by a lack of clear disease and behavioral definitions for mice (Bucan and Abel 2002, Cryan and Mombereau 2004). Furthermore, gene-phenotype studies in humans that are based on gene expression studies in mice are complicated by a lack of insight as to how the genes in question fit into larger species and tissue-specific regulatory networks that influence phenotypic expression, with no firm understanding of the issues and potential effects these forces may have had in shaping the function or impact of a gene of interest and its associated regulatory network in the human biochemical and physiologic milieu. Thus, it is important to fully characterize the genetic, regulatory, biochemical, and phenotypic backgrounds of the mouse strains used in studies of neuro-cognitive and behavioral phenotypes in an effort to not only put previous studies into context, but also direct future studies.

**Microarray Gene Expression Profiling and Globin Reduction**

Since blood is high in alpha- and beta- globin, which tend to confound detection of expression levels of non-globin genes, microarray-based gene expression profiles of peripheral whole blood suffer from poor sensitivity and high variability, hampering their utility as reliable and reproducible clinical biomarkers of disease. A variety of globin reduction methods were developed to address the needs associated with microarray-based evaluation of peripheral whole blood, including: peptide nucleic acids (Debey et al. 2006) and magnetic beads (Whitley et al. 2005). These globin reduction methods have been tested using a variety of microarray

platforms (Tian et al. 2009; Vartanian et al. 2009; Dumeaux et al. 2008; Debey et al. 2006) and often validate the importance of globin reduction in the assessment of human peripheral whole blood. These studies also indicate globin reduction may not always be beneficial or necessary. Globin reduction of human whole blood samples is known to require a relatively large amount of input RNA (Vartanian et al. 2009), may induce its own unique expression profile (Liu et al. 2006), or fail to improve reproducibility (Vartanian et al. 2009; Dumeaux et al. 2008). It is therefore necessary to continue to consider the effects of globin reduction as new microarray platforms are developed and different organisms (i.e. *Mus musculus* or *Rattus norvegicus*) are used as the source of whole blood.

Although globin reduction methods have been developed for mouse whole blood (Whitley et al. 2007), little has been done to characterize the effects of globin reduction of microarray gene expression profiling of mouse whole blood. Globin reduction of human peripheral whole blood improves detection levels of genes in various biological processes (Field et al. 2007) and genes relevant to disease (Raghavachari et al. 2009). Mouse whole blood, on the other hand, has a higher ratio of reticulocytes to lymphocytes (32:1) than human whole blood (9:1)(Fan et al. 2005). Globin reduction of mouse whole blood may not be able to overcome the significantly higher level of globin. If researchers are to use the mouse as a model of human disease it will be important to understand the limits of whole blood gene expression profiling in mice.

Researchers must also understand the need for globin reduction with various microarray platforms. Globin reduction has been shown to be effective using Affymetrix GeneChips (Vartanian et al. 2009) and the standard Illumina *in vitro*

transcription assay with (Tian et al. 2009) while globin reduction provided no benefit when used in conjunction with the Applied Biosystems AB1700 microarray system (Dumeaux et al. 2008). As microarray gene expression platforms continue to develop and become more accurate, so will the need to assess effects of globin reduction on whole blood gene expression profiling. For example, Tian et al. assessed globin reduction in the context of *in vitro* transcription amplification hybridized to the Illumina Sentrix HumanRef-6 BeadChip. The HumanRef-6 BeadChip has since been replaced by the HumanRef-8, and more recently by the HumanRef-12. Each BeadChip assesses a different number of probes with the potential to affect the background noise due to high levels of globin in whole blood. Other technologies created for the profiling of highly degraded samples, such as the Illumina Whole-Genome DASL (cDNA-mediated Annealing, Selection, extension and Ligation) (April et al. 2009), may also be potentially beneficial in the microarray gene expression profiling of peripheral whole blood.

**Finding an Accessible Surrogate Tissue for Neural Tissue**

Linkage and genome-wide association studies focusing on the identification of susceptibility genes for neural-based disorders have proven to be difficult (Gershon et al. 2011; Altshuler et al. 2008; Hovatta and Barlow 2008), most likely due to the heterogeneous and complex nature of neurodevelopmental, neuropsychiatric, and neurodegenerative diseases. One approach to disentangling these complex diseases has been the development of mouse models that mimic certain aspects of anxiety (Belzung and Griebel 2001; Crawley and Goodwin 1980), depression (Pollack et al. 2010), schizophrenia (Braff and Geyer 1990; Geyer et al. 1990), Parkinson’s disease

(Taylor et al. 2010), autism (Moy et al. 2007; Moy et al. 2004), and other neural-based diseases. These models lend themselves well to the characterization of blood gene expression, or any other tissue source, as a surrogate for brain gene expression.

Taking advantage of the natural variation manifested by behavioral phenotypes across well-characterized inbred mouse strains, previous studies combining mouse strain analysis and behavioral testing with microarray gene expression profiling have identified genes whose expression levels are associated with behavior in mice (de Jong et al. 2010; Nadler et al. 2006; Hovatta et al. 2005). In some cases, the associated genes have also been shown to be associated with disease in human populations (Donner et al. 2008), highlighting the potential of mouse models in the identification of genes relevant to human disease. By expanding this approach to blood-based gene expression, it may be possible to discover disease susceptibility genes in an easily accessible, clinically relevant tissue.

The use of mouse models enables researchers to overcome many of the difficulties in analyzing whether or not blood is a viable surrogate for brain gene expression in identifying disease susceptibility genes, particularly small sample sizes and sample degradation, as well as the inability to collect blood and brain from the same living individual. Analysis of mouse brain and spleen suggest blood gene expression is capable of acting as a surrogate for brain tissue for a subset of genes (Davies et al. 2009), while gene expression experiments in vervet monkeys also exhibit the potential of blood gene expression to act as a surrogate for brain gene expression (Jasinska et al. 2009). Nonetheless, conflicting results from the assessment of human tissues display little overlap between human blood and brain

gene expression (Cai et al. 2010). While Cai et al. took advantage of multiple large data sets, their study suffers from several pitfalls: 1.) brain and blood samples used were processed using different microarray platforms (Affymetrix and Illumina, respectively); and 2.) brain and blood samples were collected from different individuals. Thus, the results from mouse studies will need to be extended to human studies in order to assess the true clinical validity of blood-based gene expression biomarkers.

**Genetic Background Effect Analysis**

As researchers continue to study the behavior of inbred mouse strains, they must be aware of strain differences that may affect the results of any study if not accounted for properly. For example, when assessing physiological differences between strains using an activity involving sight, variation across strains with respect to visual acuity and blindness will affect behavioral test performance. In addition, genetic variation between strains could influence phenotypic expression across the strains or also contribute to assay failure. Thus, the genetic background of the strain used to develop a knockout or gene transfer investigation can have enormous effects on the study aims and hypotheses (Austin et al. 2004; Accili 2004), and traditional introgression studies have showed varying effects of a target gene's activity as a function of genetic background (Letts et al. 1995). The same holds true for microarray gene expression analyses. Sequence variation has been shown to affect hybridization and lead to an increase in false associations (de Jong S et al. 2010; Peirce et al. 2006, Radcliffe et al. 2006). Methods capable of correcting for strain-based hybridization, such as GeSNP (Greenhall et al. 2007), should be considered.

For example, the algorithm employed by GeSNP was used to identify sequence differences between three rare strains of inbred mice (Carter et al. 2005), to improve the reliability of gene expression data by masking probe pairs that cover regions with sequence differences between humans and chimpanzees (Cáceres et al. 2003), and was also applied in the expression QTL (eQTL) study described above (Hovatta et al. 2007). The results demonstrate that the GeSNP algorithm can identify sequence differences using array-based gene expression data.

## SPECIFIC AIMS

The research pursued in this dissertation focuses on the comparison of blood and brain microarray-based gene expression in mice and humans. Overall, the research focuses on: 1. the effects of globin reduction on blood gene expression levels and patterns; 2. the correlation of blood and brain gene expression to various behavioral phenotypes; and 3. the utility of blood gene expression in the development of genomic biomarkers for neural-based diseases using two existing data sets: (i) a data consisting of five brain tissues collected from six mouse strains (Hovatta et al. 2005); and (ii) data on post-mortem brain samples from the San Diego Autism Center of Excellence (Chow et al. 2011, Submitted). The specific aims are as follows:

1. The assessment of the effects of globin reduction on blood-based gene expression in mice by comparing gene expression patterns before and after globin reduction of mouse whole blood.
2. The characterization of the correlations between blood gene expression levels and behavioral phenotypes and how blood gene expression-trait correlations compare

with brain gene expression-trait correlations in order to assess the utility of blood gene expression levels for identifying neuropsychiatric and behavioral phenotypes.

3. The evaluation of gene expression profiling of human whole blood samples with two Illumina gene expression assays: (i) the *in vitro* transcription (IVT) assay; and (ii) the whole-genome cDNA-mediated Annealing, Selection, Extension, and Ligation (WG-DASL) assay.

4. The application of knowledge gained from specific aims I-III to the comparison of gene expression from brain and lymphocytes collected from individuals diagnosed with autism.

**ENUMERATION OF CHAPTERS**

Whole blood is widely recognized as an acceptable tissue source in clinically applicable gene expression studies. Nonetheless, blood-based gene expression profiling is not without challenges. Researchers must take in to account: 1. multiple cell types in the blood with varying expression patterns and cell counts; 2. intra- and inter-individual variation in cell composition of the blood; and 3. sample collection and processing-induced variation and alterations in gene expression patterns (Fan and Hegde 2005). One such challenge is the level of globin mRNA transcripts in whole blood; it is this challenge Chapter 2 serves to address in the context of mouse whole blood. Globin reduction in mouse whole blood is found to be important to improving the ability to detect genes involved in various human diseases, particularly neural-based diseases.

Chapter 3 focuses on mouse blood and mouse brain gene expression-behavioral trait correlations with an underlying emphasis on the effects of cell type

and strain-specific genetic background on blood- and brain- specific gene expression using Multivariate Distance Matrix Regression (MDMR) (Zapala et al. 2006) and Bi-variate Correlated Errors Analysis (Akritas and Bershady 1996). Strain effects on blood-specific gene expression were assessed using six commonly utilized inbred mouse strains (129S1/SvImJ, A/J, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/NJ); blood cell count effects using data from the Mouse Phenome Database (http://phenome.jax.org/pub-cgi/phenome/mpdcgi?rtn=docs/home); and brain tissue effects using regions (bed of nucleus striatum, hippocampus, hypothalamus, periaqueductal gray, and pituitary) known to play a role in neuropsychiatric phenotypes: pre-pulse inhibition, fear potentiated startle, and other anxiety phenotypes.

Chapters 4 and 5 present the results pertaining to human samples. The challenges related to globin reduction and microarray platform using human peripheral blood are addressed in Chapter 4. The fifth chapter extends the analyses pursued in specific aim 2 and the microarray platforms studied in specific aim 3 to an autism data set of lymphocyte gene expression. We find that human lymphocyte gene expression reflects similar pathways as those identified in brain gene expression. We also conclude that lymphocyte gene expression profiles are capable of discriminating between autism cases and controls.

## TABLES

**Table 1-1.** 81 genetic biomarkers approved by the US Food and Drug Administration (FDA) as of August 1, 2011.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Abacavir | Antivirals | HLA-B*5701 | Boxed Warning, Contradindications, Warnings and Precautions, Patient Counseling Information |
| Aripiprazole | Psychiatry | CYP2D6 | Clinical Pharmacology |
| Arsenic Trioxide | Oncology | PML/RARα | Boxed Warning, Clinical Pharmacology, Indications and Usage, Warnings |
| Atomoxetine | Psychiatry | CYP2D6 | Dosage and Administration, Warnings and Precautions, Drug Interactions, Clinical Pharmacology |
| Atorvastatin | Metabolic and Endocrinology | LDL receptor | Indications and Usage, Dosage and Administration, Warnings and Precautions, Clinical Pharmacology, Clinical Studies |
| Azathioprine | Rheumatology | TPMT | Dosage and Administration, Warnings and Precautions, Drug Interactions, Adverse Reactions, Clinical Pharmacology |
| Boceprevir | Antivirals | IL28B | Clinical Pharmacology |
| Busulfan | Oncology | Ph Chromosome | Clinical Studies |
| Capecitabine | Oncology | DPD | Contraindications, Precautions, Patient Information |
| Carbamazepine | Neurology | HLA-B*1502 | Boxed Warning, Warnings and Precautions |
| Carvedilol | Cardiovascular | CYP2D6 | Drug Interactions, Clinical Pharmacology |
| Celecoxib | Analgesics | CYP2C9 | Dosage and Administration, Drug Interactions, Use in Specific Populations, Clinical Pharmacology |

**Table 1-1.** Continued.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Cetuximab (1) | Oncology | EGFR | Indications and Usage, Warnings and Precautions, Description, Clinical Pharmacology, Clinical Studies |
| Cetuximab (2) | Oncology | KRAS | Indications and Usage, Clinical Pharmacology, Clinical Studies |
| Cevimeline | Dermatology and Dental | CYP2D6 | Drug Interactions |
| Chloroquine | Antiinfectives | G6PD | Precautions |
| Clopidogrel | Cardiovascular | CYP2C19 | Boxed Warning, Dosage and Administration, Warnings and Precautions, Drug Interactions, Clinical Pharmacology |
| Clozapine | Psychiatry | CYP2D6 | Drug Interactions, Clinical Pharmacology |
| Codeine | Analgesics | CYP2D6 | Warnings and Precautions, Use in Specific Populations, Clinical Pharmacology |
| Dapsone | Dermatology and Dental | G6PD | Indications and Usage, Precautions, Adverse Reactions, Patient Counseling Information |
| Dasatinib | Oncology | Ph Chromosome | Indications and Usage, Clinical Studies, Patient Counseling Information |
| Dexlansoprazole | Gastroenterology | CYP2C19 | Clinical Pharmacology |
| Dextromethorphan and Quinidine | Neurology | CYP2D6 | Clinical Pharmacology, Warnings and Precautions |
| Diazepam | Psychiatry | CYP2C19 | Drug Interactions, Clinical Pharmacology |
| Doxepin | Psychiatry | CYP2D6 | Precautions |
| Drospirenone and Ethinyl Estradiol | Reproductive | CYP2C19 | Precautions, Drug Interactions |
| Erlotinib | Oncology | EGFR | Clinical Pharmacology |
| Esomeprazole | Gastroenterology | CYP2C19 | Drug Interactions, Clinical Pharmacology |

**Table 1-1.** Continued.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Fluorouracil | Dermatology and Dental | DPD | Contraindications, Warnings |
| Fluoxetine | Psychiatry | CYP2D6 | Warnings, Precautions, Clinical Pharmacology |
| Fluoxetine and Olanzapine | Psychiatry | CYP2D6 | Drug Interactions, Clinical Pharmacology |
| Flurbiprofen | Rheumatology | CYP2C9 | Clinical Pharmacology, Special Populations |
| Fulvestrant | Oncology | ER receptor | Indications and Usage, Patient Counseling Information |
| Gefitinib | Oncology | EGFR | Clinical Pharmacology |
| Imatinib (1) | Oncology | C-Kit | Indications and Usage, Dosage and Administration Clinical Pharmacology, Clinical Studies |
| Imatinib (2) | Oncology | Ph Chromosome | Indications and Usage, Dosage and Administration, Clinical Pharmacology, Clinical Studies |
| Imatinib (3) | Oncology | PDGFR | Indications and Usage, Dosage and Administration, Clincal Studies |
| Imatinib (4) | Oncology | FIP1L1-PDGFRα | Indications and Usage, Dosage and Administration, Clinical Studies |
| Irinotecan | Oncology | UGT1A1 | Dosage and Administration, Warnings, Clinical Pharmacology |
| Isosorbide and Hydralazine | Cardiovascular | NAT1; NAT2 | Clinical Pharmacology |
| Lapatinib | Oncology | Her2/neu | Indications and Usage, Clinical Pharmacology, Patient Counseling Information |
| Lenalidomide | Hematology | 5q Chromosome | Boxed Warning, Indications and Usage |

**Table 1-1.** Continued.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Maraviroc | Antivirals | CCR5 | Indications and Usage, Warnings and Precautions, Clinical Pharmacology, Clinical Studies, Patient Counseling Information |
| Mercaptopurine | Oncology | TPMT | Dosage and Administration, Contraindications, Precautions, Adverse Reactions, Clinical Pharmacology |
| Metoprolol | Cardiovascular | CYP2D6 | Precautions, Clinical Pharmacology |
| Nelfinavir | Antivirals | CYP2C19 | Drug Interactions, Clinical Pharmacology |
| Nilotinib (1) | Oncology | Ph Chromosome | Indications and Usage, Patient Counseling Information |
| Nilotinib (2) | Oncology | UGT1A1 | Warnings and Precautions, Clinical Pharmacology |
| Panitumumab (1) | Oncology | EGFR | Indications and Usage, Warnings and Precautions, Clinical Pharmacology, Clinical Studies |
| Panitumumab (2) | Oncology | KRAS | Indications and Usage, Clinical Pharmacology, Clinical Studies |
| Peginterferon alfa-2b | Antivirals | IL28B | Clinical Pharmacology |
| Prasugrel | Cardiovascular | CYP2C19 | Use in Specific Populations, Clinical Pharmacology, Clinical Studies |
| Propafenone | Cardiovascular | CYP2D6 | Clinical Pharmacology |
| Propranolol | Cardiovascular | CYP2D6 | Precautions, Drug Interactions, Clinical Pharmacology |
| Protriptyline | Psychiatry | CYP2D6 | Precautions |
| Quinidine | Antimalarials/Antiarrhythmics | CYP2D6 | Precautions |
| Rabeprazole | Gastroenterology | CYP2C19 | Drug Interactions, Clinical Pharmacology |
| Rasburicase | Oncology | G6PD | Boxed Warning, Contraindications |

**Table 1-1.** Continued.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Rifampin, Isoniazid, and Pyrazinamide | Antiinfectives | NAT1; NAT2 | Adverse Reactions, Clinical Pharmacology |
| Risperidone | Psychiatry | CYP2D6 | Drug Interactions, Clinical Pharmacology |
| Sodium Phenylacetate and Sodium Benzoate | Gastroenterology | UCD (NAGS; CPS; ASS; OTC; ASL; ARG) | Indications and Usage, Description, Clinical Pharmacology |
| Sodium Phenylbutyrate | Gastroenterology | UCD (NAGS; CPS; ASS; OTC; ASL; ARG) | Indications and Usage, Dosage and Administration, Nutritional Management |
| Tamoxifen | Oncology | ER receptor | Indications and Usage, Precautions, Medication Guide |
| Telaprevir | Antivirals | IL28B | Clinical Pharmacology |
| Terbinafine | Antifungals | CYP2D6 | Drug Interactions |
| Tetrabenazine | Neurology | CYP2D6 | Dosage and Administration, Warnings, Clinical Pharmacology |
| Thioguanine | Oncology | TPMT | Dosage and Administration, Precautions, Warnings |
| Thioridazine | Psychiatry | CYP2D6 | Precautions, Warnings, Contraindications |
| Ticagrelor | Cardiovascular | CYP2C19 | Clinical Studies |
| Timolol | Opththalmology | CYP2D6 | Clinical Pharmacology |
| Tiotropium | Pulmonary | CYP2D6 | Clinical Pharmacology |
| Tolterodine | Reproductive and Urologic | CYP2D6 | Clinical Pharmacology, Drug Interactions, Warnings and Precautions |
| Tositumomab | Oncology | CD20 antigen | Indications and Usage, Clinical Pharmacology |
| Tramadol and Acetaminophen | Analgesics | CYP2D6 | Clinical Pharmacology |
| Trastuzumab | Oncology | Her2/neu | Indications and Usage, Precautions, Clinical Pharmacology |
| Tretinoin | Dermatology and Dental | PML/RARα | Boxed Warning, Dosage and Administration, Precautions |

**Table 1-1.** Continued.

| Drug | Therapeutic Area | Biomarker | Label Sections |
|---|---|---|---|
| Valproic Acid | Psychiatry | UCD (NAGS; CPS; ASS; OTC; ASL; ARG) | Contraindications, Precautions, Adverse Reactions |
| Venlafaxine | Psychiatry | CYP2D6 | Drug Interactions |
| Voriconazole | Antifungals | CYP2C19 | Clinical Pharmacology, Drug Interactions |
| Warfarin (1) | Hematology | CYP2C9 | Dosage and Administration, Precautions, Clinical Pharmacology |
| Warfarin (2) | Hematology | VKORC1 | Dosage and Administration, Precautions, Clinical Pharmacology |

**Table 1-2.** Example studies investigating natural variation, heritability, processing-induced variation of blood-based gene expression.

| Reference | Cell Type | Sample Size | Technology | Comments |
|---|---|---|---|---|
| Cheung et al. 2003 | LCLs | 90 subjects | cDNA | Heritability |
| Schadt et al. 2003 | LCLs | 56 subjects | Affymetrix | Heritability |
| Whitney et al. 2003 | Whole Blood PBMCs | 75 subjects | cDNA | Natural Variation |
| Debey et al. 2004 | PBMCs | 29 healthy subjects | Affymetrix | Processing-induced |
| Morley et al. 2004 | LCLs | 14 CEPH Families | Affymetrix | Heritability Natural Variation |
| Nicholson et al. 2004 | PBMCs | 12 subjects | cDNA | Natural Variation |
| Radich et al. 2004 | Leukocytes | 32 subjects | cDNA | Natural Variation |
| Cobb et al. 2005 | Whole Blood Leukocytes | 23 healthy 34 trauma/burn | Affymetrix | Natural, Heritability, Processing-induced |
| Eady et al. 2005 | PBMCs | 18 healthy subjects | cDNA | Natural Variation |
| Palmer et al. 2006 | Leukocytes | 7 healthy subjects | cDNA | Cell-type Specific Variation |
| Kim et al. 2007 | Whole Blood | 42 subjects | Affymetrix | Natural, Processing-induced |
| Karlovich et al. 2009 | Whole Blood | 20 healthy subjects | Affymetrix | Longitudinal |
| Meaburn et al. 2009 | Whole Blood | 10 subjects | Affymetrix | Natural, Heritability, Processing-induced |
| Dumeaux et al. 2010 | Whole Blood | 286 subjects | Applied Biosystems | Natural Variation |
| Min et al. 2010 | Whole Blood PBMCs LCLs | 6 subjects | Illumina | Cell-type Specific, Processing-induced |

**Table 1-3.** Online resources for the mouse (Peter et al. 2007).

| **Mouse strain information and resources (inbred and mutant)** | |
|---|---|
| International Mouse Strain Resources | http://www.informatics.jax.org/imsr/index.jsp |
| JAX Mice | http://jaxmice.jax.org/index.html |
| Federation of International Mouse Resources | http://www.fimre.org |
| Mouse Mutant Resource | http://www.jax.org/mmr/index.html |
| Mouse Mutant Regional Resource Centers | http://www.mmrrc.org |
| Riken Bioresource Center | http://www.brc.riken.jp/lab/animal/en |
| The European Mouse Archive | http://www.emmanet.org |
| Mouse Models of Human Cancer Consortium | http://http://mouse.ncifcrf.gov |
| Canadian Mouse Mutant Repository | http://www.cmmr.ca/index.html |

| **Knockout and transgenic mice** | |
|---|---|
| International Gene Trap Consortium* | http://www.genetrap.org |
| Mouse Genome Infomatics Deltagen/Lexicon | http://www.informatics.jax.org |
| Induced Mutant Resource, Jackson Laboratory | http://www.jax.org/imr/index.html |
| Samuel Lunenfeld Research Institute‡ | http://www.mshri.on.ca/nagy |
| Mouse Mutant Regional Resource Centers | http://www.mmrrc.org |
| Micer | http://www.sanger.ac.uk/PostGenomics/mousegenomics |

| **Sequence/phenotype databases** | |
|---|---|
| Ensembl | http://www.ensembl.org/Mus_musculus/index.html |
| Map Viewer at NCBI | http://www.ncbi.nlm.nih.gov/mapview |
| Genome Browser, UCSC | http://genome.ucsc.edu/cgi-bin/hgGateway |
| Mouse Genome Informatics Database | http://www.informatics.jax.org |
| Vertebrate Genome Annotation | http://vega.sanger.ac.uk/index.html |
| Panther | http://pantherdb.org |
| Mouse Phenome Database | http://www.jax.org/phenome |
| Eumorphia | http://www.eumorphia.org |
| Mouse Tumor Biology Database | http://tumor.informatics.jax.org |
| German Mouse Clinic | http://www.gsf.de/ieg/gmc |

| **Pathways analysis** | |
|---|---|
| Ingenuity | http://www.ingenuity.com |
| GenMAPP | http://www.genmapp.org |
| KEGG Pathway Database | http://www.genome.jp/kegg/pathway.html |

| **SNP databases** | |
|---|---|
| Roche | http://mousesnp.roche.com |
| GNF | http://snp.gnf.org |

**Table 1-3.** Continued.

| **SNP databases** (continued) | |
|---|---|
| NCBI | http://www.ncbi.nlm.nih.gov/SNP |
| Mouse Phenome Database | http://aretha.jax.org/pub-cgi/phenome/mpdcgi?rtn=snps/door |
| Mouse Genome Informatics Database | http://www.informatics.jax.org/menus/strain_menu.shtml |
| Perlegen | http://mouse.perlegen.com/mouse |
| Wellcome Trust Centre for Human Genetics | http://www.well.ox.ac.uk/mouse/INBREDS |
| Broad Institute | http://www.broad.mit.edu/personal/claire/MouseHapMap/Inbred.htm |
| **Expression databases** | |
| GNF SymAtlas | http://symatlas.gnf.org |
| Institute for Genomic Research | http://pga.tigr.org |
| Gene Expression Omnibus | http://www.ncbi.nlm.nih.gov/geo |
| The Jackson Laboratory | http://www.informatics.jax.org/menus/expression_menu.shtml |
| Brain Atlas | http://www.brainatlas.org |
| GenSat | http://www.gensat.org/index.html |
| EMAGE | http://genex.hgu.mrc.ac.uk/Emage/database/emageIntro.html |
| **Comparative genomics** | |
| VISTA | http://genome.lbl.gov/vista/index.shtml |
| Mouse Genome Informatics Database | http://www.informatics.jax.org/menus/homology_menu.shtml |
| Rat Genome Database | http://www.rgd.mcw.edu/VCMAP/mapview.shtml |
| **Quantitative traits analysis** | |
| The Jackson Laboratory, Churchill Laboratory | http://www.jax.org/staff/churchill/labsite |
| R/qtl | http://www.biostat.jhsph.edu/~kbroman/qtl |
| Web/QTL | http://www.genenetwork.org/home.html |
| The Jackson Laboratory PGA | http://pga.jax.org/resources/index.html |
| The Complex Trait Consortium | http:://www.complextrait.org |

* Members are: BayGenomics (USA), Centre for Modelling Human Disease (Toronto, Canada), Embryonic Stem Cell Database (University of Manitoba, Canada), Exchangeable Gene Trap Clones (Kumamoto University, Japan), German Gene Trap Consortium (Germany), Sanger Institute Gene Trap Resource (Cambridge, UK), Soriano Lab Gene Trap Database (Fred Hutchinson Cancer Research Center, Seattle, USA), TIGEM-IRBM Gene Trap (Naples, Italy). ‡ Database of *Cre*-expressing strains. GNF, Genomics Institute of the Novartis Research Foundation; NCBI, National Center for Biotechnology information; UCSC, University of California at Santa Cruz.

**FIGURES**

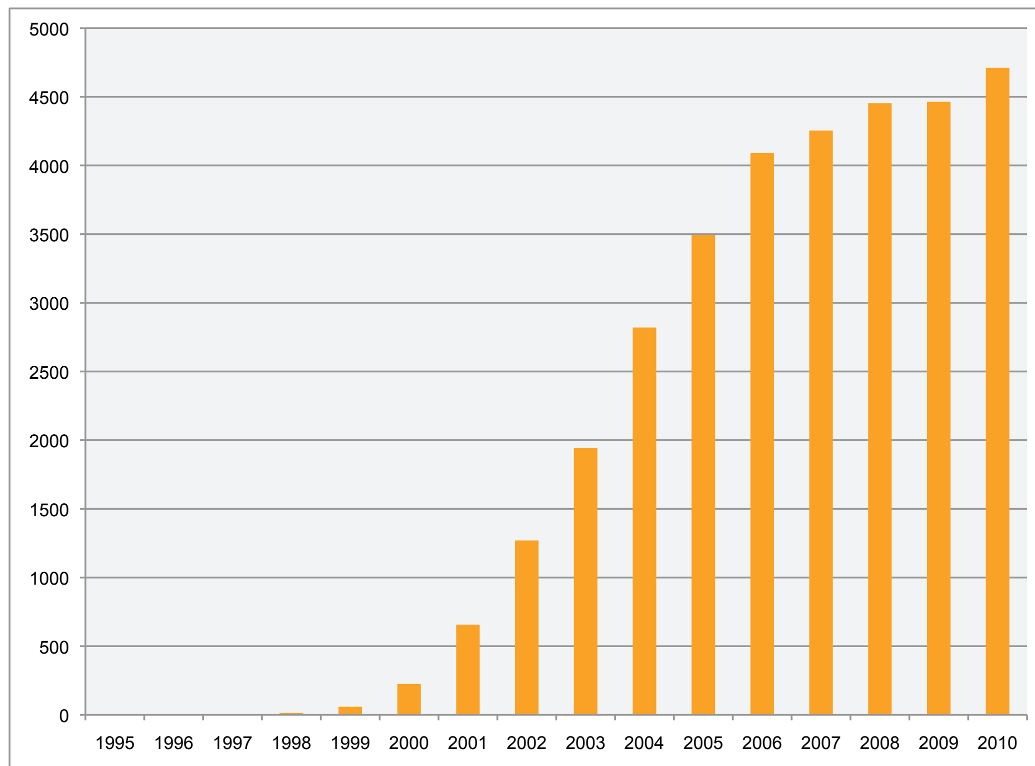


**Figure 1-1.** Number of microarray publications per year from 1995 to 2010 based on the PubMed Search, “microarray AND gene AND expression”.

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# CHAPTER 2

## The Effects of Globin on Microarray-based Gene Expression Analysis of Mouse Blood

**ABSTRACT**

The use of mouse blood as a model for human blood is often considered in the development of clinically relevant, gene expression-based disease biomarkers. However, the ability to derive biologically meaningful insights from microarray-based gene expression patterns in mouse whole blood, as in human whole blood, is hindered by high levels of globin mRNA. In order to characterize the effects of globin reduction on gene expression of peripheral mouse blood, we performed gene set enrichment analysis on genes identified as expressed in blood via microarray-based genome-wide transcriptome analysis. Depletion of globin mRNA enhanced the quality of microarray data as shown by improved gene expression detection and increased sensitivity. Compared to genes expressed in whole blood, genes detected as expressed in blood following globin reduction were enriched for low abundance transcripts implicated in many biological pathways, including development, g-protein signaling, and immune response. Broadly, globin reduction resulted in improved detection of expressed genes that serve as molecular binding proteins and enzymes in cellular metabolism, intracellular transport/localization, transcription, and translation, as well as genes that could potentially act as biomarkers for diseases such as schizophrenia. These significantly enriched pathways overlap considerably with those identified in globin reduced human blood suggesting that globin-reduced mouse blood gene expression studies may be useful for identifying genes relevant to human disease. Overall, the results of this investigation provide a better understanding of the impact of reducing globin transcripts in mouse blood and highlight the potential of microarray-based, globin-reduced, mouse blood gene expression studies in biomarker development.

**INTRODUCTION**

Microarray-based gene expression profiling is a frequently used and powerful tool in the search for molecular ‘fingerprints’ of specific diseases and thus the development of clinically relevant biomarkers for those diseases. For example, seminal investigations have considered the use of large-scale gene expression analyses to classify disease states (Alizadeh et al. 2000; Dyrskjot et al. 2003; Golub et al. 1999), develop diagnostic and prognostic gene profiles (Mirnics et al. 2000; van 't Veer et al. 2002; Welsh et al. 2001), and characterize transcriptomic fingerprints of drug efficacy and toxicity (Gunther et al. 2003). While many of these landmark studies relied heavily on primary tissue samples, recent investigations have focused on peripheral blood, a more accessible tissue (Chao et al. 2008; Coppola et al. 2008; Glatt et al. 2005; Le-Niculescu et al. 2008; Miller et al. 2007; Wang et al. 2005). Aside from being relatively easy to obtain, many of the physiological characteristics of blood cells suggest that peripheral blood gene expression is a reasonable surrogate for specific primary tissue gene expression and hence can be used in the development of clinically meaningful expression-based biomarkers for diseases whose molecular ‘lesions’ are associated with particular non-blood primary tissues (Liew et al. 2006; Fan and Hegde 2005; Mohr and Liew 2007).

One limitation of current studies designed to correlate blood gene expression patterns to primary tissue gene expression patterns is that blood and tissue samples are often not collected from the same set of individuals (Glatt et al. 2005; Liew et al. 2006; Solmi et al. 2006; Sullivan et al. 2006). The use of independent sources of blood and non-blood tissue gene expression information limits interpretability and generalizability of relevant studies and calls into question any putative blood-based

gene expression biomarker panel for the diagnosis and treatment of a disease whose primary lesions are not in blood. Nonetheless, it is often too difficult or even impossible to obtain blood and primary tissue samples from the same living individuals. The mouse provides one possible solution to this dilemma.

Unlike human subjects, blood and primary tissue samples can be easily collected from the same living mouse under highly controlled conditions. Intra- and inter-individual variation introduced by gender, age, time of day, genetic variation, and environment can be reduced in such studies (Leonardson et al. 2010; Cobb et al. 2005; Radich et al. 2004; Whitney et al. 2003), while clinically acceptable and highly standardized protocols for blood collection, RNA isolation, and globin reduction can be employed to lessen technical, assay-induced variation (Cobb et al. 2005; Debey et al. 2004; Debey et al. 2006). In addition, the fact that many different isogenic strains of mice exist suggests that, given the clone-like nature of the mice within such strains, it is possible to sample expression patterns in different tissues from different individuals within particular strains and test the expression patterns for consistencies as though they were obtained from the same individuals.

It is known that high levels of globin transcripts in the blood can confound the accurate assessment of the expression levels of genes in the blood (Wu et al. 2007), as globin mRNA represents up to 70% of the total expressed transcripts and consequently limits the ability to accurately detect genes expressed at low levels in the blood. Thus, globin reduction is often considered a necessary step in the evaluation of whole blood gene expression profiles via microarrays. GLOBINclear™, a commercially available globin reduction protocol, has been shown to improve gene expression detection sensitivity, remove upwards of 95% of alpha- and beta-globin

mRNA, and diminish globin-specific expression patterns in human whole blood samples (Field et al. 2007; Liu et al. 2006; Whitley et al. 2005; Wright et al. 2008). GLOBINclear™ is also advantageous for mouse studies in that it has been specifically developed for the mouse (Whitley et al. 2007).

In this paper we describe a study designed to: 1. evaluate and characterize the effects of globin reduction on whole blood gene expression in different mouse strains; 2. determine which pathways are enriched for genes that appear to be heavily influenced by the confounding or masking effects of globin in the blood; and 3. assess the utility of globin-reduced mouse whole blood in the identification of potential biomarkers of human disease.

## MATERIALS AND METHODS

### Sample Collection

All animal procedures were performed according to protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee. Seven-week-old male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) (129S1/SvImJ, A/J, C57BL/6J, C3H/HeJ, DBA/2J, and FVB/NJ) and individually housed for 1 week prior to blood collection. All mice were anesthetized using isoflurane in a fume hood and whole blood collected via cardiac puncture. The blood was transferred to an EDTA tube and then Trizol LS reagent immediately added (3:1 Trizol:blood) creating a solution in a 15 ml tube that was stored at -80°C for no more than two weeks.

### Processing and Globin Reduction

The extraction of total RNA from the blood was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA quantity and quality was assessed by spectrophotometer and the Agilent nano RNA chip. Alpha and beta globin mRNA were reduced from a portion of the total RNA samples using the GLOBINclear™ Mouse/Rat kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with the recommended start quantity of 10 µg of total RNA.

**Sample Amplification and Microarray Analysis**

Gene expression analysis was performed on all whole blood RNA and globin-depleted samples using Mouse 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) containing 45,101 probe sets. Sample labeling, hybridization, and scanning were performed as previously described (Zapala et al. 2005). Three biological replicate samples from independent mice were prepared for each strain for a total of 18 mice. All raw data is available on the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, GSE19282).

**Data Analysis**

Data processing and analysis was performed using R (http://www.R-project.org) and Bioconductor (http://www.bioconductor.org) (Gentleman et al. 2004). Array images were visually scanned for artifacts while quality control reports (affyQCReport) (Parman and Halling 2008) were assessed and determined to be acceptable under Affymetrix guidelines (Affymetrix). MAS5 detection calls were obtained using the Bioconductor affy package (Gautier et al. 2004). MAS5 detection

calls are used to determine whether a particular probe set is detected above background. Only probe sets called present (detection $p < 0.05$) were utilized for analysis. The associated false discovery rate (Benjamini and Hochberg 1995) for a detection p-value of 0.05 was 28.9 ± 11.3 percent over all arrays. To accommodate for false discoveries, probe sets were filtered for those present across all whole blood or globin-reduced samples prior to gene set enrichment analysis (McClintick and Endenberg 2006).

**Gene Set Enrichment Analysis**

Probe sets that were present in all 18 samples were assigned to two categories (whole blood RNA or globin-reduced RNA) (See Supplementary Materials Table 2-S1 and 2-S2) and imported into MetaCore (http://www.genego.com) for enrichment analysis in GeneGO Pathway Maps, GeneGO Diseases (by Biomarkers), GO Processes, and GO Molecular Functions. GeneGO Pathway Maps represent a set of genes participating in a consecutive set of metabolic signals, or metabolic transformations, confirmed as a whole by experimental data or by inferred relationships. GeneGO Diseases (by Biomarkers) are groups of genes implicated in certain diseases based on classifications in Medical Subject Headings (http://www.nlm.nih.gov/mesh/). The list of genes represented on the Affymetrix Mouse 430 2.0 array was used as a base gene list when calculating p-values in the MetaCore enrichment procedures. MetaCore uses a hypergeometric model to determine the significance of enrichment (Falcon and Gentleman 2007).

### Comparison to Human Whole Blood Gene Expression and Tissue Gene Expression

For mouse and human whole blood gene expression comparisons, raw human whole blood gene expression data were downloaded from the Gene Expression Omnibus (GSE2888, GSE 16728). MAS5 calls were obtained as outlined above and filtered for orthologous probe sets present in ≥ 80 percent of globin-reduced samples for each study individually. Orthologous genes and their associated Affymetrix probe sets were identified using Ensembl Biomart (http://www.www.ensembl.org/biomart). Filtered probe set lists (See Supplementary Materials Table 2-S3) were imported into MetaCore and tested for enrichment in GeneGO Pathway Maps. The list of genes represented on the Affymetrix HG U133A array was used as a base gene list when calculating p-values in the MetaCore enrichment procedures for GSE2888 and GSE16728.

## RESULTS

### Probe Detection and Microarray Sensitivity Following Globin Reduction

Consistent with previously published study results, reduction of globin mRNA in mouse whole blood resulted in a consistent increase in the number of probe sets detected and improved microarray sensitivity, particularly for low abundance genes. The average number of present calls in globin reduced samples was 12411 ± 1904 compared to 5840 ± 944 in untreated samples (**Figure 2-1**), while 5383 probe sets were present across all samples following globin reduction in contrast to 1791 present probe sets in whole blood RNA. Of the probe sets present across all arrays (n=5400), 3609 probe sets were unique to globin-reduced RNA, 17 to whole blood

RNA, and 1774 were common to both whole and globin-reduced RNA. Greater than 1/3 of all probe sets present only in globin-reduced RNA were ranked among the bottom 25 percent of all detectable probe sets, while less than 4 percent were among the top 25 percent (**Table 2-1**). In contrast, 2/3 of the probe sets detected in both whole blood and globin-reduced blood RNA were among the highest 25 percent. Again, this suggests globin reduction has a greater influence on the ability to detect genes expressed at low levels in whole blood and supports the idea that high levels of globin mRNA decrease detection sensitivity.

### Gene Set Enrichment Analysis of Consistently Present Probe Sets in Whole and Globin-Reduced RNA

In order to evaluate the potential of expression profiles generated from globin-reduced mouse blood as compared to whole mouse blood, probe sets detected as present across all samples in whole blood RNA (n=1791; See Supplementary Materials Table 2-S1) and globin-reduced RNA (n=5383; See Supplementary Materials Table 2-S2) were imported into MetaCore for gene set enrichment analysis. Globin reduction increased the ability to detect genes in peripheral whole blood involved in a variety of different biological pathways, most notably development, g-protein signaling, and immune response (**Figure 2-2**). In total, the number of significantly enriched ($p < 0.001$) GeneGO Pathway Maps increased from 43 in whole blood samples to 107 in globin-reduced samples. Globin-reduced samples were similarly enriched for GO biological processes and molecular functions (See Supplementary Materials Figure 2-S1 and 2-S2) including processes and molecular functions previously described as enriched in globin reduced human blood. This

indicates that the globin expression signal significantly weakens the ability to detect the expression levels of many genes interrogated on microarrays, not necessarily due to some biological connection with globin's involvement in particular biological processes, but rather by virtue of their globin-relative signal strength detectable via chip-based multi-probe hybridization. The genes that happen to be affected by this phenomenon collectively participate in a number of biologically meaningful functions and processes. Thus, studies that do not reduce globin yet investigate mouse blood gene expression as a way of understanding disease processes are likely to fail to implicate many important genes since their expression levels are masked by globin.

The ability to detect genes previously implicated in disease was also significantly improved following globin reduction. In general, both whole blood and globin-reduced blood RNA were significantly enriched for genes associated with diseases involving all cell types found in peripheral blood including thrombocytes/platelets (thrombocytopenia), erythrocytes (anemia), myelocytes (myeloid leukemia), and lymphocytes (lymphoma) (**Table 2-2**, See Supplementary Materials Table 2-S4 & 2-S5). Most importantly, the average potential to identify expression levels of genes involved in a given disease or biological network more than doubled following globin-reduction as seen by the proportion of disease biomarkers or disease-associated genes identified as present. To test whether the removal of globin transcripts has a statistically significant effect on the proportion of genes identified, significantly enriched GeneGO Diseases ($p \leq 0.05$) in either whole blood RNA or globin-reduced RNA were assessed by McNemar test. For the majority of significantly enriched disease networks, the reduction of globin transcripts significantly improved the ability to identify biomarkers of disease (data not shown),

including a variety of non-hematologic based diseases including neuromuscular diseases, neurodegenerative diseases and chromosome aberrations (**Table 2-3**).

### Comparisons to Human Whole Blood

In order to evaluate the relevance of mouse blood gene expression to human blood gene expression profiles, the results of our mouse expression study were compared to two human studies by gene set enrichment analysis (Lu et al. 2009) (GSE2888; GSE16728) (**Table 2-4**). Of the 325 statistically significantly enriched GeneGO Pathway Maps ($p < 0.001$), 233 were significantly enriched in our mouse study and the two human studies (**Figure 2-3**) and include 97 of the 107 pathways found to be significant after globin reduction of mouse whole blood RNA, including the development and immune response pathways. Although these results are not completely definitive due to differences in protocols used across each study, including different globin reduction methods, the number of samples, and microarray designs, the overlap in significantly enriched pathway categories suggests genes expressed in mouse peripheral blood reflect those expressed in human blood.

## DISCUSSION

Our analysis of the effects of globin reduction on mouse whole blood-derived total RNA confirms the previously observed increase in expression detection sensitivity and overall detection rate in both humans and mice (Field et al. 2007; Whitley et al. 2007; Whitley et al. 2005), and further emphasizes the importance of globin reduction in evaluating biologically significant pathways and disease processes in mouse models. Gene set enrichment analysis also indicates globin-reduced

mouse blood RNA is a reasonable and practical model for the study of blood-based gene expression correlates of human disease, as biological pathways significantly enriched in globin-reduced mouse and human blood overlap considerably.

However, globin reduction may not always be beneficial or necessary in certain disease contexts. Our results suggest that globin-reduction appears to have little effect on the ability to detect certain classes of biological pathways (**Figure 2-2**), while a small number of genes significantly decrease in expression or fall below the limits of detection following globin reduction, as noted in previous studies (Field et al. 2007). A BLAST search did not identify significant homology between the 17 probe sets whose expression level-based presence was unique to whole blood RNA samples or the globin gene family, suggesting that these probe sets are not specifically removed during globin reduction but rather decrease in expression due to a slight decline in RNA quality (Vartanian et al. 2009) or other non-specific effects. Nonetheless, these findings indicate that globin-reduced, peripheral blood-based gene expression profiling of relevant mouse models may reveal unique patterns of gene expression relevant to human disease and aid in the discovery of clinically significant biomarkers.

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## TABLES

**Table 2-1.** Distribution of probe sets detected in: 1.) globin-reduced samples only; 2.) whole blood samples only; or 3.) both whole and globin-reduced samples across all detectable probes (n = 5400). Detectable probes were ranked according to their average, normalized expression intensities in both whole and globin-reduced RNA.

| | Globin Reduced Only (Total = 3609) | | Whole Blood Only (Total = 17) | | Common (Total = 1774) | |
|---|---|---|---|---|---|---|
| | **Whole Blood RNA** | **Globin Reduced RNA** | **Whole Blood RNA** | **Globin Reduced RNA** | **Whole Blood RNA** | **Globin Reduced RNA** |
| **<5%** | 264 | 256 | 3 | 9 | 3 | 5 |
| **5 – 25%** | 1063 | 1050 | 2 | 4 | 15 | 26 |
| **25 – 50%** | 1269 | 1242 | 2 | 2 | 79 | 106 |
| **50 – 75%** | 878 | 899 | 4 | 2 | 468 | 449 |
| **75 – 95%** | 135 | 162 | 5 | 0 | 940 | 918 |
| **>95%** | 0 | 0 | 1 | 0 | 269 | 270 |

**Table 2-2.** Top 25 statistically significant GeneGO diseases categories in globin-reduced mouse blood RNA as compared to whole blood RNA. Network objects represent the proportion of disease-associated genes (biomarkers) identified per disease category with the denominator representing the number of objects assayed by the Affymetrix Mouse 430 2.0 array.

| | Whole Blood RNA | | Globin Reduced RNA | |
|---|---|---|---|---|
| **GeneGO Disease (by Biomarker)** | **Network Objects** | **p-value*** | **Network Objects** | **p-value*** |
| Hemic and Lymphatic Diseases | 251/1927 | $5.75 \times 10^{-09}$ | 597/1927 | $1.02 \times 10^{-11}$ |
| Hematologic Diseases | 195/1316 | $1.21 \times 10^{-11}$ | 424/1316 | $1.04 \times 10^{-10}$ |
| Neoplasms | 626/5827 | $3.45 \times 10^{-09}$ | 1571/5827 | $4.24 \times 10^{-10}$ |
| Anemia | 73/344 | $2.24 \times 10^{-11}$ | 135/344 | $2.25 \times 10^{-09}$ |
| Bone Marrow Diseases | 114/748 | $1.25 \times 10^{-07}$ | 256/748 | $2.60 \times 10^{-09}$ |
| Spherocytosis, Hereditary | 9/13 | $3.16 \times 10^{-07}$ | 13/13 | $1.50 \times 10^{-08}$ |
| Tay-Sachs Disease | 8/11 | $8.45 \times 10^{-07}$ | 11/11 | $2.40 \times 10^{-07}$ |
| Gangliosidoses | 8/13 | $5.53 \times 10^{-06}$ | 12/13 | $5.99 \times 10^{-07}$ |
| Anemia, Hemolytic, Congenital | 40/153 | $1.81 \times 10^{-09}$ | 66/153 | $6.44 \times 10^{-07}$ |
| Myelodysplastic Syndromes | 89/630 | $7.19 \times 10^{-05}$ | 210/630 | $7.85 \times 10^{-07}$ |

*GeneGO hypergeometric model

**Table 2-3.** Non-hematologic based diseases with a significantly improved proportion of gene expression levels detected in globin-reduced mouse blood RNA as compared to whole mouse blood RNA. All diseases are significantly enriched with a GeneGO hypergeometric p-value ≤ 0.05 in whole or globin-reduced blood RNA samples. Network objects represent the proportion of disease-associated genes identified per disease category with the denominator representing the number of objects assayed by the Affymetrix Mouse 430 2.0 array.

| GeneGO Disease (by Biomarkers) | Whole Blood Network Objects | Globin-reduced Network Objects | p-value* |
|---|---|---|---|
| Alzheimer Disease | 87/721 | 194/721 | $< 2.20 \times 10^{-16}$ |
| Amyotrophic Lateral Sclerosis | 19/167 | 54/167 | $9.08 \times 10^{-9}$ |
| Cerebellar Ataxia | 3/32 | 13/32 | $4.43 \times 10^{-3}$ |
| Dementia | 97/829 | 226/829 | $< 2.20 \times 10^{-16}$ |
| Down Syndrome | 21/133 | 41/133 | $2.15 \times 10^{-5}$ |
| Muscle Hypertonia | 5/48 | 21/48 | $1.77 \times 10^{-4}$ |
| Neurodegenerative Diseases | 161/1289 | 354/1289 | $< 2.20 \times 10^{-16}$ |
| Ophthalmoplegia | 1/34 | 12/34 | $2.57 \times 10^{-3}$ |
| Parkinson Disease | 38/283 | 64/283 | $9.44 \times 10^{-7}$ |
| Schizophrenia | 106/752 | 208/752 | $< 2.20 \times 10^{-16}$ |

*McNemar Test comparing the proportion of network objects identified before and after globin-reduction.

**Table 2-4.** Data set Characteristic for human-mouse gene set enrichment analysis.

| Study | Microarray | Globin Reduction Method | # of Globin-reduced Samples | # of Orthologous Probe Sets | Probe Sets Present in ≥80% of Samples | GeneGO Network Objects | Significant GeneGO Pathways ($p < 0.001$) |
|---|---|---|---|---|---|---|---|
| **Mouse** | Affymetrix Mouse 430 2.0 | GLOBINclear | 18 | 21287 | 5428 | 4134 | 267 |
| **GSE2888** | Affymetrix HG U133A | Affymetrix Protocol | 14 | 17042 | 6048 | 4996 | 279 |
| **GSE16728** | Affymetrix HG U133 Plus 2.0 | GLOBINclear | 10 | 17042 | 9363 | 7179 | 296 |

**FIGURES**

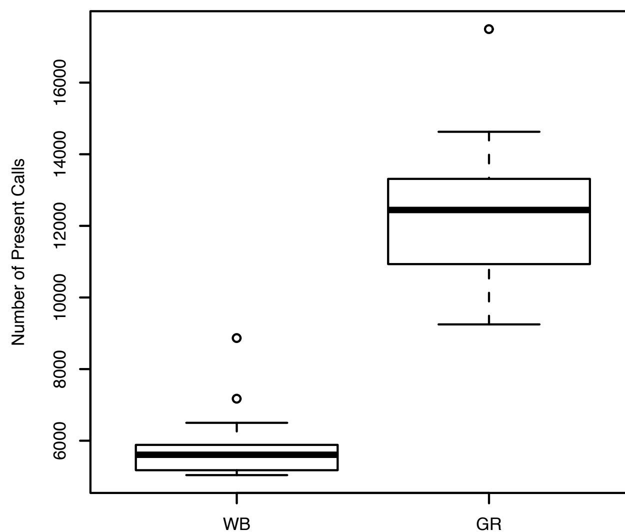


**Figure 2-1.** Box plots of present calls in whole blood RNA and globin-reduced blood RNA samples. The boxes represent the lower quartile through the upper quartile, while the whiskers extend to 1.5 times the interquartile range. Open circles denote outliers. A bold line denotes the median. WB – whole blood RNA. GR – globin-reduced blood RNA.

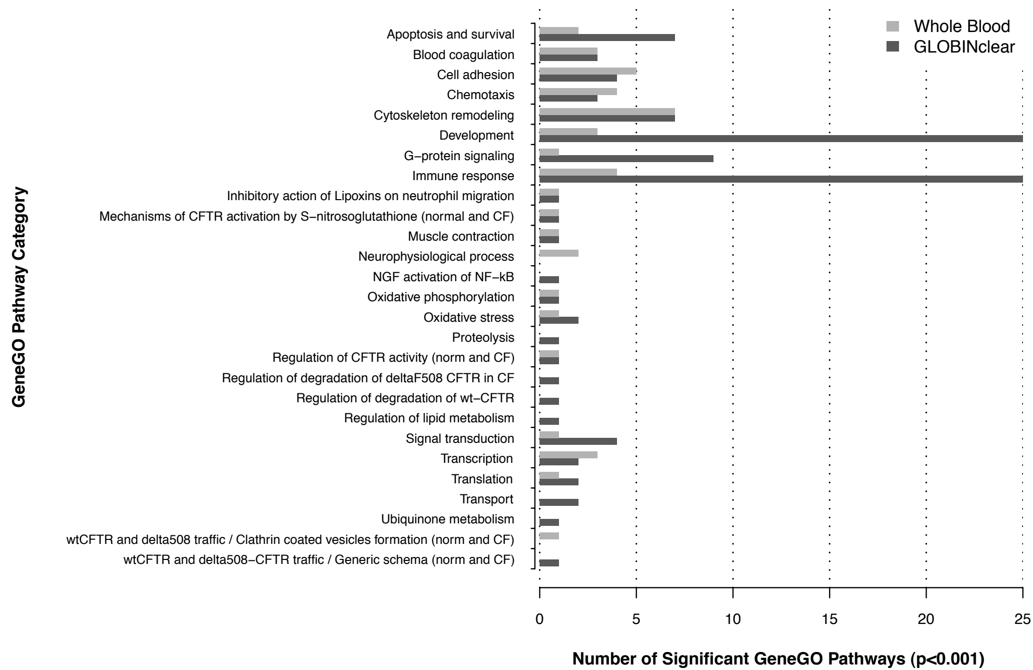


**Figure 2-2.** Number of significantly enriched GeneGO Pathways Maps at a threshold of $p \leq 0.001$ in mouse whole blood RNA (gray) and globin-reduced RNA (black). The associated false discovery rate is less than 0.01 for a p-value threshold less than or equal to 0.001.

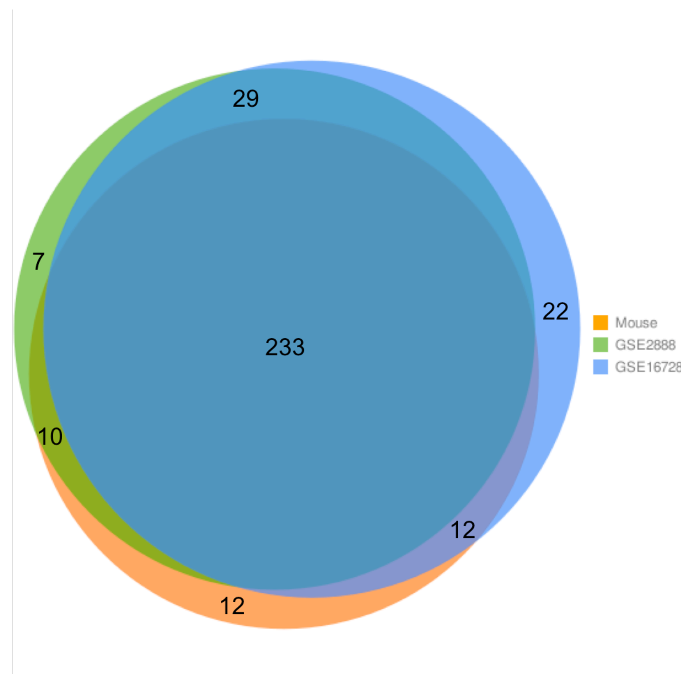


**Figure 2-3.** Venn diagram comparing significant GeneGO Pathway Maps ($p \leq 0.001$) enriched in globin-reduced mouse RNA and globin-reduced human (GSE2888 and GSE16728) RNA. The associated false discovery rate is approximately 0.01 for a p-value threshold less than or equal to 0.001.

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# CHAPTER 3

## Correlation of Blood and Brain Gene Expression in Inbred Mouse Strains

**INTRODUCTION**

The development of genomic biomarkers for neural-based diseases is hampered by the inability to collect neural tissue from living, human subjects. In order to overcome this limitation, many microarray-based gene expression studies utilize blood as the tissue source (Alter et al. 2011; Takahashi et al. 2010; Scherzer et al. 2007). Despite the increased use of blood (i.e. whole blood, lymphocytes, PBMCs, leukocytes, lymphoblastoid cell lines), it is unknown to what extent the results from these studies yield viable biomarker candidates for the diagnosis of neural-based disease or whether what blood cell-type or types most accurately reflect neural-based disease associated gene expression profiles. Whole blood has been touted as the ideal tissue source because it requires less technical processing and is believed to more directly reflect true gene expression levels at the time of sample collection (Fan and Hegde 2005). Recent studies also suggest whole blood is more amenable to long term RNA storage than other blood-derived cells such as PBMCs (Debey-Pascher et al. 2011). On the other hand, whole blood is a very heterogeneous tissue composed of a variety of cell types, which may or may not hinder the ability to reliably and/or accurately measure gene expression levels using high throughput microarray techniques. For example, the large number of reticulocytes found in whole blood samples lead to high levels of globin gene expression. The relatively high level of globin gene expression hampers microarray-based gene expression results thus requiring the need for further processing via globin reduction prior to microarray processing (Winn et al. 2010, Field et al. 2007). Furthermore, the cell types found in whole blood may respond to or reflect primary molecular disturbance of disease synergistically or in opposite directions thus mitigating true disturbance in disease-

associated gene expression profiles. Blood gene expression is also highly susceptible to time of day (Sukumaran et al. 2010; Whitney et al. 2003), diet (Leonardson et al. 2010), and other sources of intra- and inter-individual variability. Given the natural and technically induced variation associated with microarray-based gene expression profiling of peripheral whole blood along with its relative popularity in studying neural-based disease, it is necessary that we have a better understanding of its ability to accurately and reliably reflect primary perturbations in neural tissue.

There are few studies aimed at understanding the correlation of disease associated gene expression candidates in blood and disease associated gene expression candidates in brain (Cai et al. 2010; Davies et al. 2009; Jasinska et al. 2009). Davies et al. and Jasinska et al. highlight the potential of blood-based gene expression using mouse and vervet monkey models, respectively, while Cai et al. are less optimistic about the ability of blood to identify neural-based disease associated genes in humans. In an attempt to further our understanding of the overlap between blood and brain, we conducted gene expression studies on blood and 5 refined brain regions from 6 widely used inbred mouse strains. Correlations between variation in gene expression and variation in behavioral phenotypes were pursued and the results from these gene expression studies used to assess the validity of blood as a surrogate for brain tissue and possibly generate candidate genes for human studies.

The ultimate motivation for the choice of mouse strains analyzed in this study is the frequency with which the strains are used and the availability of historical phenotypic information on the strains. Strains were selected that represent phenotypic extremes of the neuropsychiatric phenotypes of interest to our research group (**Table 3-1**) as well as strains that are genealogically closely related but differ

with regard to some key behaviors. These strains include the inbred strains previously studied by us (129S6/SvImJ or 129SvEvTac, A/J, C57BL/6J, C3H/H3J, DBA/2J, and FVB/NJ) and used by others to create the BxD RI strains utilized by WebQTL (C57BL/6J and DBA/2J; Wang et al. 2003), consomic strains by Singer et al. (A/J and C57BL/6J), background strains for most knockout, transgenic, and genetrap models as well as chemically induced mutants (several 129 strains, C57BL/6J, DBA/2J, FVB/NJ, CBA/J), and most of the strains used by the eight-way RI strains of the Collaborative Cross (C57BL/6J, A/J, 129S1/SvImJ, NOD/LtJ, NZO/HILtj, CAST/EiJ, PWD/PhJ, WSB/EiJ) (Churchill et al. 2004).

The anatomical brain regions that regulate various neuropsychiatric traits are fairly well understood, particularly fear and anxiety, even though the molecular mechanisms remain to be determined. Correspondingly, previous studies in our lab show that it is very important to dissect small enough anatomical structures for gene expression analysis of brain-related phenotypes (Zapala et al. 2005, Hovatta et al. 2007, Hovatta et al. 2005). These previously employed brain regions for gene expression profiling include bed nucleus of stria terminalis (BNST), hippocampus, hypothalamus, periaqueductal gray (PAG), and pituitary gland. They were chosen for their roles in several neuropsychiatric phenotypes: prepulse inhibition, fear-potentiated startle, and anxiety.

Prepulse inhibition (PPI) is a measure of sensorimotor gating that is reduced in patients with some neuropsychiatric disorders, such as schizophrenia and obsessive-compulsive disorder (Braff et al. 2001). Neural circuits that regulate PPI have been intensively studied in rats, and it has been shown that regions such as the hippocampus, prefrontal cortex, basolateral amygdala, nucleus accumbens, striatum,

ventral tegmental area, ventral pallidum, globus pallidus, substantia nigra, thalamus, pedunculopontine nucleus, and the colliculi are implicated in the regulation of PPI (reviewed in Swerdlow et al. 2001). In this study we concentrate the gene expression profiling effort on the hippocampus as this brain region is amenable to hand dissection under a dissection microscope and enough total RNA can be obtained for gene expression profiling without the need for amplification.

Brain regions that regulate aspects of anxiety are also fairly well known. Hippocampus was chosen because studies of the behavioral effects of anxiolytic drugs used to treat human anxiety disorders including benzodiazepines, barbiturates, and selective serotonin re-uptake inhibitors suggest that anxiety is related to the septo-hippocampal system (Gray and McNaughton 2000). Using electrical stimulation of the brain it is possible to elicit escape behavior or defensive aggression in animals by stimulating the medial hypothalamus (Panksepp 1982), which controls the autonomic aspects of anxiety as well. BNST has been shown to play a role in anxiety-related processes (Somerville et al. 2010) and known to serve as a relay between the limbic system and the hypothalamic–pituitary–adrenal axis, a key regulator in response to stress (Choi et al. 2007). Fear-potentiated startle, a measure of conditioned fear and a well-studied phenotype in the context of anxiety (Davis et al. 1993), has been shown to be blocked by chemical lesions of the PAG (Fendt et al. 1996).

**METHODS**

The data sets employed here were previously described (Winn et al. 2010, Hovatta et al. 2007). 10 mice per strain were used for the assessment of the

behavioral phenotypes: anxiety-like behavior (light-dark box and open field test), fear potentiated startle, and prepulse inhibition; 3 mice per strain were used for the assessment of blood gene expression profiles; 2 mice per strain were used for the assessment of neural tissue gene expression profiles. As behavioral testing and brain gene expression were collected earlier (Hovatta et al. 2007, Hovatta et al. 2005), different animals were used for blood gene expression profiling (Winn et al. 2010). This approach is appropriate for several reasons. First, behavioral testing of animals is likely to change their brain gene expression levels. If we first measure the behavior of an animal, the brain and blood gene expression pattern is altered and consequently we cannot reliably measure the baseline gene expression levels. The reciprocal is likely also true due to stress induced by blood collection procedures. Second, a key component of our approach is based on the analysis of multiple inbred strains. Because each animal within an inbred strain is genetically identical, measurements from any individual of the same sex and age handled similarly is meaningful (and could be considered a repeated measure), thus abrogating the need to perform different types of analyses on a single animal and risking altering the baseline gene expression patterns.

**Blood Sample Collection**

All animal procedures were performed according to protocols approved by the University of California, San Diego Institutional Animal Care and Use Committee. Seven-week-old male mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) (129S1/SvImJ, A/J, C57BL/6J, C3H/HeJ, DBA/2J, and FVB/NJ) and individually housed for 1 week prior to blood collection. All mice were

anesthetized using isoflurane in a fume hood and whole blood collected via cardiac puncture. The blood was transferred to an EDTA tube and then Trizol LS reagent immediately added (3:1 Trizol:blood) creating a solution in a 15 ml tube that was stored at -80°C for no more than two weeks.

**Blood Sample Processing and Globin Reduction**

The extraction of total RNA from the blood was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA quantity and quality was assessed by spectrophotometer and the Agilent nano RNA chip. Alpha and beta globin mRNA were reduced from a portion of the total RNA samples using the GLOBINclear™ Mouse/Rat kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with the recommended start quantity of 10 µg of total RNA.

**Brain Sample Collection and RNA Processing**

All animal procedures were performed according to protocols approved by the Salk Institute for Biological Studies Institutional Animal Care and Use Committee. Seven-week-old male inbred mice were received from the Jackson Laboratory (Bar Harbor, ME, USA) (A/J, C3H/HeJ, C57BL/6J, DBA/2J, and FVB/NJ) or from Taconic Farms (Germantown, NY, USA) (129S6/SvEvTac) and individually housed for 1 week before dissections were conducted. All brain dissections were done between 11:00 and 17:00 hours on a petri dish filled with ice using a dissection microscope. The dissected brain regions for gene expression analysis included hypothalamus (Hypo), hippocampus (Hippo), pituitary gland (Pit), periaqueductal gray (PAG), and bed

nucleus of the stria terminalis (BNST). Hippocampus samples were directly frozen on dry ice and stored at -80°C. The smaller brain structures were collected in RNA Later buffer (Ambion, Austin, TX, USA) and samples from two to five animals were pooled and stored at -80°C. At least two independent replicate samples for each strain and brain region using independent animals were dissected. If samples were pooled, at least two independent pools were collected. The extraction of total RNA from the tissues was performed using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions.

**Sample Amplification and Microarray Hybridization**

Gene expression analysis was performed on all globin-depleted blood samples and brain tissue samples using Mouse 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) containing 45,101 probe sets. Sample labeling, hybridization, and scanning were performed as previously described (Zapala et al. 2005). For blood samples, three biological replicate samples from independent mice were prepared for each strain for a total of 18 mice. For brain tissue samples, two replicate samples from independent animals were prepared for each strain and each tissue (analysis of BNST for C3H/HeJ was performed in triplicate).

**Microarray Data Processing and Analysis**

Data processing and analysis was performed using R (http://www.R-project.org) and Bioconductor (http://www.bioconductor.org) (Gentleman et al. 2004). Array images were visually scanned for artifacts while quality control reports (affyQCReport) (Parman and Halling 2008) were assessed and determined to be

acceptable under Affymetrix guidelines (Affymetrix). Raw expression values for each set of tissue-specific microarrays were individually GCRMA normalized via the Bioconductor *gcrma* package (Wu et al. 2004). MAS5 detection calls were obtained using the Bioconductor *affy* package (Gautier et al. 2004). MAS5 detection calls are used to determine whether a particular probe set is detected above background. Only probe sets called present in 2 out 3 blood samples per strain or 2 out 2 brain tissue samples per strain were utilized for analysis (**Table 3-2**).

In order to address hybridization artifacts due to strain-specific differences, GeSNP (http://porifera.ucsd.edu/~cabney/cgi-bin/geSNP.cgi) was used to identify possible sequence differences between strains (Greenhall et al. 2007). Identified probes were removed from the chip description file (cdf) prior to normalization and MAS5 calling.

### Statistical Data Analysis

Multivariate distance matrix regression (MDMR) was used to assess the proportion of variance explained by strain, tissue source (brain) or cell counts (blood), and individual mouse (http://polymorphism.scripps.edu/~cabney/cgi-bin/mmr.cgi) (Zapala et al. 2006). MDMR correlation analyses compare phenotype data to gene expression data in order to identify genes whose expression mirrors the phenotypic differences across inbred strains. Blood cell counts were collected from the Mouse Phenome Database (http://phenome.jax.org/) (**Table 3-3**). We used hierarchical clustering, as performed in R, to assess: 1.) the relationship between microarray gene expression profiles across the 6 strains in the 6 tissues, 2.) the relationship between microarray gene expression profiles across genes displaying a strain-specific effect in

blood, and 3.) the expression patterns of genes significantly associated with behavioral phenotypes in brain and one or more of the neural tissues. Gene expression profiles from each tissue were individually assessed by Bivariate Correlated Errors Scatter Analysis (Akritas et al. 1996) to identify genes associated with behavioral phenotypes. Bivariate Correlated Errors Scatter Analysis takes into account error in the dependent and independent variable, in this case error associated with the collection of the microarray gene expression profiles and the behavioral phenotypes. Lastly, genes identified as significant by Bivariate Correlated Errors Scatter Analysis were imported into MetaCore™ Gene Expression and Pathway Analysis (version 5.0, St. Joseph, MI, USA) for gene network analysis.

**Behavioral Tests**

Each strain was assessed for anxiety and related phenotypes using the light-dark box (LD), open field test (OF), fear potentiated startle (FPS), and prepulse inhibition (PPI). Light-dark box variables studied included: time to emerge, latency to emerge, time in dark, percent in dark, time in light, percent in light, and number of transitions. The control phenotype, distance travelled in dark, was used as a covariate for LD behavioral phenotypes. The control phenotype, distance travelled, was used as a covariate for OF behavioral phenotypes. Covariates for FPS and PPI included context, shock response, and startle. Locomotor activity served as a negative control phenotype. Due to the high correlation between the behavioral phenotypes (i.e. light-dark phenotypes and open field phenotypes) (**Figure 3-1**) and no significant covariates as assessed by linear regression, statistical data analyses were restricted to LD_% time in light, LD_% time in dark, FPS, and PPI.

*Open Field/Locomotor Activity*

Each mouse was placed in the bottom, left hand corner of their respective enclosure at the start of the test session. The movements of the mice were tracked for 5-60 min, with data being stored in 5-min blocks, respectively (Polytrack digitizer (San Diego Instruments). To analyze the locomotor data, an arbitrary maze was created that consists of a center (20 x 20 cm; or 40 x 40 pixels), 4 corners (10 x 10 cm; or 20 x 20 pixels), and 4 rectangular areas along the walls (20 x 10 cm; or 40 x 20 pixels). Four dependent measures were calculated. To assess the overall amount of locomotor activity, transitions between the 9 regions of the maze were counted. The geometric patterns of locomotor activity was quantified by the spatial scaling exponent, d, as described in detail elsewhere (Paulus and Geyer 1991). Briefly, the spatial scaling exponent, d, quantifies the extent to which a sequence of movements are along a straight line (d=1) or within a circumscribed area (d=2). The time spent in the center (min) was also calculated as the primary variable used to quantify anxiety-like responses. In addition, the mean duration per response was defined as the average time (s) spent in the center during each entry into the center and was also calculated to normalize for locomotor differences (for further details see Dulawa et al 1999; Ralph-Williams et al 2003; Ralph et al 2001).

*Startle and Prepulse Inhibition Testing*

Animals were always tested according to a pre-determined sequence that counterbalances groups with respect to time of day and stabilimeter chamber. Animals were brought to an adjacent room to the startle testing room 1 h before testing, weighed, and placed in the stabilimeter at the appropriate time. A constant

background noise of 65 dB was provided to avoid uncontrolled variations and to enable comparable tests by other laboratories. For assessing PPI in mice, both prepulse intensity (67 – 81 dB) and interstimulus interval (ISI) between (range from 20-1080 ms) prepulse to pulse onsets were varied (SR-LAB Startle System (SDI). A short block of varied intensity startle stimuli (90, 105, 120 dB) were also included. Prepulse stimuli were 20 ms in duration and startle stimuli were 40 ms in duration. Unless otherwise specified, the ISI was 100 ms (onset-onset). All acoustic stimuli were broadband, thereby avoiding complications due to standing waves associated with sine wave tones of specific frequencies. A typical test session began with a 5-min acclimation period, followed by 5 consecutive blocks of test trials. Blocks 1 and 5 consisted of 120 dB pulse alone trials (5 each). Block 2 consisted of 5 each of 90, 105, and 120 dB startle pulses in a pseudorandom order. Block 3 contained 5 each of 120 dB and 105 dB startle trials, 5 each of 120 dB startle pulse preceded 100 ms by either a 69 or 73 dB prepulse, and 5 each of 105 dB startle pulse preceded 100 ms by either a 69 or 73 dB prepulse. Block 4 contained 6 startle trials (120 dB pulse alone) and 5 each of prepulse (73 dB preceding 120 dB pulse) trials with varied prepulse- pulse onset intervals (ISI; 20, 50, 100, 200, 500 ms). "No stimulus" trials, in which data are recorded without any stimuli were presented between each stimulus trial. No prepulse-alone trials were required, because any potentially detectable responses to the prepulse stimuli were recorded by the system during the ISI period. The intertrial interval (ITI) between stimulus-containing trials ranged from 7-23 s with an average of 15 s. Extensive details can be found elsewhere (Geyer and Dulawa 2003).

*Fear Potentiated Startle*

Fear potentiated startle was assessed as described previously (Risbrough et al 2009). Separated training and test sessions were used, with a training day followed 24 hrs later by a testing day. All mice were exposed to 5 training and testing days, with the 5$^{th}$ testing day providing the final assessment of FPS. During the training session mice were exposed to 20 CS-US pairings (the CS was a combined 30 sec light and 80 dB 4 KHz tone ending with the 0.4 mA 0.5 s foot shock). During the testing session mice were presented with 24 acoustic startle pulse trials (110 dB startle pulse, 40 ms) with half of the pulses presented at the end of a CS presentation (cue trials) and the other half presented with no CS (no cue trials). %Fear potentiated startle was calculated as [(startle magnitude during Cue trial/Startle Magnitude during No Cue Trial)*100]-100. Details can be found in Risbrough et al. 2009. Data used for gene expression association was from the final test day after the 5th training session.

*Home Cage Activity*

The activity of the mice in their home cage was measured as a behavioral control phenotype. Increased anxiety-like behavior is associated with lower activity in the brightly lit open-field chamber. However, the activity of the animals in a home cage is not associated with anxiety-like behavior. Different inbred strains differ with regard to home cage activity (Carter et al. 2001), and the strain order for this phenotype is different from the observed anxiety-related behavioral phenotype, and the strain order for PPI (Willott et al. 2003).

**RESULTS**

**Influence of Strain, Cell Count, and Tissue Type on Gene Expression Profiles**

To understand the effects of strain, blood cell counts, and tissue type on natural variation in blood and neural tissues, we analyzed blood and brain gene expression levels using MDMR. Each tissue type (blood and neural) was assessed individually due to batch differences in the raw intensity levels unable to be addressed by microarray pre-processing methods. Blood cell counts (**Table 3-4**) or neural tissue (**Table 3-5**) explained the most significant portion of variance (HGB: PVE = 0.317, p-value < 0.0001; PIT: PVE = 0.881, p-value < 0.0001). On average, cell type explained the most variation in blood (0.1798) and neural tissue (0.2474) followed by strain (0.1452, 0.0092) and individual (0.0588, 0.0167). The individuals that explained the most variation in blood (S3, S7, and S17) were most often from the strains that explained the most variation (S3 = 129Sv/ImJ; S7 = C57BL/6J; S17 = FVB). In brain, individuals that explained the most variation were from the two tissues that explained the most variation, pituitary and hippocampus. Overall, there was very little difference between strains within each tissue type as demonstrated by hierarchical clustering analyses (**Figure 3-2**) while the relationship between each strain was not maintained across blood and/or neural tissue.

Next, we performed an analysis of variance (ANOVA) using blood gene expression to identify strain-specific genes and to compare them to the previous brain gene expression results reported by Hovatta et al. 478 probe sets (6.7% of the 7108 probe sets present in blood) displayed a significant ($p < 0.01$, $q < 0.1$) strain-specific effect. To see if strain-specific effects in blood recapitulate known genetic relationships between inbred mouse strains, we constructed a dendrogram of gene

expression relatedness using the 478 strain-specific probe sets (**Figure 3-3**), which was able to partially recapitulate strain relatedness as captured by Hovatta et al. Specifically, a total of 36 strain-specific probe sets overlapped between blood and brain (**Table 3-6**); 338 of the probe sets exhibiting strain-specific effects in blood exhibited region-specific effects in brain. These data suggest that although genetic differences between inbred mouse strains account for a portion of gene expression differences in both blood and brain, blood-specific differences are more likely to capture variation due to ‘secondary’ perturbations or other sources rather than the primary neural insult.

**Gene Expression-Behavioral Phenotype Correlations**

We went on to evaluate the overlap between gene expression-behavioral phenotype correlations in blood and brain employing Bivariate Correlated Errors Scatter analysis to identify strain-specific expression patterns associated with anxiety. Bivariate Correlated Error Scatter analysis takes into account the measurement error associated with not only gene expression intensities but also behavioral phenotype testing. As seen in **Figure 3-4** and **Figure 3-5**, there was quite a bit of variance associated with the collection of behavioral phenotypes. Nonetheless, significant ($p<0.05$) anxiety-specific effects were identified in all 6 tissues (**Table 3-7**). None of the probe sets were identified as significant across all 6 tissues. Only 1 probe set (1425858_at) was significant across blood and 3 neural tissues, while 30 other probe sets were significant across blood and 1 or 2 neural tissues (**Table 3-8**). Closer inspection of individual gene expression intensities (**Figure 3-6**) showed differences

between the strains were often slight and not correlated in the same direction in blood and brain.

Finally, pathway-level analyses were pursued to determine the extent to which the genes associated with behavioral phenotypes in blood and brain are associated with known biological pathways and diseases. First, GeneGo Pathway Maps enriched among genes identified in blood were identified and evaluated in each neural tissue (**Table 3-9**, **Table 3-10**, **Table 3-11**, and **Table 3-12**). Many pathways significantly enriched in blood were significantly enriched in neural tissues (% Time in Dark = 6/18; FPS = 13/16; % Time in Light = 12/24; PPI = 8/16). Those pathways identified in both blood and brain included pathways involving apoptosis and survival, cell adhesion, cell cycle, cytoskeleton remodeling, development, g-protein signaling, muscle contraction, regulation of lipid metabolism, signal transduction, transcription, and transport. Second, GeneGo Pathway Maps enriched among genes identified in neural tissues were identified and evaluated in blood, specifically neurophysiological pathways (**Table 3-13**). 19 neurophysiological pathways were significantly associated with one or more behavioral phenotypes and significantly enriched in one or more neural tissues. None of the pathways were significantly enriched in blood and only 5 of those 19 pathways contained 1 gene identified in blood (RHOA; 1437628_s_at). Lastly, GeneGo Diseases (by Biomarker) enriched among genes identified in neural tissues were identified and evaluated in blood (**Table 3-14**). In MetaCore, GeneGo Diseases (by Biomarker) are organized into a hierarchical structure starting with broad disease categories and moving down into specific diseases. The broad category Psychiatry and Psychology and diseases falling under it were assessed here. 25 diseases/disease categories were significantly identified in one or more

behavioral phenotypes and one or more neural tissues. Although not significant, 18, 9, 10, and 11 Psychiatry and Pyschology genes were associated with % Time in Dark, FPS, % Time in Light, and PPI, respectively, in blood. Only two diseases were significantly associated with a behavioral phenotype in blood; *Schizophrenia* and *Schizophrenia and Disorders with Psychotic Features* were associated with fear potentiated startle (p = 0.0068 and 0.0069).

## DISCUSSION

The relevance of blood-based gene expression biomarkers depends on the strength of the correlation between gene expression levels in the blood and disease-related phenotypes and/or gene expression levels in the primary tissue involved in the pathogenesis of the disease. Here we assessed whole blood gene expression in regards to anxiety-related phenotypes in mice and evaluated the ability of blood to identify genes and pathways associated with anxiety-related phenotypes in neural tissues. We demonstrate blood gene expression profiles only capture a very small subset of genes associated with inbred mouse strains (1.6%) (Hovatta et al. 2007) and behavioral phenotypes (0.6% on average) in neural tissue, while in regards to neurophysiological pathways there was no overlap. This is likely due to a significant difference in the number of probe sets detected as present between blood and neural tissues (**Table 3-2**). Overall, 5153 genes were detected as present across all 6 tissues, while 11828 were present in all 5 neural tissues. Regardless of these differences, looking at the results from the perspective of blood gene expression profiles, 7.5% of genes associated with inbred mouse strains in blood (36/478) overlapped with neural associated genes, while 33% (% Time in Dark), 81% (FPS),

and 50% (% Time in Light and PPI) of pathways associated with behavioral phenotypes in blood were also significantly identified in one or more neural tissue. These results suggest blood gene expression profiles struggle to effectively capture 'primary' disease perturbations despite the fact many of the genes and pathways identified in blood are relevant to the phenotypes and diseases of interest.

Biologically relevant genes identified in blood and brain include many genes associated with the GABAergic neurotransmitter system such as pyridoxine 5'-phosphate oxidase (*Pnpo*). Significantly associated with PPI in blood and PAG and shown to be associated with schizophrenia in a Japanese population (Song et al. 2007), *Pnpo* is the rate limiting enzyme in vitamin B6 synthesis which in turn plays a key role in serotonin, epinephrine, norepinephrine and gamma-aminobutyric acid (GABA) biosynthesis. Vitamin B6 treatment has shown to upregulate the GABAergic system in mice (Yoo et al. 2011). GABA(A) receptors, the primary target of the psychoactive drug benzodiazepine, are known to be reduced in the hippocampus of patients with panic disorders as well as a mouse models of anxiety (Crestani et al. 1999) while GABA(A) receptor agonists elimination fear potentiated startle in mice (Risbrough et al . 2003). *Gabarap*, associated with % Time in Dark in blood and pituitary, clusters neurotransmitter receptors by mediating interactin with the cytoskeleton. Reduction of *Vps13a* (1440146_at), associated with % Time in Dark in blood and hippocampus, leads to the upregulation of GABA(A) receptors in the mouse hippocampus (Kurano et al. 2006), while *Sept11*, also associated with % Time in Dark but in blood and PAG, is known to play a role in GABAergic synaptic connectivity (Li et al. 2009).

A closer look at the significantly enriched pathways and diseases validates the utility of blood gene expression not directly highlighted by the lack of overlap between blood and brain enriched pathways. For example, although only 7 of 932 network objects in the Schizophrenia disease ontology in the MetaCore database are associated with FPS in blood, these 932 network objects are associated with 52/55 of the GeneGo Pathway Maps associated with behavioral phenotypes in blood.

Despite the potential, or lack there of, to identify biologically relevant, neural-based disease genes and pathways using blood gene expression profiles, this study is not without its limitations. One of the most significant limitations is the fact blood and neural-tissues were not collected at the same time. This is more important from a technical standpoint rather than a biological standpoint (Bryant et al. 2011), as processing each tissue type in batches leads to the inability to correct for differences in microarray intensity and may have led to the significant difference between the number of genes detected as present in blood and the number of genes detected as present in neural tissues. Another limitation is the number of mice and the strains of mice utilized for gene expression profiling (3 per strain) and behavioral testing (10 per strain). Increasing the number of mice screened should reduce variability thus improving the ability to identify small differences in gene expression levels and behavioral phenotypes in such highly related, inbred mouse strains while including more genetically and phenotypically diverse strains enhance the differences between strains.

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**TABLES**

**Table 3-1.** Mouse strains studied and their associated anxiety levels (Mozhui et al. 2010, Hovatta et al. 2005).

| Strain | Phenotype |
|---|---|
| 129S1/SvImJ | High Anxiety |
| A/J | High Anxiety |
| C3H/HeJ | Intermediate Anxiety |
| C57BL/6J | Low Anxiety, Low Pre-pulse Inhibition |
| DBA/2J | High Anxiety, High Pre-pulse Inhibition |
| FVB/nJ | Low Anxiety |

**Table 3-2.** Probe sets present across each tissue type. Probe sets were considered present in blood if scored as P in 2 out of 3 replicates per strain. Probe sets were considered present in each brain tissue if scored P in 2 out of 2 replicates per strain.

| | Blood | BNST | Hippo | Hypo | PAG | Pit |
|---|---|---|---|---|---|---|
| **Blood** | 7108 | | | | | |
| **BNST** | 5726 | 16544 | | | | |
| **Hippo** | 5735 | 15256 | 16902 | | | |
| **Hypo** | 5811 | 15482 | 15580 | 17321 | | |
| **PAG** | 5590 | 14673 | 14610 | 15060 | 15568 | |
| **Pituitary** | 5739 | 13093 | 13193 | 13602 | 12653 | 15416 |

**Table 3-3.** Blood hematology parameters from the Mouse Phenome Database Graubert 1 dataset collected from 9-week-old male mice.

| | 129S1/SvImJ | A/J | C57Bl/6J | C3H/HeJ | DBA/2J | FVB/NJ |
|---|---|---|---|---|---|---|
| Total WBC* | 10.3 | 5.60 | 7.57 | 8.77 | 7.53 | 6.69 |
| Neutrophils* | 3.41 | 1.07 | 2.43 | 2.91 | 1.58 | 0.945 |
| Lymphocytes* | 6.51 | 4.33 | 4.89 | 5.03 | 5.44 | 5.54 |
| % Neutrophils | 31.4 | 19.4 | 33.0 | 34.2 | 20.0 | 13.5 |
| % Lymphocytes | 64.5 | 77.1 | 63.5 | 56.6 | 72.4 | 83.5 |
| Platelets* | 492 | 929 | 687 | 702 | 590 | 795 |
| Hemoglobin$ | 15.5 | 13.9 | 15.7 | 15.3 | 14.9 | 13.9 |

* units/vol x$10^3$
$ g/dL

**Table 3-4.** Results from Multivariate Distance Martix Regression (MDMR) analysis using mouse whole blood samples. N: number of samples; NPERMS: number of permutations; SS(TRACE): sum of squares; FSTAT: F-statistic; PVAL: p-value; PVE: portion of variance explained. Predictors of variance included cell counts as collected from the Mouse Phenome Database (purple), strain (blue), and individual mouse (white).

| | Strain | N | NPERMS | SS(TRACE) | FSTAT | PVAL | PVE |
|---|---|---|---|---|---|---|---|
| **HGB** | | 18 | 10000 | 0.003 | 7.438 | $< 1x10^{-4}$ | 0.317 |
| **129S1/SvImJ** | | 18 | 10000 | 0.003 | 5.987 | $< 1x10^{-4}$ | 0.272 |
| **WBC** | | 18 | 10000 | 0.002 | 5.834 | $< 1x10^{-4}$ | 0.267 |
| **HCT** | | 18 | 10000 | 0.002 | 5.543 | $< 1x10^{-4}$ | 0.257 |
| **S3** | 129 | 18 | 10000 | 0.002 | 4.621 | 0.006 | 0.224 |
| **LYM** | | 18 | 10000 | 0.002 | 4.490 | $< 1x10^{-4}$ | 0.219 |
| **NEUT** | | 18 | 10000 | 0.002 | 4.216 | $< 1x10^{-4}$ | 0.209 |
| **PLT** | | 18 | 10000 | 0.002 | 4.142 | 0.0001 | 0.206 |
| **C57BL/6J** | | 18 | 10000 | 0.002 | 4.061 | 0.0008 | 0.202 |
| **EOS** | | 18 | 10000 | 0.002 | 3.986 | 0.0001 | 0.199 |
| **BASO** | | 18 | 10000 | 0.002 | 3.735 | 0.0010 | 0.189 |
| **S7** | C57 | 18 | 10000 | 0.002 | 3.734 | 0.0620 | 0.189 |
| **MPV** | | 18 | 10000 | 0.002 | 3.323 | 0.0044 | 0.172 |
| **RBC** | | 18 | 10000 | 0.002 | 3.095 | 0.0059 | 0.162 |
| **A/J** | | 18 | 10000 | 0.001 | 2.817 | 0.0190 | 0.150 |
| **FVB/nJ** | | 18 | 10000 | 0.001 | 2.602 | 0.0349 | 0.140 |
| **RDW** | | 18 | 10000 | 0.001 | 2.420 | 0.0381 | 0.131 |
| **MONO** | | 18 | 10000 | 0.001 | 2.318 | 0.0510 | 0.127 |
| **MCHC** | | 18 | 10000 | 0.001 | 2.055 | 0.0903 | 0.114 |
| **RETIC** | | 18 | 10000 | 0.001 | 1.940 | 0.1166 | 0.108 |
| **S17** | FVB | 18 | 10000 | 0.001 | 1.908 | 0.1325 | 0.107 |
| **MCH** | | 18 | 10000 | 0.001 | 1.802 | 0.1624 | 0.101 |
| **MCV** | | 18 | 10000 | 0.001 | 1.732 | 0.1771 | 0.098 |
| **S6** | AJ | 18 | 10000 | 0.001 | 1.455 | 0.2092 | 0.083 |
| **S9** | C57 | 18 | 10000 | 0.001 | 1.304 | 0.2502 | 0.075 |
| **S1** | 129 | 18 | 10000 | 0.001 | 1.282 | 0.2572 | 0.074 |
| **DBA/2J** | | 18 | 10000 | 0.001 | 1.028 | 0.4802 | 0.060 |
| **C3H/HeJ** | | 18 | 10000 | 0.000 | 0.793 | 0.6065 | 0.047 |
| **S8** | C57 | 18 | 10000 | 0.000 | 0.743 | 0.4586 | 0.044 |
| **S5** | AJ | 18 | 10000 | 0.000 | 0.664 | 0.4951 | 0.040 |
| **S18** | FVB | 18 | 10000 | 0.000 | 0.556 | 0.5922 | 0.034 |
| **S10** | C3H | 18 | 10000 | 0.000 | 0.516 | 0.6221 | 0.031 |
| **S2** | 129 | 18 | 10000 | 0.000 | 0.485 | 0.6612 | 0.029 |
| **S14** | DBA | 18 | 10000 | 0.000 | 0.457 | 0.6804 | 0.028 |
| **S4** | AJ | 18 | 10000 | 0.000 | 0.336 | 0.7955 | 0.021 |
| **S16** | FVB | 18 | 10000 | 0.000 | 0.299 | 0.8293 | 0.018 |
| **S13** | DBA | 18 | 10000 | 0.000 | 0.264 | 0.8580 | 0.016 |
| **S15** | DBA | 18 | 10000 | 0.000 | 0.261 | 0.8541 | 0.016 |
| **S11** | C3H | 18 | 10000 | 0.000 | 0.259 | 0.8639 | 0.016 |
| **S12** | C3H | 18 | 10000 | 0.000 | 0.205 | 0.8964 | 0.013 |

**Table 3-5.** Results from Multivariate Distance Martix Regression (MDMR) analysis using mouse brain samples. N: number of samples; NPERMS: number of permutations; SS(TRACE): sum of squares; FSTAT: F-statistic; PVAL: p-value; PVE: portion of variance explained. Predictors of variance included brain tissue source (purple), strain (blue), and individual mouse (white).

| | Tissue | Strain | NOBS | NPERMS | SS(TRACE) | FSTAT | PVAL | PVE |
|---|---|---|---|---|---|---|---|---|
| **PIT** | | | 61 | 10000 | 0.229 | 434.953 | $< 1\text{x}10^{-4}$ | 0.881 |
| **HIPPO** | | | 61 | 10000 | 0.049 | 13.640 | $< 1\text{x}10^{-4}$ | 0.188 |
| **PAG** | | | 61 | 10000 | 0.022 | 5.425 | 0.0012 | 0.084 |
| **BNST** | | | 61 | 10000 | 0.017 | 4.233 | 0.0081 | 0.067 |
| **S54** | C57 | Pit | 61 | 10000 | 0.017 | 4.016 | 0.0188 | 0.064 |
| **S55** | C57 | Pit | 61 | 10000 | 0.016 | 3.945 | 0.0376 | 0.063 |
| **S60** | FVB | Pit | 61 | 10000 | 0.016 | 3.866 | 0.0499 | 0.061 |
| **S61** | FVB | Pit | 61 | 10000 | 0.016 | 3.828 | 0.0628 | 0.061 |
| **S52** | AJ | Pit | 61 | 10000 | 0.016 | 3.793 | 0.0693 | 0.060 |
| **S58** | DBA | Pit | 61 | 10000 | 0.016 | 3.776 | 0.0721 | 0.060 |
| **S56** | C3H | Pit | 61 | 10000 | 0.016 | 3.762 | 0.0763 | 0.060 |
| **S53** | AJ | Pit | 61 | 10000 | 0.016 | 3.749 | 0.0794 | 0.060 |
| **S59** | DBA | Pit | 61 | 10000 | 0.015 | 3.739 | 0.0812 | 0.060 |
| **S50** | 129 | Pit | 61 | 10000 | 0.015 | 3.612 | 0.1228 | 0.058 |
| **S51** | 129 | Pit | 61 | 10000 | 0.015 | 3.610 | 0.1176 | 0.058 |
| **S57** | C3H | Pit | 61 | 10000 | 0.015 | 3.589 | 0.1318 | 0.057 |
| **HYPO** | | | 61 | 10000 | 0.004 | 1.022 | 0.4912 | 0.017 |
| **S18** | C57 | Hippo | 61 | 10000 | 0.004 | 0.840 | 0.2783 | 0.014 |
| **S24** | FVB | Hippo | 61 | 10000 | 0.004 | 0.814 | 0.2933 | 0.014 |
| **S25** | FVB | Hippo | 61 | 10000 | 0.003 | 0.783 | 0.2981 | 0.013 |
| **S20** | C3H | Hippo | 61 | 10000 | 0.003 | 0.777 | 0.3055 | 0.013 |
| **S14** | 129 | Hippo | 61 | 10000 | 0.003 | 0.771 | 0.3045 | 0.013 |
| **S17** | AJ | Hippo | 61 | 10000 | 0.003 | 0.771 | 0.3124 | 0.013 |
| **S23** | DBA | Hippo | 61 | 10000 | 0.003 | 0.760 | 0.3137 | 0.013 |
| **S19** | C57 | Hippo | 61 | 10000 | 0.003 | 0.758 | 0.3179 | 0.013 |
| **S16** | AJ | Hippo | 61 | 10000 | 0.003 | 0.753 | 0.3223 | 0.013 |
| **S22** | DBA | Hippo | 61 | 10000 | 0.003 | 0.742 | 0.3206 | 0.012 |
| **S15** | 129 | Hippo | 61 | 10000 | 0.003 | 0.742 | 0.3136 | 0.012 |
| **S21** | C3H | Hippo | 61 | 10000 | 0.003 | 0.739 | 0.3248 | 0.012 |
| **C57BL/6J** | | | 61 | 10000 | 0.003 | 0.725 | 0.5855 | 0.012 |
| **129S6/SvEvTac** | | | 61 | 10000 | 0.003 | 0.610 | 0.612 | 0.010 |
| **A/J** | | | 61 | 10000 | 0.002 | 0.527 | 0.643 | 0.009 |
| **DBA/2J** | | | 61 | 10000 | 0.002 | 0.504 | 0.6538 | 0.008 |
| **FVB/nJ** | | | 61 | 10000 | 0.002 | 0.497 | 0.6458 | 0.008 |
| **C3H/HeJ** | | | 61 | 10000 | 0.002 | 0.489 | 0.6515 | 0.008 |
| **S43** | C57 | PAG | 61 | 10000 | 0.002 | 0.383 | 0.5726 | 0.006 |
| **S39** | 129 | PAG | 61 | 10000 | 0.002 | 0.383 | 0.5704 | 0.006 |
| **S42** | C57 | PAG | 61 | 10000 | 0.002 | 0.370 | 0.582 | 0.006 |
| **S38** | 129 | PAG | 61 | 10000 | 0.002 | 0.370 | 0.5908 | 0.006 |
| **S45** | C3H | PAG | 61 | 10000 | 0.002 | 0.364 | 0.5881 | 0.006 |
| **S40** | AJ | PAG | 61 | 10000 | 0.002 | 0.355 | 0.5956 | 0.006 |
| **S49** | FVB | PAG | 61 | 10000 | 0.002 | 0.354 | 0.5927 | 0.006 |
| **S41** | AJ | PAG | 61 | 10000 | 0.002 | 0.351 | 0.6099 | 0.006 |
| **S8** | C3H | BNST | 61 | 10000 | 0.002 | 0.347 | 0.602 | 0.006 |

**Table 3-5.** Continued.

| | Tissue | Strain | NOBS | NPERMS | SS(TRACE) | FSTAT | PVAL | PVE |
|---|---|---|---|---|---|---|---|---|
| **S44** | C3H | PAG | 61 | 10000 | 0.001 | 0.327 | 0.6259 | 0.006 |
| **S47** | DBA | PAG | 61 | 10000 | 0.001 | 0.303 | 0.642 | 0.005 |
| **S46** | DBA | PAG | 61 | 10000 | 0.001 | 0.300 | 0.6493 | 0.005 |
| **S2** | 129 | BNST | 61 | 10000 | 0.001 | 0.295 | 0.6582 | 0.005 |
| **S48** | FVB | PAG | 61 | 10000 | 0.001 | 0.287 | 0.6584 | 0.005 |
| **S6** | C57 | BNST | 61 | 10000 | 0.001 | 0.282 | 0.6748 | 0.005 |
| **S4** | AJ | BNST | 61 | 10000 | 0.001 | 0.281 | 0.6737 | 0.005 |
| **S5** | C57 | BNST | 61 | 10000 | 0.001 | 0.264 | 0.6897 | 0.004 |
| **S11** | DBA | BNST | 61 | 10000 | 0.001 | 0.251 | 0.7003 | 0.004 |
| **S10** | DBA | BNST | 61 | 10000 | 0.001 | 0.249 | 0.7091 | 0.004 |
| **S7** | C3H | BNST | 61 | 10000 | 0.001 | 0.237 | 0.7169 | 0.004 |
| **S9** | C3H | BNST | 61 | 10000 | 0.001 | 0.228 | 0.718 | 0.004 |
| **S3** | AJ | BNST | 61 | 10000 | 0.001 | 0.227 | 0.7264 | 0.004 |
| **S1** | 129 | BNST | 61 | 10000 | 0.001 | 0.215 | 0.7384 | 0.004 |
| **S13** | FVB | BNST | 61 | 10000 | 0.001 | 0.206 | 0.7424 | 0.003 |
| **S12** | FVB | BNST | 61 | 10000 | 0.001 | 0.198 | 0.7558 | 0.003 |
| **S30** | C57 | Hypo | 61 | 10000 | 0.000 | 0.105 | 0.8357 | 0.002 |
| **S26** | 129 | Hypo | 61 | 10000 | 0.000 | 0.101 | 0.8369 | 0.002 |
| **S27** | 129 | Hypo | 61 | 10000 | 0.000 | 0.092 | 0.8443 | 0.002 |
| **S31** | C57 | Hypo | 61 | 10000 | 0.000 | 0.089 | 0.8472 | 0.002 |
| **S37** | FVB | Hypo | 61 | 10000 | 0.000 | 0.083 | 0.8542 | 0.001 |
| **S33** | C3H | Hypo | 61 | 10000 | 0.000 | 0.078 | 0.8516 | 0.001 |
| **S32** | C3H | Hypo | 61 | 10000 | 0.000 | 0.068 | 0.8603 | 0.001 |
| **S36** | FVB | Hypo | 61 | 10000 | 0.000 | 0.067 | 0.8609 | 0.001 |
| **S35** | DBA | Hypo | 61 | 10000 | 0.000 | 0.065 | 0.8659 | 0.001 |
| **S28** | AJ | Hypo | 61 | 10000 | 0.000 | 0.062 | 0.8639 | 0.001 |
| **S29** | AJ | Hypo | 61 | 10000 | 0.000 | 0.049 | 0.8758 | 0.001 |
| **S34** | DBA | Hypo | 61 | 10000 | 0.000 | 0.048 | 0.8744 | 0.001 |

**Table 3-6.** Strain-specific genes identified in blood and neural tissues.

| Probe Set | Gene Symbol | Gene Name |
|---|---|---|
| 1427077_a_at | AP2B1 | adaptor-related protein complex 2, beta 1 subunit |
| 1433860_at | C5orf22 | chromosome 5 open reading frame 22 |
| 1415796_at | DAZAP2 | DAZ associated protein 2 |
| 1420862_at | DCTN4 | dynactin 4 (p62) |
| 1424324_at | ESCO1 | establishment of cohesion 1 homolog 1 (S. cerevisiae) |
| 1431020_a_at | FGFR1OP2 | FGFR1 oncogene partner 2 |
| 1417714_x_at | HBA1 | hemoglobin, alpha 1 |
| 1417714_x_at | HBA2 | hemoglobin, alpha 2 |
| 1419964_s_at | HDGF | hepatoma-derived growth factor |
| 1419041_at | ITFG1 | integrin alpha FG-GAP repeat containing 1 |
| 1455905_at | KIAA0100 | KIAA0100 |
| 1450740_a_at | MAPRE1 | microtubule-associated protein, RP/EB family, member 1 |
| 1419909_at | MPHOSPH9 | M-phase phosphoprotein 9 |
| 1434396_a_at | MYL6 | myosin, light chain 6, alkali, smooth muscle and non-muscle |
| 1435914_at | NCOR1 | nuclear receptor corepressor 1 |
| 1432332_a_at | NUDT19 | nudix (nucleoside diphosphate linked moiety X)-type motif 19 |
| 1441937_s_at | PINK1 | PTEN induced putative kinase 1 |
| 1428381_a_at | PPDPF | pancreatic progenitor cell differentiation and proliferation factor homolog (zebrafish) |
| 1442148_at | PSIP1 | PC4 and SFRS1 interacting protein 1 |
| 1438390_s_at | PTTG1 | pituitary tumor-transforming 1 |
| 1438069_a_at | RBM5 | RNA binding motif protein 5 |
| 1434933_at | RC3H1 | ring finger and CCCH-type domains 1 |
| 1460670_at | RIOK3 | RIO kinase 3 (yeast) |
| 1416779_at | SDPR | serum deprivation response |
| 1452439_s_at | SRSF2 | serine/arginine-rich splicing factor 2 |
| 1419741_at | SUPT16H | suppressor of Ty 16 homolog (S. cerevisiae) |
| 1438963_s_at | TFPT | TCF3 (E2A) fusion partner (in childhood Leukemia) |
| 1452686_s_at | TMEM222 | transmembrane protein 222 |
| 1417912_at | TMEM93 | transmembrane protein 93 |
| 1419738_a_at | TPM2 | tropomyosin 2 (beta) |
| 1437666_x_at<br>1420494_x_at | UBC | ubiquitin C |
| 1416156_at | VCL | vinculin |
| 1433748_at | ZDHHC18 | zinc finger, DHHC-type containing 18 |
| 1449552_at | ZFR | zinc finger RNA binding protein |

**Table 3-7.** Number of significant genes ($p<0.05$) identified by Bivariate Correlated Errors Scatter Analysis. Numbers in () represent the percentage of present probe sets.

| Tissue | % Time in Dark | % Time in Light | Fear Potentiated Startle | Pre-Pulse Inhibition |
|---|---|---|---|---|
| **Blood** | 113 (1.5) | 56 (0.8) | 35 (0.5) | 52 (0.7) |
| **BNST** | 479 (2.9) | 255 (1.5) | 281 (1.7) | 491 (3.0) |
| **Hippocampus** | 319 (1.9) | 239 (1.4) | 264 (1.6) | 341 (2.0) |
| **Hypothalamus** | 408 (2.4) | 291 (1.7) | 250 (1.4) | 263 (1.5) |
| **PAG** | 333 (2.1) | 227 (1.5) | 216 (1.4) | 290 (1.9) |
| **Pituitary** | 526 (3.4) | 213 (1.4) | 505 (3.3) | 510 (3.3) |

**Table 3-8.** Genes shared between blood and at least 1 neural tissue (p≤0.05)

| Brain Tissue | Probe Set | p-value (Blood) | p-value (Brain) | Gene Symbol | Gene Name |
|---|---|---|---|---|---|
| *% Time in Dark* | | | | | |
| BNST<br>Hippo | 1419112_at | 0.0404 | <0.01<br>0.05 | Nlk | nemo like kinase |
| BNST<br>Hypo<br>Pit | 1425858_at | 0.01 | <0.01<br><0.01<br><0.01 | Ube2m | ubiquitin-conjugating enzyme E2M |
| BNST | 1453207_at | 0.02 | 0.03 | | |
| BNST | 1453784_at | 0.02 | 0.02 | Ilkap | integrin-linked kinase-associated serine/threonine phosphatase 2C |
| Hippo | 1428836_at | 0.01 | 0.04 | | |
| Hippo | 1440146_at | <0.01 | 0.03 | Vps13a | vacuolar protein sorting 13A (yeast) |
| Hypo | 1429004_at | 0.01 | 0.03 | Phip | pleckstrin homology domain interacting protein |
| Hypo<br>PAG | 1447883_x_at | 0.03 | 0.04<br><0.01 | Map1lc3a | microtubule-associated protein 1 light chain 3 alpha |
| PAG | 1451259_at | 0.05 | 0.05 | Rexo2 | REX2, RNA exonuclease 2 homolog (S. cerevisiae) |
| PAG | 1451290_at | <0.01 | 0.01 | Map1lc3a | microtubule-associated protein 1 light chain 3 alpha |
| PAG | 1460626_at | 0.02 | 0.03 | Sept11 | septin 11 |
| Pit | 1416937_at | 0.02 | 0.05 | Gabarap | gamma-aminobutyric acid receptor associated protein |
| Pit | 1422807_at | 0.04 | 0.03 | Arf5 | ADP-ribosylation factor 5 |
| Pit | 1425837_a_at | 0.01 | 0.04 | Ccrn4l | CCR4 carbon catabolite repression 4-like (S. cerevisiae) |
| Pit | 1428068_at | 0.02 | 0.02 | Samm50 | sorting and assembly machinery component 50 homolog (S. cerevisiae) |
| Pit | 1442989_at | 0.04 | 0.01 | | |
| Pit | 1449579_at | 0.05 | 0.05 | Shy3yl1 | Sh3 domain YSC-like 1 |
| *% Time in Light* | | | | | |
| BNST | 1419112_at | 0.03 | 0.01 | Nlk | nemo like kinase |
| BNST<br>Hypo<br>Pit | 1425858_at | 0.02 | <0.01<br><0.01<br><0.01 | Ube2m | ubiquitin-conjugating enzyme E2M |
| Hippo | 1428181_at | 0.05 | 0.04 | Etfb | electron transferring flavoprotein, beta polypeptide |
| Hippo | 1448020_at | 0.05 | 0.02 | Rap1a | RAS-related protein-1a |
| PAG | 1447883_x_at | 0.03 | 0.01 | Map1lc3a | microtubule-associated protein 1 light chain 3 alpha |
| PAG | 1451290_at | 0.02 | 0.02 | Map1lc3a | microtubule-associated protein 1 light chain 3 alpha |
| Pit | 1424598_at | 0.02 | 0.04 | Ddx6 | DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 |
| Pit | 1453784_at | 0.03 | <0.01 | Ilkap | integrin-linked kinase-associated serine/threonine phosphatase 2C |

**Table 3-8.** Continued.

| Brain Tissue | Probe Set | p-value (Blood) | p-value (Brain) | Gene Symbol | Gene Name |
|---|---|---|---|---|---|
| *Fear Potentiated Startle* | | | | | |
| Hypo | 1423746_at | 0.02 | 0.04 | Txndc5 | thioredoxin domain containing 5 |
| PAG<br>Pit | 1452077_at | 0.03 | 0.03<br>0.03 | Ddx3y | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked |
| Pit | 1416614_at | 0.05 | 0.03 | Eid1 | EP300 interacting inhibitor of differentiation 1 |
| Pit | 1428534_at | 0.05 | 0.05 | Nr2c2ap | nuclear receptor 2C2-associated protein |
| *Pre-pulse Inhibition* | | | | | |
| BNST<br>Hypo<br>Pit | 1425858_at | 0.03 | <0.01<br><0.01<br><0.01 | Ube2m | ubiquitin-conjugating enzyme E2M |
| Hippo | 1440880_at | <0.01 | 0.05 | Mppe1 | metallophosphoesterase 1 |
| Hypo | 1416034_at | 0.05 | 0.05 | Cd24a | nectadrin |
| PAG | 1415793_at | 0.02 | 0.01 | Pnpo | pyridoxine 5'-phosphate oxidase |
| PAG | 1415856_at | <0.01 | <0.01 | Emb | embigin |
| Pit | 1427060_at | 0.05 | 0.01 | Mapk3 | mitogen-activated protein kinase 3 |
| Pit | 1437615_s_at | 0.05 | 0.03 | Vps37c | vacuolar protein sorting 37C (yeast) |
| Pit | 1448204_at | 0.03 | 0.05 | Sav1 | salvador homolog 1 (Drosophila) |

**Table 3-9.** GeneGO Pathway Maps significantly enriched ($p<0.05$) among genes associated with % Time in Dark in blood.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Oxidative phosphorylation | Blood | 1 | $<1\times10^{-3}$ | 4 | 105 |
| | BNST | 145 | NS | 3 | 105 |
| | Hippo | 290 | NS | 1 | 105 |
| | Hypo | 297 | NS | 1 | 105 |
| | PAG | 232 | NS | 1 | 105 |
| | Pit | 73 | NS | 4 | 105 |
| Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | Blood | 2 | 0.002 | 2 | 23 |
| | BNST | 185 | NS | 1 | 23 |
| | Pit | 190 | NS | 1 | 23 |
| Muscle contraction_S1P2 receptor-mediated smooth muscle contraction | Blood | 3 | 0.004 | 2 | 30 |
| | BNST | 216 | NS | 1 | 30 |
| | Hypo | 141 | NS | 1 | 30 |
| | Pit | 228 | NS | 1 | 30 |
| Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling | Blood | 4 | 0.004 | 3 | 111 |
| | BNST | 8 | 0.001 | 7 | 111 |
| | Hippo | 45 | 0.044 | 3 | 111 |
| | Hypo | 10 | 0.005 | 5 | 111 |
| | PAG | 233 | NS | 1 | 111 |
| | Pit | 1 | $<1\times10^{-3}$ | 11 | 111 |
| Development_MAG-dependent inhibition of neurite outgrowth | Blood | 5 | 0.006 | 2 | 37 |
| | BNST | 11 | 0.001 | 4 | 37 |
| | Hypo | 45 | 0.052 | 2 | 37 |
| | PAG | 124 | NS | 1 | 37 |
| Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity | Blood | 6 | 0.008 | 2 | 45 |
| | BNST | 293 | NS | 1 | 45 |
| | Hypo | 223 | NS | 1 | 45 |
| | Pit | 121 | NS | 2 | 45 |
| Cell adhesion_Integrin-mediated cell adhesion and migration | Blood | 7 | 0.009 | 2 | 48 |
| | BNST | 309 | NS | 1 | 48 |
| | Hypo | 240 | NS | 1 | 48 |
| | Pit | 8 | 0.001 | 5 | 48 |
| Pentose phosphate pathway/ Rodent version | Blood | 8 | 0.01 | 2 | 51 |
| | BNST | 316 | NS | 1 | 51 |
| | PAG | 185 | NS | 1 | 51 |
| | Pit | 147 | NS | 2 | 51 |
| Pentose phosphate pathway | Blood | 9 | 0.011 | 2 | 52 |
| | BNST | 321 | NS | 1 | 52 |
| | PAG | 188 | NS | 1 | 52 |
| | Pit | 153 | NS | 2 | 52 |

**Table 3-9.** Continued.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Cell cycle_Influence of Ras and Rho proteins on G1/S Transition | Blood | 10 | 0.011 | 2 | 53 |
| | BNST | 53 | 0.03 | 3 | 53 |
| | Hippo | 55 | NS | 2 | 53 |
| | PAG | 193 | NS | 1 | 53 |
| | Pit | 22 | 0.009 | 4 | 53 |
| Development_WNT signaling pathway. Part 2 | Blood | 11 | 0.011 | 2 | 53 |
| | BNST | 144 | NS | 2 | 53 |
| | Hippo | 225 | NS | 1 | 53 |
| | Hypo | 81 | NS | 2 | 53 |
| | Pit | 157 | NS | 2 | 53 |
| Apoptosis and survival_Endoplasmic reticulum stress response pathway | Blood | 12 | 0.011 | 2 | 53 |
| | BNST | 324 | NS | 1 | 53 |
| | Hippo | 222 | NS | 1 | 53 |
| | Hypo | 80 | NS | 2 | 53 |
| | PAG | 18 | 0.015 | 3 | 53 |
| | Pit | 155 | NS | 2 | 53 |
| Blood coagulation_GPVI-dependent platelet activation | Blood | 13 | 0.012 | 2 | 55 |
| | BNST | 149 | NS | 2 | 55 |
| | Hypo | 261 | NS | 1 | 55 |
| | PAG | 199 | NS | 1 | 55 |
| Muscle contraction_ACM regulation of smooth muscle contraction | Blood | 14 | 0.012 | 2 | 56 |
| | BNST | 334 | NS | 1 | 56 |
| | Hippo | 238 | NS | 1 | 56 |
| | Hypo | 263 | NS | 1 | 56 |
| | Pit | 162 | NS | 2 | 56 |
| Development_Role of IL-8 in angiogenesis | Blood | 15 | 0.013 | 2 | 58 |
| | BNST | 9 | 0.001 | 5 | 58 |
| Regulation of lipid metabolism_Alpha-1 adrenergic receptors signaling via arachidonic acid | Blood | 16 | 0.019 | 2 | 70 |
| | BNST | 361 | NS | 1 | 70 |
| | Hypo | 278 | NS | 1 | 70 |
| | Pit | 383 | NS | 1 | 70 |
| Blood coagulation_GPCRs in platelet aggregation | Blood | 17 | 0.019 | 2 | 71 |
| | BNST | 78 | NS | 3 | 71 |
| | Hippo | 264 | NS | 1 | 71 |
| | Hypo | 109 | NS | 2 | 71 |
| Ubiquinone metabolism | Blood | 18 | 0.021 | 2 | 74 |
| | BNST | 86 | NS | 3 | 74 |
| | Hippo | 270 | NS | 1 | 74 |
| | Hypo | 284 | NS | 1 | 74 |
| | PAG | 223 | NS | 1 | 74 |
| | Pit | 94 | NS | 3 | 74 |

**Table 3-9.** Continued.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Immune response _CCR3 signaling in eosinophils | Blood | 19 | 0.023 | 2 | 77 |
| | BNST | 367 | NS | 1 | 77 |
| | Hippo | 273 | NS | 1 | 77 |
| | PAG | 68 | NS | 2 | 77 |
| | Pit | 391 | NS | 1 | 77 |
| HETE and HPETE biosynthesis and metabolism | Blood | 20 | 0.024 | 2 | 80 |
| | Pit | 392 | NS | 1 | 80 |
| Muscle contraction_GPCRs in the regulation of smooth muscle tone | Blood | 21 | 0.026 | 2 | 83 |
| | BNST | 370 | NS | 1 | 83 |
| | Hippo | 12 | 0.021 | 3 | 83 |
| | Hypo | 41 | 0.049 | 3 | 83 |
| | Pit | 110 | NS | 3 | 83 |
| Transport_Intracellular cholesterol transport in norm | Blood | 22 | 0.027 | 2 | 85 |
| | BNST | 113 | NS | 3 | 85 |
| | Hippo | 276 | NS | 1 | 85 |
| | Hypo | 289 | NS | 1 | 85 |
| Cytoskeleton remodeling_Cytoskeleton remodeling | Blood | 23 | 0.038 | 2 | 102 |
| | BNST | 4 | $<1x10^{-3}$ | 7 | 102 |
| | Hippo | 99 | NS | 2 | 102 |
| | Hypo | 68 | NS | 3 | 102 |
| | PAG | 97 | NS | 2 | 102 |
| | Pit | 4 | $<1x10^{-3}$ | 8 | 102 |

**Table 3-10.** GeneGO Pathway Maps significantly enriched ($p<0.05$) among genes associated with FPS in blood.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Immune response_Role of DAP12 receptors in NK cells | Blood | 1 | $<1x10^{-3}$ | 3 | 54 |
| | BNST | 222 | NS | 1 | 54 |
| | Hippo | 256 | NS | 2 | 54 |
| | Pit | 118 | 0.009 | 4 | 54 |
| Apoptosis and survival_Endoplasmic reticulum stress response pathway | Blood | 2 | 0.002 | 2 | 53 |
| | Hippo | 82 | 0.001 | 4 | 53 |
| | Hypo | 165 | NS | 1 | 53 |
| | PAG | 176 | NS | 1 | 53 |
| | Pit | 116 | 0.008 | 4 | 53 |
| Cell adhesion_Role of CDK5 in cell adhesion | Blood | 3 | 0.012 | 1 | 9 |
| | Hypo | 5 | 0.002 | 2 | 9 |
| IL-1 beta-dependent CFTR expression | Blood | 4 | 0.02 | 1 | 16 |
| | BNST | 88 | NS | 1 | 16 |
| | Hippo | 272 | NS | 1 | 16 |
| Cytoskeleton remodeling_CDC42 in cellular processes | Blood | 5 | 0.028 | 1 | 22 |
| | Pit | 293 | NS | 1 | 22 |
| Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | Blood | 6 | 0.029 | 1 | 23 |
| | BNST | 101 | NS | 1 | 23 |
| | Pit | 102 | 0.005 | 3 | 23 |
| Cell adhesion_Endothelial cell contacts by non-junctional mechanisms | Blood | 7 | 0.03 | 1 | 24 |
| | BNST | 104 | NS | 1 | 24 |
| | Hypo | 13 | 0.012 | 2 | 24 |
| | Pit | 299 | NS | 1 | 24 |
| Cytoskeleton remodeling_Neurofilaments | Blood | 8 | 0.032 | 1 | 25 |
| | BNST | 9 | 0.001 | 3 | 25 |
| | Hippo | 301 | NS | 1 | 25 |
| | Hypo | 15 | 0.013 | 2 | 25 |
| | PAG | 10 | 0.012 | 2 | 25 |
| | Pit | 304 | NS | 1 | 25 |
| Cell adhesion_Cadherin-mediated cell adhesion | Blood | 9 | 0.033 | 1 | 26 |
| | Hypo | 16 | 0.014 | 2 | 26 |
| Cell adhesion_Endothelial cell contacts by junctional mechanisms | Blood | 10 | 0.033 | 1 | 26 |
| | BNST | 26 | 0.016 | 2 | 26 |
| | Hippo | 306 | NS | 1 | 26 |
| | Hypo | 1 | $<1x10^{-3}$ | 4 | 26 |
| | Pit | 310 | NS | 1 | 26 |

**Table 3-10.** Continued.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Immune response_Antigen presentation by MHC class I | Blood | 11 | 0.035 | 1 | 28 |
| | BNST | 28 | 0.019 | 2 | 28 |
| | Hippo | 311 | NS | 1 | 28 |
| | Hypo | 19 | 0.016 | 2 | 28 |
| | Pit | 318 | NS | 1 | 28 |
| Development_Osteopontin signaling in osteoclasts | Blood | 12 | 0.038 | 1 | 30 |
| | BNST | 29 | 0.022 | 2 | 30 |
| | Hippo | 317 | NS | 1 | 30 |
| Cell cycle_Spindle assembly and chromosome separation | Blood | 13 | 0.042 | 1 | 33 |
| | BNST | 32 | 0.026 | 2 | 33 |
| | Hippo | 332 | NS | 1 | 33 |
| | Pit | 130 | 0.013 | 3 | 33 |
| Cell adhesion_Alpha-4 integrins in cell migration and adhesion | Blood | 14 | 0.043 | 1 | 34 |
| | Hippo | 201 | 0.035 | 2 | 34 |
| | Hypo | 26 | 0.023 | 2 | 34 |
| | Pit | 132 | 0.015 | 3 | 34 |
| Development_Role of CDK5 in neuronal development | Blood | 15 | 0.043 | 1 | 34 |
| | BNST | 137 | NS | 1 | 34 |
| | Hippo | 339 | NS | 1 | 34 |
| | Hypo | 94 | NS | 1 | 34 |
| | PAG | 97 | NS | 1 | 34 |
| | Pit | 138 | 0.015 | 3 | 34 |
| Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion | Blood | 16 | 0.047 | 1 | 37 |
| | Hippo | 350 | NS | 1 | 37 |
| | Hypo | 103 | NS | 1 | 37 |
| | Pit | 358 | NS | 1 | 37 |

**Table 3-11.** GeneGO Pathway Maps significantly enriched ($p<0.05$) among genes associated with % Time in Light in blood.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | Blood | 1 | 0.001 | 2 | 23 |
| | Pit | 45 | NS | 1 | 23 |
| Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling | Blood | 2 | 0.001 | 3 | 111 |
| | BNST | 35 | 0.046 | 3 | 111 |
| | Hippo | 80 | 0.033 | 3 | 111 |
| | Hypo | 5 | 0.010 | 4 | 111 |
| | PAG | 63 | NS | 2 | 111 |
| | Pit | 2 | $<1x10^{-3}$ | 6 | 111 |
| Muscle contraction_S1P2 receptor-mediated smooth muscle contraction | Blood | 3 | 0.002 | 2 | 30 |
| | Pit | 60 | NS | 1 | 30 |
| Development_MAG-dependent inhibition of neurite outgrowth | Blood | 4 | 0.002 | 2 | 37 |
| | Hippo | 180 | NS | 1 | 37 |
| | Hypo | 103 | NS | 1 | 37 |
| Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier integrity | Blood | 5 | 0.003 | 2 | 45 |
| | Pit | 25 | 0.030 | 2 | 45 |
| Cell adhesion_Integrin-mediated cell adhesion and migration | Blood | 6 | 0.004 | 2 | 48 |
| | Hypo | 145 | NS | 1 | 48 |
| | Pit | 6 | 0.003 | 3 | 48 |
| Pentose phosphate pathway/ Rodent version | Blood | 7 | 0.004 | 2 | 51 |
| | BNST | 158 | NS | 1 | 51 |
| | PAG | 154 | NS | 1 | 51 |
| Pentose phosphate pathway | Blood | 8 | 0.005 | 2 | 52 |
| | BNST | 160 | NS | 1 | 52 |
| | PAG | 158 | NS | 1 | 52 |
| Cell cycle_Influence of Ras and Rho proteins on G1/S Transition | Blood | 9 | 0.005 | 2 | 53 |
| | BNST | 164 | NS | 1 | 53 |
| | Hippo | 240 | NS | 1 | 53 |
| | Pit | 27 | 0.041 | 2 | 53 |
| Development_WNT signaling pathway. Part 2 | Blood | 10 | 0.005 | 2 | 53 |
| | BNST | 42 | 0.056 | 2 | 53 |
| | Hippo | 243 | NS | 1 | 53 |
| | Hypo | 26 | 0.061 | 2 | 53 |
| | PAG | 29 | 0.058 | 2 | 53 |
| Blood coagulation_GPVI-dependent platelet activation | Blood | 11 | 0.005 | 2 | 55 |
| | BNST | 44 | NS | 2 | 55 |
| | Hippo | 249 | NS | 1 | 55 |
| | Hypo | 168 | NS | 1 | 55 |
| | PAG | 166 | NS | 1 | 55 |

**Table 3-11.** Continued.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Muscle contraction_ACM regulation of smooth muscle contraction | Blood | 12 | 0.005 | 2 | 56 |
| | Pit | 30 | 0.045 | 2 | 56 |
| Regulation of lipid metabolism_Insulin regulation of glycogen metabolism | Blood | 13 | 0.005 | 2 | 56 |
| | Hippo | 96 | 0.048 | 2 | 56 |
| | PAG | 167 | NS | 1 | 56 |
| | Pit | 117 | NS | 1 | 56 |
| Blood coagulation_GPCRs in platelet aggregation | Blood | 14 | 0.009 | 2 | 71 |
| | BNST | 50 | NS | 2 | 71 |
| | Hippo | 40 | 0.010 | 3 | 71 |
| | Hypo | 194 | NS | 1 | 71 |
| Immune response _CCR3 signaling in eosinophils | Blood | 15 | 0.010 | 2 | 77 |
| | Hippo | 286 | NS | 1 | 77 |
| | PAG | 191 | NS | 1 | 77 |
| | Pit | 139 | NS | 1 | 77 |
| Muscle contraction_GPCRs in the regulation of smooth muscle tone | Blood | 16 | 0.011 | 2 | 83 |
| | Hippo | 18 | 0.002 | 4 | 83 |
| | Hypo | 203 | NS | 1 | 83 |
| | Pit | 16 | 0.014 | 3 | 83 |
| Cytoskeleton remodeling_Cytoskeleton remodeling | Blood | 17 | 0.017 | 2 | 102 |
| | BNST | 203 | NS | 1 | 102 |
| | Hippo | 117 | NS | 2 | 102 |
| | Hypo | 60 | NS | 2 | 102 |
| | PAG | 52 | NS | 2 | 102 |
| | Pit | 1 | $<1x10^{-3}$ | 6 | 102 |
| Cytoskeleton remodeling_Alpha-1A adrenergic receptor-dependent inhibition of PI3K | Blood | 18 | 0.037 | 1 | 19 |
| | Pit | 38 | NS | 1 | 19 |
| Development_FGF2-dependent induction of EMT | Blood | 19 | 0.039 | 1 | 20 |
| | Hippo | 112 | NS | 1 | 20 |
| | PAG | 45 | NS | 1 | 20 |
| Cytoskeleton remodeling_Role of Activin A in cytoskeleton remodeling | Blood | 20 | 0.039 | 1 | 20 |
| | BNST | 61 | NS | 1 | 20 |
| Cytoskeleton remodeling_ESR1 action on cytoskeleton remodeling and cell migration | Blood | 21 | 0.043 | 1 | 22 |
| | Hippo | 115 | NS | 1 | 22 |
| | Hypo | 49 | NS | 1 | 22 |
| Development_S1P4 receptor signaling pathway | Blood | 22 | 0.043 | 1 | 22 |
| G-protein signaling_Cross-talk between Ras-family GTPases | Blood | 23 | 0.045 | 1 | 23 |
| | BNST | 66 | NS | 1 | 23 |
| | Hippo | 7 | $<1x10^{-3}$ | 3 | 23 |
| Cytoskeleton remodeling_Role of PDGFs in cell migration | Blood | 24 | 0.047 | 1 | 24 |
| | Hippo | 123 | NS | 1 | 24 |

**Table 3-12.** GeneGO Pathway Maps significantly enriched ($p<0.05$) among genes associated with PPI in blood.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Development_MAG-dependent inhibition of neurite outgrowth | Blood | 1 | 0.001 | 2 | 37 |
| | BNST | 111 | NS | 2 | 37 |
| | Hippo | 202 | NS | 1 | 37 |
| Regulation of lipid metabolism_Insulin regulation of glycogen metabolism | Blood | 2 | 0.002 | 2 | 56 |
| | BNST | 166 | NS | 2 | 56 |
| | Hippo | 311 | NS | 1 | 56 |
| | PAG | 245 | NS | 1 | 56 |
| | Pit | 277 | NS | 2 | 56 |
| Cell adhesion_Chemokines and adhesion | Blood | 3 | 0.007 | 2 | 100 |
| | BNST | 5 | $<1x10^{-3}$ | 8 | 100 |
| | Hippo | 167 | NS | 2 | 100 |
| | Hypo | 76 | NS | 2 | 100 |
| | PAG | 60 | 0.039 | 3 | 100 |
| | Pit | 40 | 0.004 | 6 | 100 |
| Transport_Rab-9 regulation pathway | Blood | 4 | 0.013 | 1 | 10 |
| | BNST | 24 | 0.010 | 2 | 10 |
| | PAG | 90 | NS | 1 | 10 |
| Cytoskeleton remodeling_CDC42 in cellular processes | Blood | 5 | 0.028 | 1 | 22 |
| | BNST | 194 | NS | 1 | 22 |
| | Pit | 132 | 0.043 | 2 | 22 |
| Cytoskeleton remodeling_Regulation of actin cytoskeleton by Rho GTPases | Blood | 6 | 0.029 | 1 | 23 |
| | BNST | 67 | 0.049 | 2 | 23 |
| | Hippo | 131 | NS | 1 | 23 |
| | Hypo | 79 | NS | 1 | 23 |
| | Pit | 312 | NS | 1 | 23 |
| Transcription_Transcription regulation of aminoacid metabolism | Blood | 7 | 0.032 | 1 | 25 |
| | BNST | 74 | 0.057 | 2 | 25 |
| | Hippo | 30 | 0.026 | 2 | 25 |
| | Hypo | 91 | NS | 1 | 25 |
| | Pit | 49 | 0.006 | 3 | 25 |
| Development_S1P2 and S1P3 receptors in cell proliferation and differentiation | Blood | 8 | 0.033 | 1 | 26 |
| | BNST | 213 | NS | 1 | 26 |
| | Pit | 152 | NS | 2 | 26 |
| Development_Thrombospondin-1 signaling | Blood | 9 | 0.035 | 1 | 28 |
| | BNST | 79 | NS | 2 | 28 |
| | Hippo | 154 | NS | 1 | 28 |
| Vitamin B6 metabolism | Blood | 10 | 0.038 | 1 | 30 |
| | Hypo | 104 | NS | 1 | 30 |
| | PAG | 136 | NS | 1 | 30 |

**Table 3-12.** Continued.

| Maps | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|
| Development_Slit-Robo signaling | Blood | 11 | 0.038 | 1 | 30 |
| | BNST | 233 | NS | 1 | 30 |
| | Hippo | 163 | NS | 1 | 30 |
| Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility | Blood | 12 | 0.039 | 1 | 31 |
| | BNST | 85 | NS | 2 | 31 |
| | Hippo | 168 | NS | 1 | 31 |
| | Hypo | 107 | NS | 1 | 31 |
| | Pit | 67 | 0.011 | 3 | 31 |
| Cell adhesion_Alpha-4 integrins in cell migration and adhesion | Blood | 13 | 0.043 | 1 | 34 |
| | BNST | 251 | NS | 1 | 34 |
| | Hippo | 181 | NS | 1 | 34 |
| | Hypo | 119 | NS | 1 | 34 |
| | PAG | 152 | NS | 1 | 34 |
| | Pit | 370 | NS | 1 | 34 |
| Cell adhesion_Role of tetraspanins in the integrin-mediated cell adhesion | Blood | 14 | 0.047 | 1 | 37 |
| | BNST | 110 | NS | 2 | 37 |
| | Hippo | 201 | NS | 1 | 37 |
| | Hypo | 17 | 0.020 | 2 | 37 |
| | Pit | 389 | NS | 1 | 37 |
| Signal transduction_cAMP signaling | Blood | 15 | 0.048 | 1 | 38 |
| | BNST | 284 | NS | 1 | 38 |
| | Hippo | 55 | 0.057 | 2 | 38 |
| | Hypo | 18 | 0.021 | 2 | 38 |
| | Pit | 395 | NS | 1 | 38 |
| Blood coagulation_Blood coagulation | Blood | 16 | 0.049 | 1 | 39 |
| | BNST | 291 | NS | 1 | 39 |

**Table 3-13.** Neurophysiological Process GeneGo Pathway Maps significantly enriched ($p<0.05$) in neural tissues. Neurophysiological GeneGO Pathway Maps identified in blood are shown (gray), whether or not they were significant.

| Maps | Phenotype | Tissue | Rank | pValue | Ratio |
|---|---|---|---|---|---|
| Neurophysiological process_ACM regulation of nerve impulse | Dark | Blood | 83 | 0.131 | 1 46 |
| | FPS | Hippo | 27 | $<1x10^{-3}$ | 5 46 |
| | | PAG | 27 | 0.037 | 2 46 |
| | Light | Blood | 67 | 0.088 | 1 46 |
| | | Pit | 26 | 0.032 | 2 46 |
| | PPI | Hippo | 13 | 0.011 | 3 46 |
| | | Hypo | 36 | 0.029 | 2 46 |
| Neurophysiological process_ACM regulation of nerve impulse | PPI | Pit | 8 | $<1x10^{-3}$ | 6 46 |
| Neurophysiological process_ACM1 and ACM2 in neuronal membrane polarization | Dark | Blood | 68 | 0.115 | 1 40 |
| | FPS | Hippo | 134 | 0.005 | 3 40 |
| | Light | Blood | 56 | 0.077 | 1 40 |
| Neurophysiological process_Circadian rhythm | Light | BNST | 34 | 0.045 | 2 47 |
| | | PAG | 25 | 0.047 | 2 47 |
| | PPI | PAG | 73 | 0.048 | 2 47 |
| Neurophysiological process_Corticoliberin signaling via CRHR1 | Dark | Hippo | 48 | 0.049 | 2 50 |
| | FPS | Hippo | 75 | 0.001 | 4 50 |
| | Light | Hippo | 5 | $<1x10^{-3}$ | 4 50 |
| | PPI | Hypo | 42 | 0.034 | 2 50 |
| | | Pit | 51 | 0.007 | 4 50 |
| Neurophysiological process_Dopamine D2 receptor transactivation of PDGFR in CNS | FPS | Hippo | 177 | 0.021 | 2 26 |
| | | Pit | 113 | 0.007 | 3 26 |
| | Light | BNST | 13 | 0.015 | 2 26 |
| | PPI | BNST | 1 | $<1x10^{-3}$ | 6 26 |
| | | Hippo | 32 | 0.028 | 2 26 |
| | | Hypo | 9 | 0.010 | 2 26 |
| Neurophysiological process_EphB receptors in dendritic spine morphogenesis and synaptogenesis | Light | Hippo | 52 | 0.020 | 2 35 |
| | PPI | Hippo | 52 | 0.049 | 2 35 |
| | | Hypo | 15 | 0.018 | 2 35 |
| Neurophysiological process_GABA-A receptor life cycle | Dark | Blood | 37 | 0.079 | 1 27 |
| | | Hypo | 32 | 0.029 | 2 27 |
| | FPS | Hippo | 90 | 0.002 | 3 27 |
| | Light | Blood | 27 | 0.052 | 1 27 |
| | PPI | BNST | 10 | 0.001 | 4 27 |

**Table 3-13.** Continued.

| Maps | Phenotype | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|---|
| Neurophysiological process_Glutamate regulation of Dopamine D1A receptor signaling | Dark | Hypo | 2 | 0.001 | 4 | 45 |
| | FPS | BNST | 55 | 0.046 | 2 | 45 |
| | | Pit | 52 | 0.001 | 5 | 45 |
| | Light | BNST | 7 | 0.004 | 3 | 45 |
| | | PAG | 20 | 0.043 | 2 | 45 |
| | PPI | BNST | 9 | 0.001 | 5 | 45 |
| | | PAG | 67 | 0.044 | 2 | 45 |
| Neurophysiological process_HTR1A receptor signaling in neuronal cells | FPS | BNST | 49 | 0.040 | 2 | 42 |
| | Light | Hippo | 68 | 0.029 | 2 | 42 |
| Neurophysiological process_Kappa-type opioid receptor in transmission of nerve impulses | FPS | Hippo | 189 | 0.027 | 2 | 30 |
| | Light | BNST | 16 | 0.020 | 2 | 30 |
| Neurophysiological process_Long-term depression in cerebellum | FPS | PAG | 30 | 0.042 | 2 | 49 |
| | PPI | Pit | 124 | 0.037 | 3 | 49 |
| Neurophysiological process_Melatonin signaling | FPS | BNST | 50 | 0.042 | 2 | 43 |
| | Light | Hippo | 70 | 0.030 | 2 | 43 |
| Neurophysiological process_Netrin-1 in regulation of axon guidance | FPS | Pit | 156 | 0.024 | 3 | 41 |
| Neurophysiological process_NMDA-dependent postsynaptic long-term potentiation in CA1 hippocampal neurons | Dark | BNST | 34 | 0.019 | 4 | 80 |
| | | Hypo | 39 | 0.045 | 3 | 80 |
| | | PAG | 30 | 0.044 | 3 | 80 |
| | FPS | BNST | 3 | <1x10-3 | 5 | 80 |
| | | Hippo | 135 | 0.005 | 4 | 80 |
| | FPS | PAG | 4 | 0.002 | 4 | 80 |
| | Light | Hippo | 16 | 0.002 | 4 | 80 |
| | PPI | PAG | 5 | <1x10-3 | 5 | 80 |
| | | Pit | 115 | 0.032 | 4 | 80 |
| Neurophysiological process_nNOS signaling in neuronal synapses | PPI | PAG | 35 | 0.020 | 2 | 29 |
| Neurophysiological process_PGE2-induced pain processing | Dark | Blood | 74 | 0.123 | 1 | 43 |
| | | Hippo | 32 | 0.037 | 2 | 43 |
| | Light | Hippo | 20 | 0.002 | 3 | 43 |
| Neurophysiological process_Receptor-mediated axon growth repulsion | Dark | Blood | 80 | 0.128 | 1 | 45 |
| | | Hippo | 33 | 0.041 | 2 | 45 |
| | FPS | BNST | 54 | 0.046 | 2 | 45 |
| | | PAG | 25 | 0.036 | 2 | 45 |
| | Light | Blood | 65 | 0.086 | 1 | 45 |
| | | Hippo | 21 | 0.003 | 3 | 45 |
| | PPI | Blood | 20 | 0.056 | 1 | 45 |

**Table 3-13.** Continued.

| Maps | Phenotype | Tissue | Rank | pValue | Ratio | |
|---|---|---|---|---|---|---|
| Neurophysiological process_Thyroliberin in cell hyperpolarization and excitability | Dark | Blood | 54 | 0.101 | 1 | 35 |
| | FPS | Hippo | 119 | 0.003 | 3 | 35 |
| | Light | Blood | 42 | 0.067 | 1 | 35 |
| | PPI | Hippo | 49 | 0.049 | 2 | 35 |
| | | Hypo | 14 | 0.018 | 2 | 35 |
| | | Pit | 31 | 0.002 | 4 | 35 |

**Table 3-14.** GeneGO Diseases (by Biomarkers) significantly enriched (p<0.05) in neural tissues. GeneGO Diseases (by Biomarkers) identified in blood are shown (gray), whether or not they were significant.

| Disease | Phenotype | Tissue | Rank | p-value | Ratio | |
|---|---|---|---|---|---|---|
| Affective Disorders, Psychotic | Dark | Hippo | 2 | <1x10-4 | 21 | 632 |
| | | Hypo | 3 | <1x10-4 | 29 | 632 |
| | | PAG | 90 | 0.0095 | 18 | 632 |
| | FPS | Blood | 455 | NS | 1 | 632 |
| | | BNST | 92 | 0.0075 | 17 | 632 |
| | | Hippo | 16 | <1x10-4 | 24 | 632 |
| | | Hypo | 100 | 0.0162 | 13 | 632 |
| | Light | Hippo | 8 | <1x10-4 | 18 | 632 |
| | | Hypo | 14 | 0.0002 | 20 | 632 |
| | | PAG | 104 | 0.0064 | 14 | 632 |
| | PPI | Blood | 279 | NS | 1 | 632 |
| | | BNST | 212 | 0.0166 | 24 | 632 |
| | | Hippo | 8 | 0.0014 | 20 | 632 |
| | | Hypo | 73 | 0.0095 | 15 | 632 |
| | | PAG | 71 | 0.0420 | 14 | 632 |
| | | Pit | 47 | 0.0004 | 30 | 632 |
| Agoraphobia | Dark | Hippo | 62 | 0.0061 | 2 | 10 |
| | | Hypo | 89 | 0.0130 | 2 | 10 |
| | FPS | BNST | 94 | 0.0082 | 2 | 10 |
| | | Hippo | 129 | 0.0070 | 2 | 10 |
| | | Hypo | 47 | 0.0047 | 2 | 10 |
| | | Pit | 15 | 0.0015 | 3 | 10 |
| | PPI | Pit | 202 | 0.0235 | 2 | 10 |
| | | Hippo | 54 | 0.0095 | 2 | 10 |
| | | Hypo | 55 | 0.0061 | 2 | 10 |
| | Light | Hippo | 62 | 0.0039 | 2 | 10 |
| | | Hypo | 88 | 0.0070 | 2 | 10 |
| Anxiety Disorders | Dark | Blood | 289 | NS | 1 | 174 |
| | | Hippo | 30 | 0.0012 | 8 | 174 |
| | | Hypo | 161 | 0.0366 | 7 | 174 |
| | FPS | BNST | 206 | 0.0357 | 6 | 174 |
| | Light | Blood | 197 | NS | 1 | 174 |
| | | Hippo | 78 | 0.0066 | 6 | 174 |
| | | Hypo | 92 | 0.0076 | 7 | 174 |
| | PPI | Blood | 141 | NS | 1 | 174 |
| | | Hippo | 83 | 0.0166 | 7 | 174 |
| | | Hypo | 95 | 0.0190 | 6 | 174 |
| | | PAG | 27 | 0.0086 | 7 | 174 |
| Bipolar Disorder | Dark | Hippo | 1 | <1x10-4 | 21 | 630 |
| | | Hypo | 7 | <1x10-4 | 27 | 630 |
| | | PAG | 89 | 0.0092 | 18 | 630 |
| | FPS | Blood | 454 | NS | 1 | 630 |
| | | BNST | 90 | 0.0072 | 17 | 630 |
| | | Hippo | 14 | <1x10-4 | 24 | 630 |
| | | Hypo | 98 | 0.0158 | 13 | 630 |

**Table 3-14.** Continued.

| Disease | Phenotype | Tissue | Rank | p-value | Ratio | |
|---|---|---|---|---|---|---|
| Bipolar Disorder (continued) | Light | Hippo | 7 | <1x10-4 | 18 | 630 |
| | | Hypo | 33 | 0.0014 | 18 | 630 |
| | | PAG | 103 | 0.0063 | 14 | 630 |
| | PPI | Blood | 278 | NS | 1 | 630 |
| | | BNST | 207 | 0.0160 | 24 | 630 |
| | | Hippo | 7 | 0.0014 | 20 | 630 |
| | | Hypo | 70 | 0.0092 | 15 | 630 |
| | | PAG | 69 | 0.0410 | 14 | 630 |
| | | Pit | 46 | 0.0004 | 30 | 630 |
| Borderline Personality Disorder | Dark | Pit | 110 | 0.0247 | 2 | 10 |
| | FPS | BNST | 97 | 0.0082 | 2 | 10 |
| Depression | FPS | Hippo | 274 | 0.0428 | 3 | 60 |
| | Light | Hypo | 212 | 0.0428 | 3 | 60 |
| | PPI | Hypo | 136 | 0.0359 | 3 | 60 |
| Depression, Postpartum | FPS | BNST | 31 | 0.0006 | 2 | 3 |
| Depressive Disorder | Dark | Blood | 433 | NS | 2 | 751 |
| | | Hippo | 73 | 0.0092 | 17 | 751 |
| | | Hypo | 21 | 0.0009 | 26 | 751 |
| | FPS | BNST | 46 | 0.0019 | 21 | 751 |
| | | Hippo | 11 | <1x10-4 | 27 | 751 |
| | | Hypo | 87 | 0.0129 | 15 | 751 |
| | Light | Hippo | 73 | 0.0055 | 15 | 751 |
| | | Hypo | 15 | 0.0003 | 22 | 751 |
| | PPI | Blood | 304 | NS | 1 | 751 |
| | | Hippo | 90 | 0.0192 | 19 | 751 |
| | | Hypo | 96 | 0.0193 | 16 | 751 |
| | | Pit | 79 | 0.0015 | 32 | 751 |
| Depressive Disorder, Major | Dark | Blood | 432 | NS | 2 | 744 |
| | | Hippo | 100 | 0.0178 | 16 | 744 |
| | | Hypo | 27 | 0.0017 | 25 | 744 |
| | FPS | BNST | 43 | 0.0017 | 21 | 744 |
| | | Hippo | 10 | <1x10-4 | 27 | 744 |
| | | Hypo | 84 | 0.0119 | 15 | 744 |
| | Light | Hippo | 107 | 0.0119 | 14 | 744 |
| | | Hypo | 23 | 0.0006 | 21 | 744 |
| | PPI | Blood | 302 | NS | 1 | 744 |
| | | Hippo | 84 | 0.0176 | 19 | 744 |
| | | Hypo | 93 | 0.0178 | 16 | 744 |
| | | Pit | 74 | 0.0013 | 32 | 744 |
| Mental Disorders | Dark | Blood | 116 | NS | 18 | 2945 |
| | | BNST | 12 | 0.0010 | 82 | 2945 |
| | | Hippo | 35 | 0.0016 | 52 | 2945 |
| | | Hypo | 14 | 0.0002 | 76 | 2945 |
| | | PAG | 127 | 0.0245 | 58 | 2945 |
| | | Pit | 164 | 0.0450 | 87 | 2945 |

**Table 3-14.** Continued.

| Disease | Phenotype | Tissue | Rank | p-value | Ratio | |
|---|---|---|---|---|---|---|
| Mental Disorders (continued) | FPS | Blood | 402 | NS | 9 | 2945 |
| | | BNST | 40 | 0.0014 | 59 | 2945 |
| | | Hippo | 42 | 0.0005 | 57 | 2945 |
| | | Hypo | 151 | 0.0292 | 41 | 2945 |
| | | PAG | 39 | 0.0009 | 50 | 2945 |
| | | Pit | 83 | 0.0265 | 86 | 2945 |
| | Light | Blood | 211 | NS | 10 | 2945 |
| | | Hippo | 190 | 0.0408 | 37 | 2945 |
| | | Hypo | 20 | 0.0005 | 57 | 2945 |
| | | PAG | 160 | 0.0162 | 42 | 2945 |
| | | Pit | 34 | 0.0199 | 38 | 2945 |
| | PPI | Blood | 42 | NS | 11 | 2945 |
| | | BNST | 28 | 0.0001 | 98 | 2945 |
| | | Hippo | 53 | 0.0094 | 59 | 2945 |
| | | Hypo | 97 | 0.0199 | 47 | 2945 |
| | | PAG | 15 | 0.0018 | 56 | 2945 |
| | | Pit | 25 | 0.0001 | 101 | 2945 |
| Mood Disorders | Dark | Blood | 469 | NS | 2 | 982 |
| | | Hippo | 12 | 0.0003 | 25 | 982 |
| | | Hypo | 4 | <1x10-4 | 38 | 982 |
| | | PAG | 152 | 0.0309 | 23 | 982 |
| | FPS | Blood | 461 | NS | 1 | 982 |
| | | BNST | 30 | 0.0005 | 27 | 982 |
| | | Hippo | 9 | <1x10-4 | 32 | 982 |
| | | Hypo | 93 | 0.0151 | 18 | 982 |
| | Light | Hippo | 13 | 0.0001 | 22 | 982 |
| | | Hypo | 6 | <1x10-4 | 29 | 982 |
| | | PAG | 205 | 0.0276 | 17 | 982 |
| | PPI | Blood | 177 | NS | 3 | 982 |
| | | BNST | 223 | 0.0177 | 34 | 982 |
| | | Hippo | 97 | 0.0237 | 23 | 982 |
| | | Hypo | 116 | 0.0276 | 19 | 982 |
| | | Pit | 82 | 0.0017 | 39 | 982 |
| Neurotic Disorders | PPI | Pit | 181 | 0.0174 | 4 | 41 |
| Obsessive-Compulsive Disorder | Dark | Hippo | 33 | 0.0015 | 5 | 69 |
| | FPS | BNST | 138 | 0.0159 | 4 | 69 |
| Panic Disorder | Dark | Blood | 186 | NS | 1 | 94 |
| | | Hippo | 127 | 0.0271 | 4 | 94 |
| | | PAG | 98 | 0.0155 | 5 | 94 |
| | FPS | BNST | 50 | 0.0020 | 6 | 94 |
| | Light | Blood | 134 | NS | 1 | 94 |
| | | Hippo | 37 | 0.0021 | 5 | 94 |
| | | Hypo | 186 | 0.0338 | 4 | 94 |
| | PPI | Blood | 83 | NS | 1 | 94 |
| | | Hippo | 20 | 0.0030 | 6 | 94 |
| | | Hypo | 53 | 0.0056 | 5 | 94 |
| | | PAG | 6 | 0.0003 | 7 | 94 |
| | | Pit | 219 | 0.0274 | 6 | 94 |
| Personality Disorders | FPS | BNST | 57 | 0.0044 | 3 | 24 |

**Table 3-14.** Continued.

| Disease | Phenotype | Tissue | Rank | p-value | Ratio | |
|---|---|---|---|---|---|---|
| Psychiatry and Psychology | Dark | Blood | 130 | NS | 18 | 2980 |
| | | BNST | 19 | 0.0014 | 82 | 2980 |
| | | Hippo | 39 | 0.0020 | 52 | 2980 |
| | | Hypo | 18 | 0.0003 | 76 | 2980 |
| | | PAG | 140 | 0.0305 | 58 | 2980 |
| | FPS | Blood | 404 | NS | 9 | 2980 |
| | | BNST | 47 | 0.0019 | 59 | 2980 |
| | | Hippo | 46 | 0.0007 | 57 | 2980 |
| | | Hypo | 124 | 0.0223 | 42 | 2980 |
| | | PAG | 45 | 0.0012 | 50 | 2980 |
| | | Pit | 101 | 0.0346 | 86 | 2980 |
| | Light | Blood | 216 | NS | 10 | 2980 |
| | | Hippo | 215 | 0.0478 | 37 | 2980 |
| | | Hypo | 26 | 0.0007 | 57 | 2980 |
| | | PAG | 171 | 0.0196 | 42 | 2980 |
| | | Pit | 38 | 0.0238 | 38 | 2980 |
| | PPI | Blood | 44 | NS | 11 | 2980 |
| | | BNST | 32 | 0.0002 | 98 | 2980 |
| | | Hippo | 60 | 0.0121 | 59 | 2980 |
| | | Hypo | 110 | 0.0243 | 47 | 2980 |
| | | PAG | 13 | 0.0014 | 57 | 2980 |
| | | Pit | 23 | 0.0001 | 102 | 2980 |
| Psychoses, Substance-Induced | FPS | BNST | 145 | 0.0182 | 2 | 15 |
| Schizophrenia | Dark | Blood | 263 | NS | 5 | 932 |
| | | Hippo | 20 | 0.0008 | 23 | 932 |
| | | Hypo | 1 | <1x10-4 | 38 | 932 |
| | | PAG | 10 | <1x10-4 | 32 | 932 |
| | FPS | Blood | 288 | 0.0068 | 7 | 932 |
| | | Hippo | 38 | 0.0004 | 25 | 932 |
| | | PAG | 22 | 0.0001 | 24 | 932 |
| | Light | Blood | 252 | NS | 3 | 932 |
| | | Hippo | 119 | 0.0165 | 16 | 932 |
| | | Hypo | 7 | <1x10-4 | 28 | 932 |
| | | PAG | 29 | <1x10-4 | 24 | 932 |
| | PPI | Blood | 81 | NS | 4 | 932 |
| | | BNST | 104 | 0.0028 | 36 | 932 |
| | | Hippo | 47 | 0.0072 | 24 | 932 |
| | | Hypo | 46 | 0.0041 | 21 | 932 |
| | | PAG | 1 | <1x10-4 | 29 | 932 |
| | | Pit | 286 | 0.0483 | 31 | 932 |
| Schizophrenia and Disorders with Psychotic Features | Dark | Blood | 264 | NS | 5 | 936 |
| | | Hippo | 21 | 0.0008 | 23 | 936 |
| | | Hypo | 2 | <1x10-4 | 38 | 936 |
| | | PAG | 11 | <1x10-4 | 32 | 936 |
| | FPS | Blood | 289 | 0.0069 | 7 | 936 |
| | | Hippo | 39 | 0.0004 | 25 | 936 |
| | | PAG | 23 | 0.0001 | 24 | 936 |

**Table 3-14.** Continued.

| Disease | Phenotype | Tissue | Rank | p-value | Ratio | |
|---|---|---|---|---|---|---|
| Schizophrenia and Disorders with Psychotic Features (continued) | Light | Blood | 254 | NS | 3 | 936 |
| | | Hippo | 123 | 0.0171 | 16 | 936 |
| | | Hypo | 8 | <1x10-4 | 28 | 936 |
| | | PAG | 30 | <1x10-4 | 24 | 936 |
| | PPI | Blood | 84 | NS | 4 | 936 |
| | | BNST | 108 | 0.0030 | 36 | 936 |
| | | Hippo | 49 | 0.0075 | 24 | 936 |
| | | Hypo | 47 | 0.0043 | 21 | 936 |
| | | PAG | 2 | <1x10-4 | 29 | 936 |
| Schizophrenia, Paranoid | Dark | PAG | 173 | 0.0452 | 2 | 22 |
| | | Pit | 90 | 0.0169 | 3 | 22 |
| Self-Injurious Behavior | Dark | BNST | 158 | 0.0432 | 5 | 94 |
| | | Hippo | 128 | 0.0271 | 4 | 94 |
| | | Hypo | 55 | 0.0067 | 6 | 94 |
| | | PAG | 99 | 0.0155 | 5 | 94 |
| | FPS | BNST | 24 | 0.0003 | 7 | 94 |
| | | Hippo | 134 | 0.0075 | 5 | 94 |
| | | PAG | 107 | 0.0217 | 4 | 94 |
| | | Pit | 41 | 0.0077 | 7 | 94 |
| | Light | BNST | 91 | 0.0150 | 4 | 94 |
| | | Hippo | 1 | <1x10-4 | 8 | 94 |
| | PPI | Pit | 131 | 0.0081 | 7 | 94 |
| Stress Disorders, Post-Traumatic | Dark | Pit | 137 | 0.0296 | 2 | 11 |
| | FPS | Pit | 87 | 0.0277 | 2 | 11 |
| Stress Disorders, Traumatic | Dark | Pit | 148 | 0.0350 | 2 | 12 |
| | FPS | Pit | 96 | 0.0328 | 2 | 12 |
| Stress, Psychological | Dark | Pit | 115 | 0.0251 | 1 | 1 |
| Suicide, Attempted | PPI | Pit | 12 | <1x10-4 | 4 | 8 |

**FIGURES**

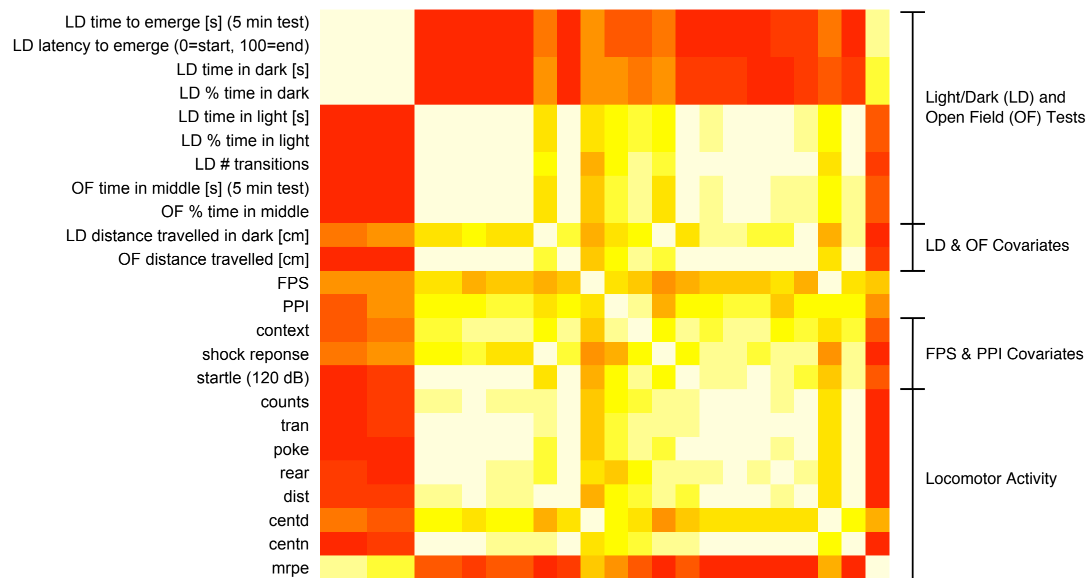


**Figure 3-1.** Correlations between the behavioral phenotypes. Light yellow represent near perfect positive correlation while bright red represents near perfect negative correlation.

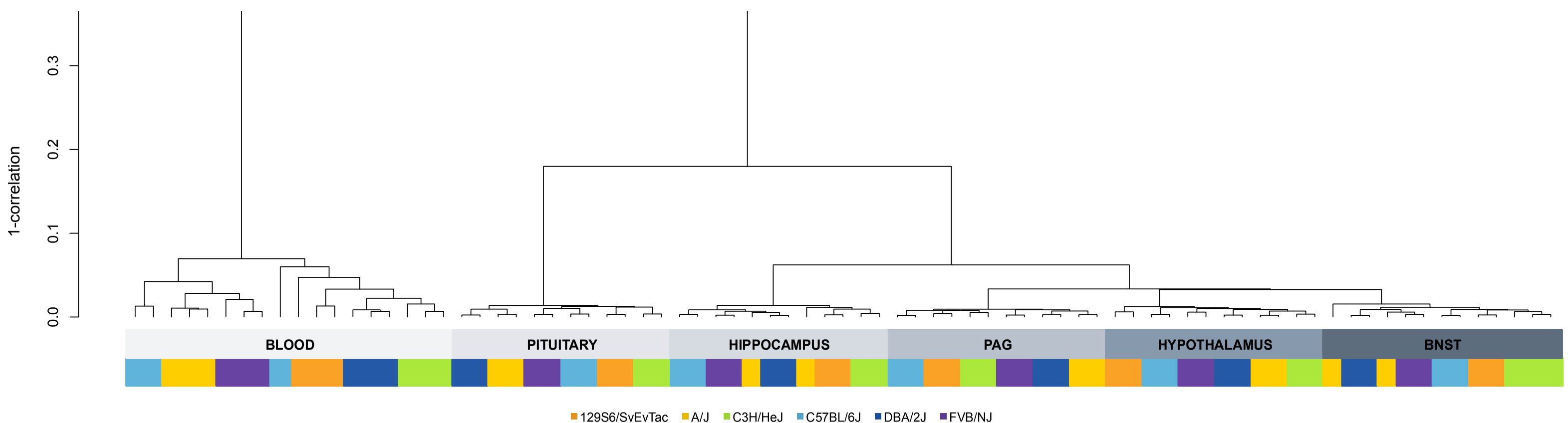


**Figure 3-2.** Unsupervised hierarchical clustering of gene expression profiles in blood and neural tissues. Gold: 129S1/SvImJ(blood) or 129S6/SvEvTac (neural); Yellow: A/J; Green: C3H/HeJ; Light blue: C57BL/6J; Dark blue: DBA/2J; Purple: FVB/nJ.

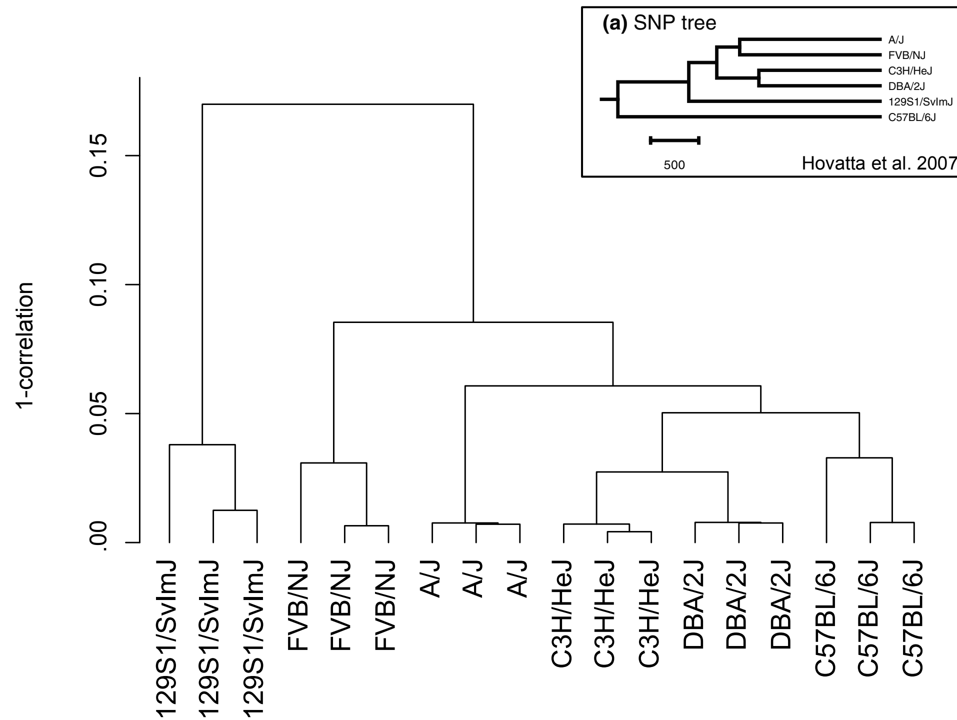


**Figure 3-3.** Unsupervised hierarchical clustering of strain-specific gene expression profiles in blood (n=478 probe sets exhibiting significant strain-effects). Inset dendrogram from Hovatta et al. 2007 highlighting the strain relationships between inbred mouse strains.

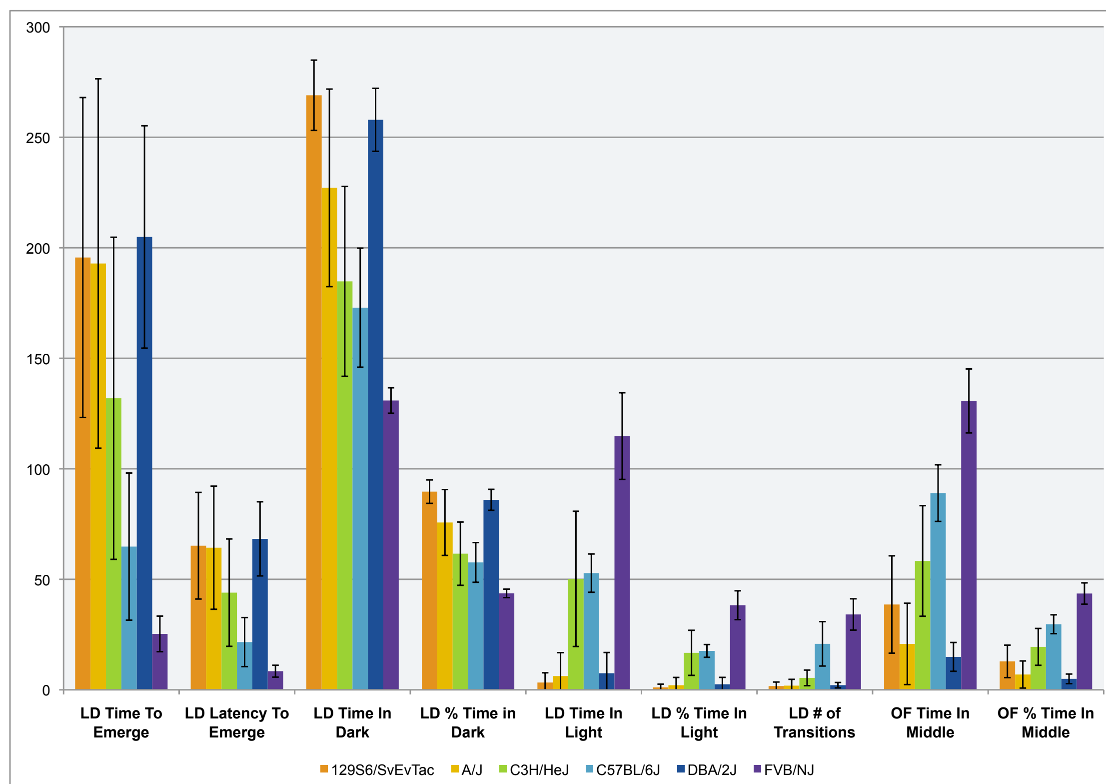


**Figure 3-4.** Light-dark box (LD) and open field (OF) behavioral phenotypes of inbred mouse strains. Error bars = 95% Confidence Interval. Gold: 129S1/SvImJ(blood) or 129S6/SvEvTac (neural); Yellow: A/J; Green: C3H/HeJ; Light blue: C57BL/6J; Dark blue: DBA/2J; Purple: FVB/nJ.

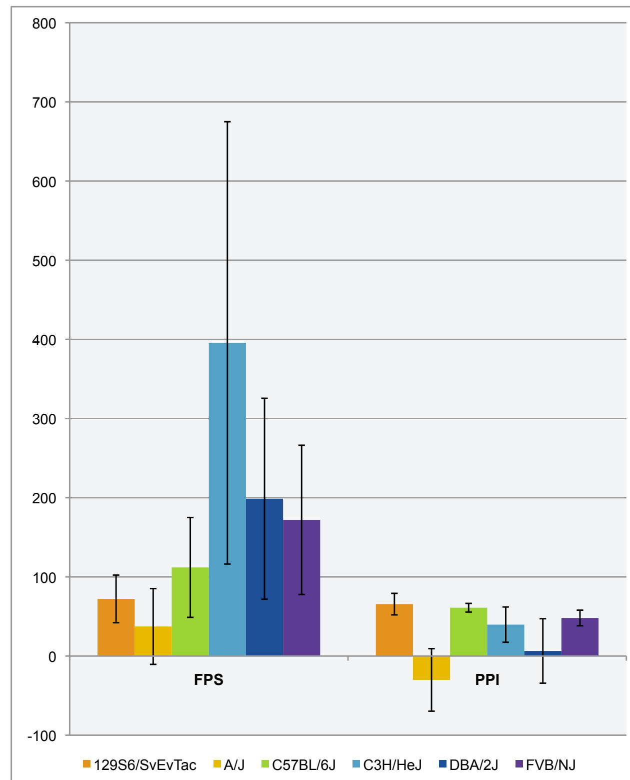


**Figure 3-5.** Fear potentiated startle (FPS) and pre-pulse inhibition (PPI) behavioral phenotypes of inbred mouse strains. Error bars = 95% Confidence Interval. Gold: 129S1/SvImJ(blood) or 129S6/SvEvTac (neural); Yellow: A/J; Green: C3H/HeJ; Light blue: C57BL/6J; Dark blue: DBA/2J; Purple: FVB/nJ.

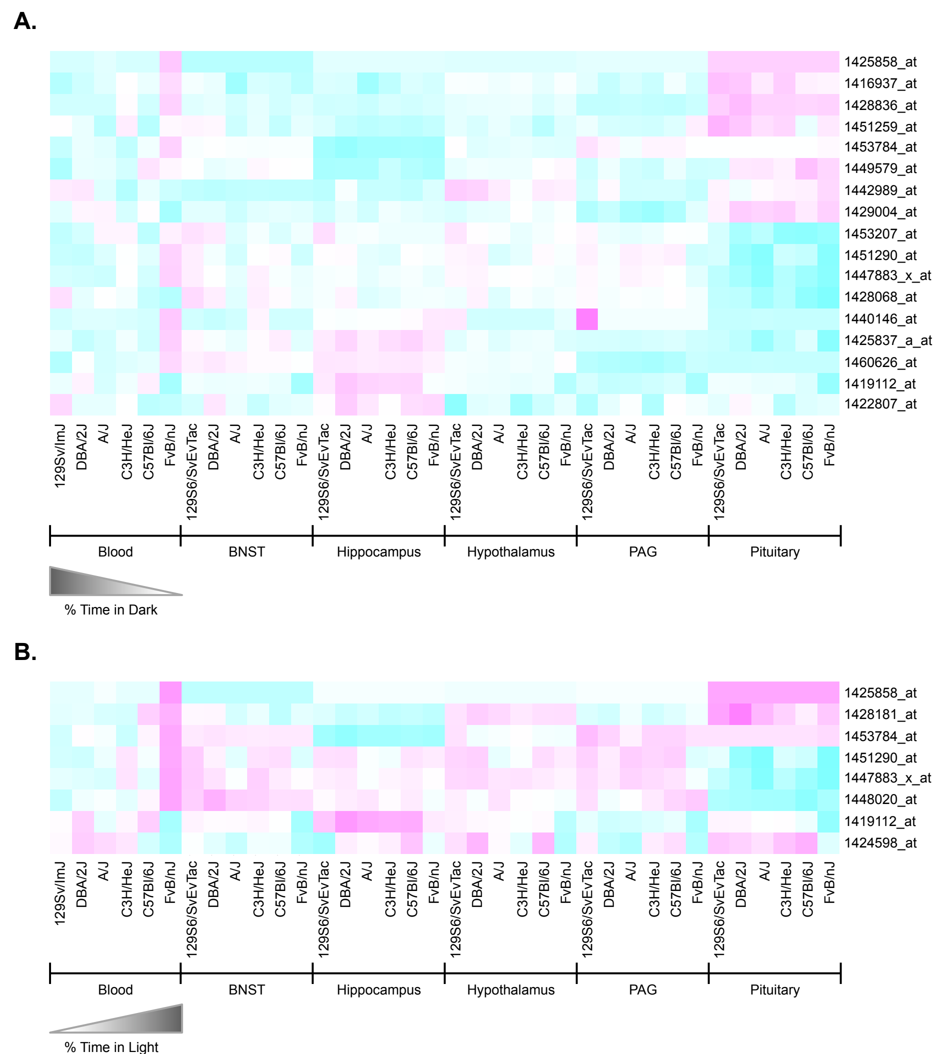


**Figure 3-6.** Heat maps of probe sets significantly associated with four anxiety-related phenotypes in blood and at least one neural tissue: A.) % time in dark, B.) % time in light, C.) FPS, and D.) PPI. The heat maps are clustered according to the y-axis. The y-axis shows the probe set identifiers. The x-axis is organized by strain and tissue. Strains are organized by phenotype, as shown by scale. Blue represents low signal intensity and pink represents high signal intensity; a more intense color means the relatively higher or lower the signal intensity.

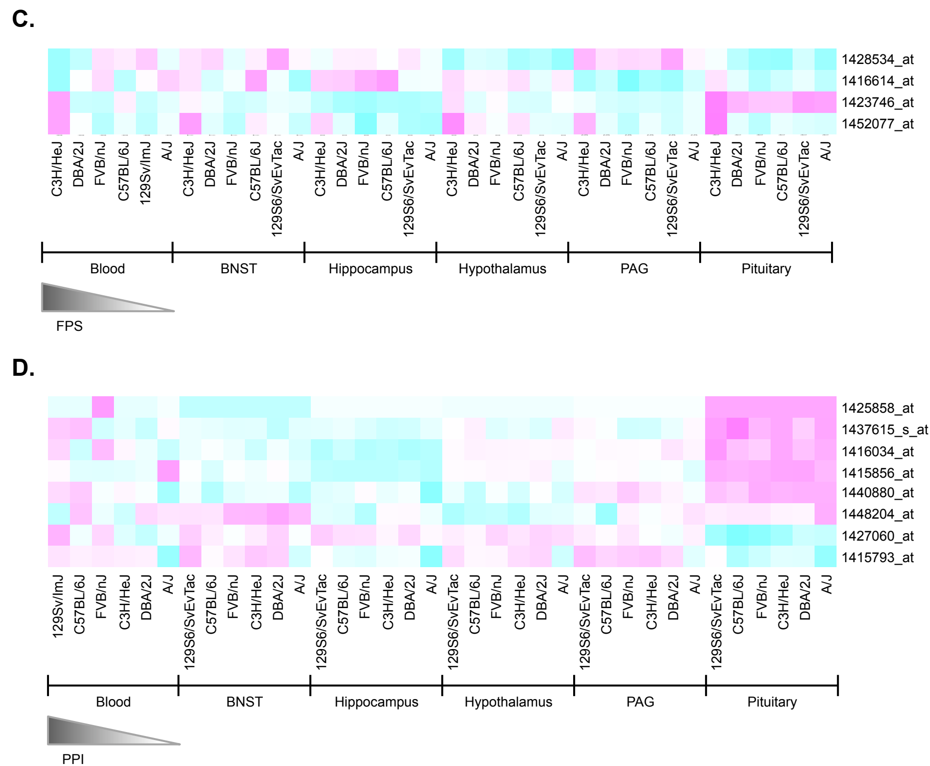


**Figure 3-6.** Heat maps of probe sets significantly associated with four anxiety-related phenotypes in blood and at least one neural tissue: A.) % time in dark, B.) % time in light, C.) FPS, and D.) PPI. The heat maps are clustered according to the y-axis. The y-axis shows the probe set identifiers. The x-axis is organized by strain and tissue. Strains are organized by phenotype, as shown by scale. Blue represents low signal intensity and pink represents high signal intensity; a more intense color means the relatively higher or lower the signal intensity. Continued.

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# CHAPTER 4

## Gene Expression Profiling of Human Whole Blood Samples with the Illumina WG-DASL Assay

**ABSTRACT**

**Background**

Microarray-based gene expression analysis of peripheral whole blood is a common strategy in the development of clinically relevant biomarker panels for a variety of human diseases. However, the results of such an analysis are often plagued by decreased sensitivity and reliability due to the effects of relatively high levels of globin mRNA in whole blood. Globin reduction assays have been shown to overcome such effects, but they require large amounts of total RNA and may induce distinct gene expression profiles. The Illumina whole genome DASL assay can detect gene expression levels using partially degraded RNA samples and has the potential to detect rare transcripts present in highly heterogeneous whole blood samples without the need for globin reduction. We assessed the utility of the whole genome DASL assay in an analysis of peripheral whole blood gene expression profiles.

**Results**

We find that gene expression detection is significantly increased with the use of whole genome DASL compared to the standard IVT-based direct hybridization. Additionally, globin-probe negative whole genome DASL did not exhibit significant improvements over globin-probe positive whole genome DASL. Globin reduction further increases the detection sensitivity and reliability of both whole genome DASL and IVT-based direct hybridization with little effect on raw intensity correlations. Raw intensity correlations between total RNA and globin reduced RNA were 0.955 for IVT-based direct hybridization and 0.979 for whole genome DASL.

**Conclusions**

Overall, the detection sensitivity of the whole genome DASL assay is higher than the IVT-based direct hybridization assay, with or without globin reduction, and should be considered in conjunction with globin reduction methods for future blood-based gene expression studies.

**BACKGROUND**

Peripheral whole blood is an attractive source of mRNA for the identification, examination, and development of disease biomarkers via microarray-based gene expression (Rockett et al. 2004). In fact, many studies have explored the utility of gene expression patterns in whole blood for the purposes of classifying or predicting clinical conditions (Hoang et al. 2010; Lin et al. 2009; Takahashi et al. 2010). However, the sensitivity and specificity of microarray assays using peripheral whole blood are reduced due to the relatively high proportion of globin mRNA present in total RNA, which obscures the detection of transcripts expressed at low levels in whole blood (Fan et al. 2004; Wright et al. 2008). While globin reduction assays have been shown to overcome these effects when used in conjunction with Affymetrix microarrays (Vartanian et al. 2009) and the standard Illumina direct hybridization assay (Debey et al. 2006; Tian et al. 2009), globin reduction assays require large amounts of total RNA (Vartanian et al. 2009), fail to completely eliminate globin transcripts (Vartanian et al. 2009), and may induce distinct gene expression profiles (Liu et al. 2006). Consequently, methods of developing blood-based gene expression biomarker panels that do not involve globin reduction are needed. Developing a microarray-based gene expression assay that does not rely on globin reduction or other methods of sample fractionation, such as the isolation of PBMCs or other cell types from the blood, should reduce sample variability introduced by sample handling and preparation. This will result in a more accurate reflection of the transcriptome at the time of blood draw, and will reduce time and cost.

There are ways to eliminate the need for globin reduction including 1.) the removal of globin probes from the microarray; and 2.) the elimination of globin

transcript amplification. Originally developed for the profiling of partially degraded and fixed RNA samples, the highly sensitive and reproducible Illumina cDNA-mediated annealing, selection, extension and ligation (DASL) assay (Fan et al. 2004; April et al. 2009) uses random priming and a modifiable oligo pool for cDNA synthesis. Random priming in conjunction with PCR amplification may allow for the increased detection of low abundance transcripts. In addition, removing globin-specific oligos from the DASL Assay Oligo Pool (DAP) should decrease noise associated with the high abundance of globin mRNA transcripts and potentially eliminate the necessity of globin reduction. Currently, the DAP is available with and without globin-specific oligos. In order to assess the need for globin reduction with the Illumina DASL assay, we compared microarray gene expression profiles of peripheral blood total RNA and globin-reduced RNA amplified via in vitro transcription (IVT)-based direct hybridization, DASL with globin-specific oligos, and DASL without globin-specific oligos.

**METHODS SUMMARY**

Peripheral whole blood samples were collected from eight human donors in PAXGene blood RNA tubes. RNA was isolated after freezing and storage and then prepared for gene expression analysis using the Illumina Human-Ref8 v3.0 Beadchip. Alpha and beta globin were reduced from a portion of the total RNA using the GLOBINclear assay (Ambion, Austin, TX, USA). Two methods of microarray target preparation were examined: Illumina IVT-based direct hybridization (IVT) and Illumina Whole-Genome DASL (WG-DASL) (**Figure 4-1**). The differences between IVT and WG-DASL are outlined in **Table 4-1**. Two DASL Assay Oligo pools (DAP) were utilized for DASL target preparation: the DASL Assay Oligo Pool with globin probes

(DAP +) and the DASL Asssay Oligo Pool without globin probes (DAP-). Comparisons involving the number of genes whose expression levels were detected and the actual levels of expression of the genes were made across the different platforms. A more complete description of the methods is provided in the Methods section.

## RESULTS

### Comparison between IVT and WG-DASL with and without globin reduction

Following target amplification as outlined in **Figure 4-1**, samples were hybridized with the Illumina Human-Ref8 v 3.0 following the manufacturer's instructions. Each target preparation method was assessed for performance by the number of probes detected as present (Detection p-value < 0.05) (**Figure 4-2**). Probes are generally detected as present if the probe intensity is significantly increased in comparison to the array background intensity. As noted, high levels of background due to the presence of globin transcripts in whole blood are known to decrease the number of significantly detected probes. The WG-DASL target preparation method significantly improved detection sensitivity compared to IVT (p-value = $2.13 \times 10^{-9}$ from an analysis of variance (ANOVA)). Globin reduction decreased probe detection variability with both IVT and WG-DASL target preparation methods. The removal of globin probes from the DASL assay oligo pool (DAP-) resulted in a moderate increase in the number of probes detected but had no significant affect on detection variability (p-value = 0.680, ANOVA) as compared to the DAP+ target preparation method. Overall, 8677 probes were detected across all samples by the

five target preparation methods (**Figure 4-3**), but only 867 probes were detected by IVT alone. 2604 probes were detected by WG-DASL alone.

NanoDrop Spectrophotometer 260/280 ratios were moderately decreased following globin reduction with an average ratio equal to 2.06 prior and 1.97 post globin reduction (**Table 4-2**). However, raw intensity correlations indicate that whole and globin-reduced blood yield similar expression profiles with both IVT and DASL DAP+ assays. Overall raw intensity values increased in globin reduced samples (**Figure 4-4: A, C-D**) despite the failure of GLOBINclear to completely eliminate the two most abundant globin transcripts, hemoglobin alpha (HBA2) and hemoglobin beta (HBB). The removal of globin probes from the DASL Assay Oligo Pool (DAP-) (**Figure 4-4: B**) had little effect on gene expression profiles compared to DAP+ (R2 = 0.993) despite the near complete elimination of HBA2 and HBB.

**Expression patterns maintained across target preparation methods**

IVT target amplification is approximately linear while WG-DASL is approximately logarithmic, making it difficult to compare expression intensities directly. Thus, it was important in our analyses that the sample-to-sample relations are maintained among each target preparation method. Despite the differences in target amplification, sample relations were preserved across the five target preparation methods as shown by unsupervised hierarchical clustering (**Figure 4-5**). For example, with both IVT and WG-DASL, expression profiles for Sample 3 and Sample 7 exhibited the greatest differences from the other six samples, while for the IVT or WG-DASL whole blood RNA clustered separately from globin reduced RNA.

**DISCUSSION**

The analysis of whole blood, microarray-based gene expression profiles is often hindered by low sensitivity and high variability due to high levels of globin mRNA transcripts. These issues have been addressed by the development of globin-reduction methods, which specifically target and remove globin transcripts prior to array hybridization. However, studies have shown that globin reduction, like other methods of sample fractionation, may alter expression profiles (Liu et al. 2006), require large amounts of sample input, increase sample variability (Vartanian et al. 2009), and lead to increased costs. Thus, the ability to assay whole blood without sample fractionation or globin reduction may result in improved gene expression profile quality and decrease cost.

Here we describe the utility of a highly sensitive, whole-genome assay in the assessment of whole blood gene expression. Our results suggest that gene expression detection sensitivity is significantly increased with the whole-genome cDNA-mediated annealing, selection, extension and ligation (WG-DASL) assay as compared to IVT-based direct hybridization (IVT). The increased detection sensitivity of WG-DASL may be due to, 1.) random priming allowing for cDNA synthesis along the length of mRNA transcripts, or 2.) the ability to produce larger amounts of cDNA with PCR amplification. Regardless, attempts to further improve detection sensitivity and decrease expression variability through the selective removal of globin probes from the DASL assay oligo pool (DAP-) did not exhibit any large improvements over globin-probe positive DASL (DAP+). Our study also confirms the positive effect of globin reduction on microarray quality when used in conjunction with the Illumina BeadChip and standard IVT-based hybridization (Tian et al. 2009), while showing that

the positive effect of globin reduction extends to WG-DASL as well. However, as shown by unsupervised hierarchical clustering analysis, globin reduction appears to mildly influence gene expression profiles produced by both IVT and WG-DASL assays. Whether this is due to the induction of a globin reduction-specific profile (Liu et al. 2006), reduced RNA quality due to globin reduction, or the result of decreased noise is unknown, and should be taken into consideration while planning blood-based gene expression experiments.

## CONCLUSIONS

Overall, our results suggest that the detection sensitivity of the WG-DASL assay is higher than the IVT-based direct hybridization assay, with or without globin reduction, and should be considered in conjunction with globin reduction methods for future blood-based gene expression studies. However, further investigation into the ability of the WG-DASL assay to distinguish between disease populations using whole blood is needed, as our study was not designed to address such issues.

## METHODS

### Blood collection and RNA isolation

For each sample, 2.5 ml whole blood was collected in a PAXgene Blood RNA collection tube (Qiagen, Valencia, CA, USA) and stored frozen at -80ºC prior to RNA isolation. RNA isolation was performed using the PAXGene Blood RNA Isolation System (Qiagen, Valencia, CA, USA). RNA quantity and quality were assessed by NanoDrop® Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) before and after globin reduction as well as before and after RNA amplification. For the 8

samples isolated, the total RNA yield ranged from 5.8 – 13.8 ug (average 7.9 ug +/- 1.0 ug), while A260/A280 ratios revealed all samples appeared to be of sufficient quality for microarray analysis (1.93 – 2.10) (**Table 4-2**), despite a moderate decrease in quality following globin reduction.

**Globin Reduction**

Alpha and beta globin mRNA were reduced from a portion of the total RNA samples using the GLOBINclear™ Human kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions with the recommended start quantity of 2 µg of total RNA. Each sample was processed twice then globin-reduced RNA pooled prior to RNA amplification and hybridization.

**RNA amplification and hybridization**

Whole blood total RNA and globin-reduced samples were assayed at both Scripps Genomic Medicine (La Jolla, CA, USA) and Illumina (San Diego, CA, USA) for IVT and DASL-based labelling, hybridization, and scanning, respectively (**Table 4-1**). Briefly, the WG-DASL method utilizes biotinylated random nonamer and oligo (dT) primers to convert 10-200 ng input RNA to cDNA. The biotinylated cDNA is then immobilized to a streptavidin-coated solid support and annealed to a pool of gene-specific oligonucleotides (DAP) for extension and ligation followed by PCR amplification with a biotinylated and a fluorophore-labeled universal primer. Finally, the single-stranded PCR products are eluted and hybridized to an Illumina BeadChip. For this study, 250 ng and 100 ng input RNA were utilized for IVT and DASL, respectively.

Gene expression analysis was performed on all whole blood RNA and globin-reduced samples using Human-Ref8 v3.0 Beadchips (Illumina, San Diego, CA, USA) containing 24,526 probes. All arrays were scanned with the Illumina BeadArray Reader and read into Illumina GenomeStudio® software (version 1.1.1). Individual samples were assayed once for all IVT analyses and twice for all DASL analyses. Given the limited amount of mRNA, replicates were only performed for the DASL assay due to its relative novelty as compared to the IVT assay. All replicates were highly correlated (average R2 = .9925). All raw data is available on the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo, [GSE 28064]).

**Microarray data analysis**

Raw intensities values were exported from GenomeStudio® software (version 1.1.1) for data processing and analysis in R (http://www.R-project.org) and Bioconductor (http://www.bioconductor.org) (Gentleman et al. 2004). Data quality and sample relations were assessed using the Bioconductor lumi package (Du et al. 2008). Probes with a Detection p-value less than 0.05 were considered present. Analysis of Variance (ANOVA) was used to assess the consistency of present/absent calls across the different sample preparation methods. Correlation coefficients were calculated from the raw intensity levels to assess the similarity of expression profiles.

**ABBREVIATIONS**

cDNA: complementary deoxyribonucleic acid; DAP+: DASL Assay Oligo Pool with globin probes; DAP-: DASL Assay Oligo Pool without globin probes; DASL: cDNA-mediated annealing, selection, extension and ligation; GR: RNA following

globin reduction by GLOBINclear; HBA2: hemoglobin, alpha 2; HBB: hemoglobin, beta; HBD: hemoglobin, delta; HBE1: hemoglobin, epsilon; HBG1: hemoglobin, gamma A; HBG2: hemoglobin, gamma G ;HBM: hemoglobin, mu; HBQ: hemoglobin, theta 1; HBZ: hemoglobin, zeta; IVT: in vitro-transcription; mRNA: messenger RNA; WB: total RNA from peripheral whole blood; WG: whole genome.

**ACKNOWLEDGMENTS**

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## TABLES

**Table 4-1.** Summary of IVT and WG-DASL Methods.

| | IVT | WG-DASL |
|---|---|---|
| **Target Preparation Protocol Name** | *In Vitro* Transcription | cDNA-mediated annealing, selection, extension and ligation |
| **Total RNA Input Amount** | 50-100 ng | 10-200 ng |
| **Priming Method** | Reverse Transcription off polyA tail | Poly(T) and random priming with biotinylated nonamers |
| **Amplification** | *In Vitro* Transcription (Linear) | PCR (Exponential) |
| **Hybridization** | Illumina BeadChip | |

**Table 4-2.** RNA quality as assessed by 260/280 ratio. RNA quality was assessed before and after globin reduction as well as before and after amplification. tRNA; total RNA; GC RNA; GLOBINclear treated RNA or globin reduced RNA.

| Sample ID | Before Amplification | Amplified | 1st Globin Reduction | 2nd Globin Reduction | Pooled Globin Reduction | Diluted to 20 ng/ul | Average |
|---|---|---|---|---|---|---|---|
| **C00023 (tRNA)** | 2.03 | 2.01 | | | | 2.07 | 2.04 |
| **C00023 (GC RNA)** | 1.98 | 1.99 | 2.02 | 1.89 | 2.00 | 1.94 | 1.97 |
| **C00027 (tRNA)** | 2.06 | 2.01 | | | | 2.06 | 2.04 |
| **C00027 (GC RNA)** | 1.98 | 2.01 | 2.02 | 1.95 | 1.94 | 1.89 | 1.97 |
| **C00169 (tRNA)** | 2.04 | 2.12 | | | | 1.89 | 2.02 |
| **C00169 (GC RNA)** | 1.96 | 1.95 | 1.88 | 1.90 | 2.02 | 1.84 | 1.93 |
| **C00179 (tRNA)** | 2.04 | 1.99 | | | | 2.12 | 2.05 |
| **C00179 (GC RNA)** | 1.95 | 1.98 | 1.90 | 2.01 | 2.01 | 2.02 | 1.98 |
| **C00275 (tRNA)** | 2.03 | 1.95 | | | | 2.14 | 2.04 |
| **C00275 (GC RNA)** | 1.99 | 1.99 | 1.87 | 1.91 | 1.93 | 2.01 | 1.95 |
| **C00304 (tRNA)** | 2.05 | 1.99 | | | | 2.25 | 2.10 |
| **C00304 (GC RNA)** | 1.98 | 2.00 | 1.89 | 2.11 | 1.91 | 1.96 | 1.98 |
| **C00311 (tRNA)** | 2.04 | 2.10 | | | | 2.12 | 2.09 |
| **C00311 (GC RNA)** | 2.02 | 2.00 | 2.04 | 1.93 | 1.97 | 2.01 | 2.00 |
| **C00342 (tRNA)** | 2.01 | 2.07 | | | | 2.21 | 2.10 |
| **C00342 (GC RNA)** | 2.03 | 2.03 | 1.97 | 1.99 | 1.93 | 1.97 | 1.99 |

**FIGURES**

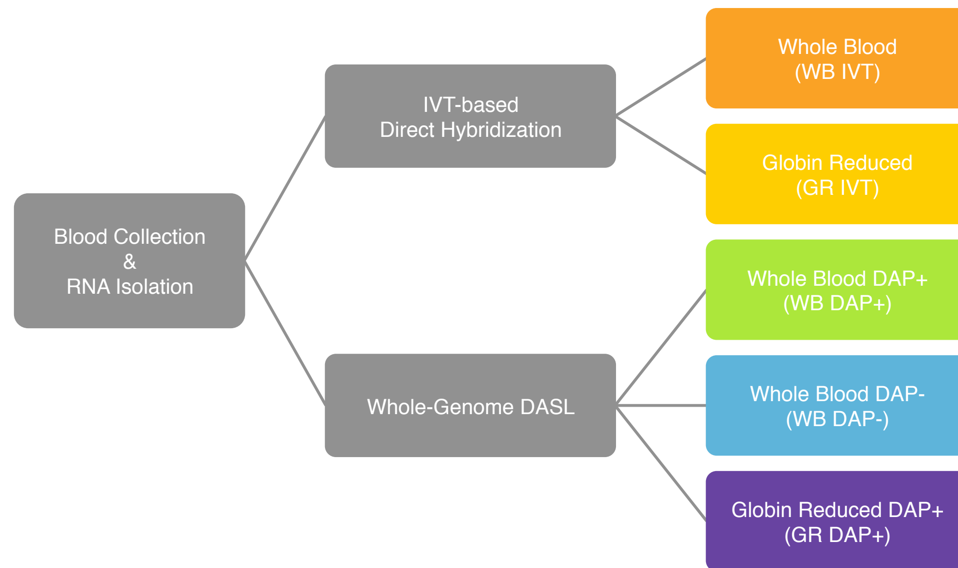


**Figure 4-1.** Flow diagram of study design. A PAXGene blood tube was collected from 8 individuals then frozen and stored for later processing. RNA was isolated and microarray targets prepared by one of five different methods: IVT-based direct hybridization with total RNA (WB IVT), IVT-based direct hybridization with globin-reduced RNA (GR IVT), whole-genome DAP+ DASL with total RNA (WB DAP+), whole-genome DAP- DASL with total RNA (WB DAP-), and whole-genome DAP+ DASL with globin-reduced RNA (GR DAP+).

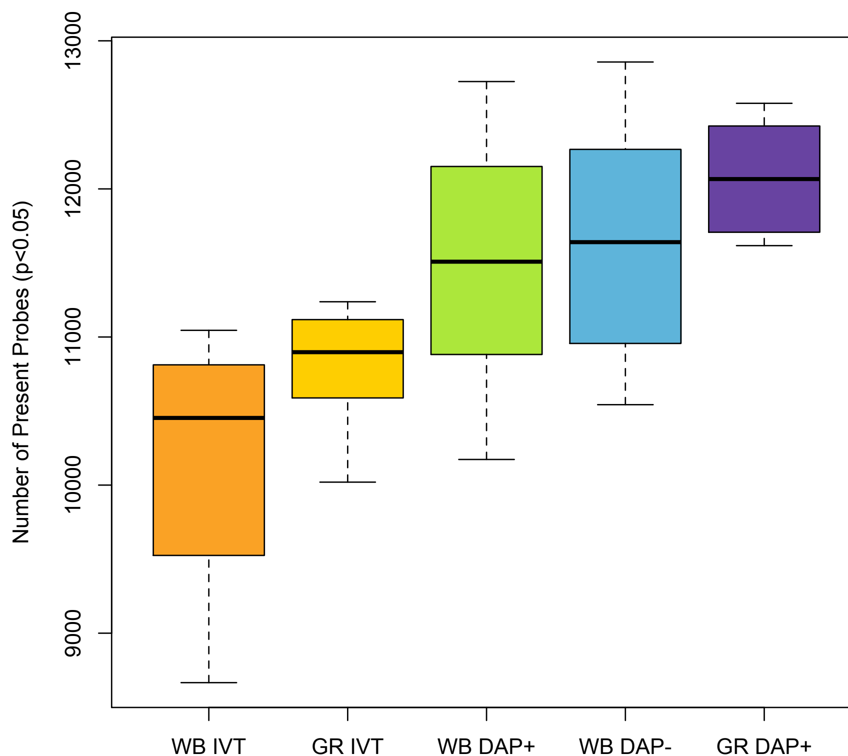


**Figure 4-2**. Box plots of present calls**.** The number of detected probes (detection p-value < 0.05) per target preparation method are shown. The boxes represent the lower quartile through the upper quartile, while the whiskers extend to 1.5 times the interquartile range. A bold line denotes the median. WB IVT and GR IVT (n=8). WB DASL+, WB DASL-, and GR DAP+ (n=16).

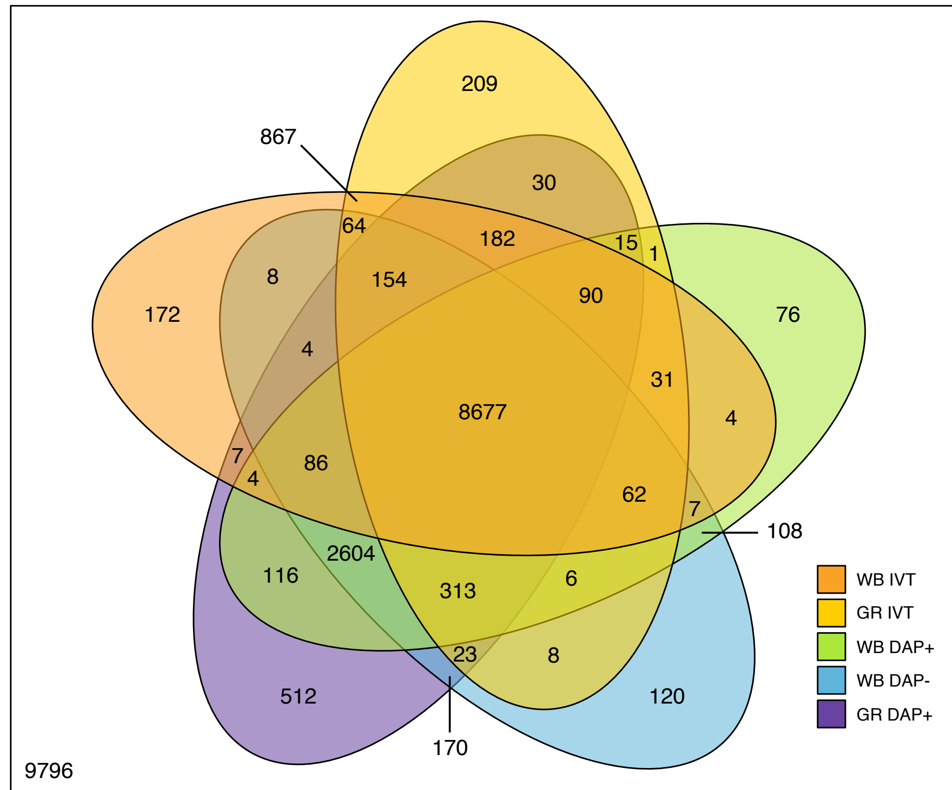


**Figure 4-3.** Overlap of detected probes**.** Probes detected as present across all eight samples per target preparation method are compared. WB IVT: IVT-based direct hybridization with total RNA, GR IVT: IVT-based direct hybridization with globin-reduced RNA, WB DAP+: whole-genome DAP+ DASL with total RNA, WB DAP-: whole-genome DAP- DASL with total RNA, and GR DAP+: whole-genome DAP+ DASL with globin-reduced RNA.

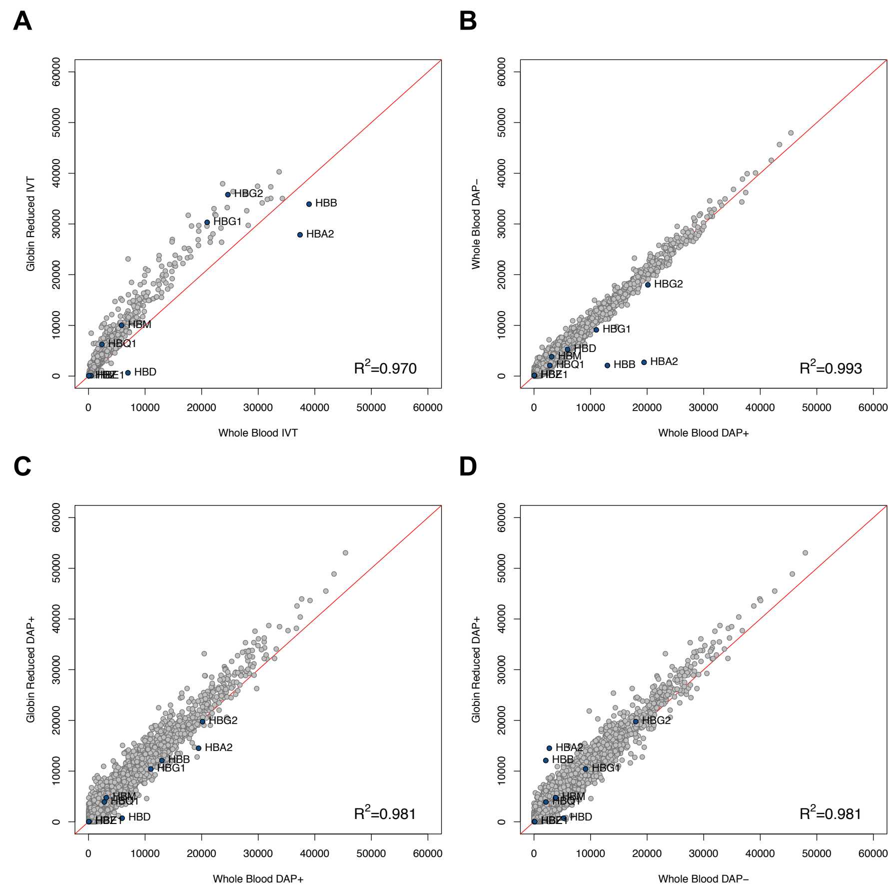


**Figure 4-4.** Raw intensity scatter plots**.** Raw intensities for all probes (n=24526) were compared for (A) whole blood RNA and globin reduced RNA with IVT, (B) whole blood RNA with DAP+ and whole blood RNA with DAP-, (C) whole blood RNA and globin reduced RNA with DAP+, and (D) whole blood RNA with DAP- and globin reduced RNA with DAP-. Correlations for sample 1 are depicted. Average correlations for paired WB IVT versus GR IVT, WB DAP+ versus WB DAP-, WB DAP+ versus GR DAP+, and WB DAP- versus GR DAP- samples are 0.955, 0.992, 0.976, and 0.979, respectively. All 8 hemoglobin genes assayed on Illumina BeadChip Human-Ref v3.0 are labelled: HBA2, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ, and HBZ. GLOBINclear specifically targets only HBA2 and HBB for reduction.

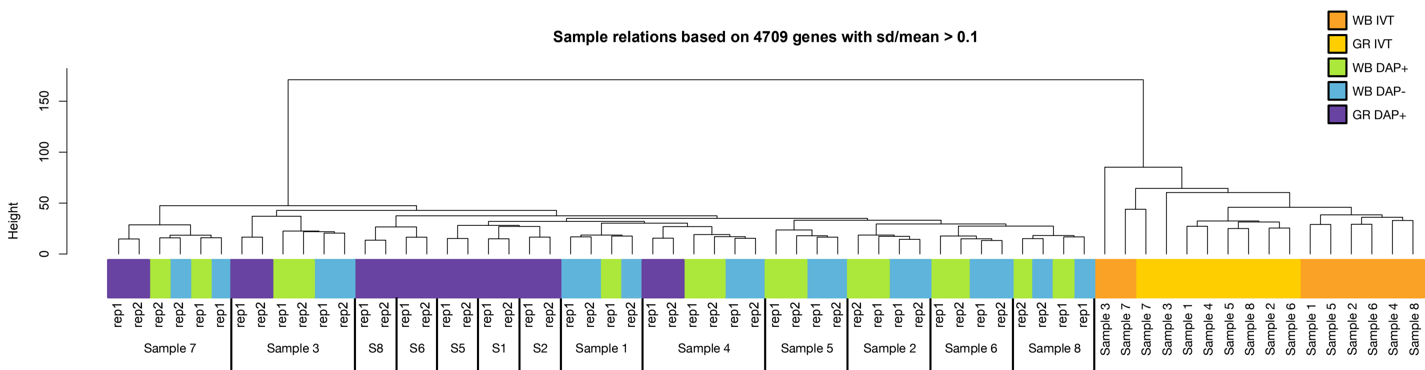


**Figure 4-5.** Sample relations as assessed by unsupervised hierarchical clustering**.** Dendrogram reflecting the clustering of the individual samples and the different sample preparation methods. The dendrogram was constructed using hierarchical clustering methods as implemented in the Bioconductor lumi package.

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# CHAPTER 5

## Comparison of Lymphocyte and Brain Gene Expression Patterns in Young Autistic Cases and Controls

**ABSTRACT**

There has been great interest in the identification of gene expression differences in whole blood, or cell types extracted from blood, between diseased and non-diseased individuals. Such differences might reflect underlying molecular pathologies associated with a disease or act, either, as clinically accessible biomarkers of disease susceptibility or surrogate endpoints for pathogenic processes. It is therefore important to not only assess the strength of associations between gene expression patterns and a disease, but also their potential biological relevance to the disease. We assessed gene expression differences in lymphocytes obtained from young autistic cases and controls to explore the potential blood-based cell type analyses have in producing biologically relevant biomarkers and surrogate endpoints for autism. We contrast our results with gene expression differences recently reported in an expression study involving neural tissues in autism as well as the literature as a whole. We find evidence for gene expression differences in pathways of relevance to autism pathology, but these differences are not entirely consistent with those found in the brain. In particular we find development, transcription, translation, and apoptosis and survival pathways to be among the most enriched in lymphocytes and brain. Our evaluation of the clinical utility of a lymphocyte-based classifier of autism suggests that there is potential, but replication studies are in order.

## INTRODUCTION

There is growing interest in the identification of biomarkers of disease susceptibility, clinical outcomes, and drug response that are relatively easy to interrogate (Addona et al. 2011; Perlis 2011). Many diseases, such as autism and related neurodevelopmental or neuropsychiatric diseases, have primary lesions in tissues that are difficult to access (e.g., neural tissues), hence motivating researchers to consider the utility of biomarkers in more accessible tissues such as blood (Noelker et al. 2011; Le-Niculescu et al. 2009; Rockett et al. 2004). Assaying blood for potential biomarkers with modern transcriptomic, proteomic, and epigenomic technologies has, in fact, led to a number of recent notable successes (Feinberg et al. 2010; Rosenberg et al. 2010; Teschendorff et al. 2008). Despite these successes, there are a number of issues surrounding the use of blood-based assays for differentiating individuals with and without, e.g., autism or another clinical condition.

Blood is composed of a number of cell types known to manifest unique gene and protein expression, as well as epigenomic, profiles (Miao et al. 2008; Jacobsen et al. 2006; Palmer et al. 2006). These unique profiles are not only influenced by genetic factors (Yang et al. 2010; Göring et al. 2007) but also environmental factors, such as diet (Leonardson et al. 2010). Thus, not only is it the case that variation in the fraction of blood that is composed of different cell types across individuals can impact, e.g., blood gene expression profiles (Palmer et al. 2006; Whitney et al. 2003), but the degree to which different blood cell types express common genes amongst themselves and with respect to different tissues raises questions about the biological coherence or relevance of expression patterns in the blood with respect to expression patterns in other tissues.

One way to avoid issues associated with the heterogeneity of cell types in the blood is to focus on one particular blood cell type or cell family. Peripheral blood mononuclear cells (PBMCs) and lymphocytes have been studied widely in this regard (Baine et al. 2011; Gupta et al 2011; Bowden et al. 2006; Vawter et al. 2004). Given their role in immune system activity, the interrogation of perturbations or genomic alterations in lymphocytes is seen as logical given that lymphocytes more or less act as 'sentinels' of immune system dysregulation and other disturbances possibly associated with a disease (Fan et al. 2005). In addition, since many genes are known to be expressed in both lymphocytes and other tissues – and possibly ubiquitously expressed across all cell types and tissues – it may be the case that pathogenic molecular genetic disturbances that manifest in lymphocytes are also present in cell types more directly relevant to the fundamental lesions associated with a disease (Rollins et al. 2010). Such common disturbances may not necessarily occur at the individual gene level, but possibly at higher levels of biological organization, such as at the pathway or molecular physiologic process level (Subramanian et al. 2005).

Ultimately, however, even if a biologically-sound lymphocyte or whole blood-based biomarker profile is found to differentiate individuals with and without a particular condition such as autism or autism-related clinical phenotypes for diagnostic, prognostic, or therapeutic purposes, a good question is whether or not such differentiation can be achieved through other, possibly less invasive and less costly, procedures (Rosenberg et al. 2010). This question, though not directly related to the biological relevance of blood cell profiles, or processes in blood cells, to other cell types, does bear on the clinical utility and motivation for blood-based biomarker assays.

We assessed lymphocyte gene expression patterns in young individuals with and without autism. We compared the results of our study with those of a previous report investigating brain gene expression patterns in autism (Chow et al. 2011, submitted) as well as genes implicated in previous genome-wide association, copy number variation, and gene expression studies. We considered not only individual gene expression differences, but also pathway level differences. Finally, we compared the classification accuracy of lymphocyte-based gene expression patterns with the accuracy of classifiers based on brain gene expression.

## RESULTS

### Differential gene expression and gene set enrichment analysis between autism cases and controls in lymphocytes

We analyzed lymphocyte gene expression profiles from 290 individuals (total number of arrays = 347) collected by the Autism Center of Excellence (La Jolla, CA, USA). This is a diverse data set containing both single or longitudinal time points as well as single (child only), duo (mother- or father-child), or trio (mother, father, and child) samples for young children (12-24 months of age) at risk for autism spectrum disorder (ASD), developmental delay (DD), or language delay (LD). Given the heterogeneous nature of the data set, this study focuses on 76 male, first time-point samples (cases=45, controls=31; **Table 5-1**).

We identified 2321 genes as differentially expressed in autism cases relative to controls (p-value < 0.05; FDR ~ 0.163)(See Supplementary Material Table 5-S1). Among the top 20 up- and/or down-regulated genes were genes potentially involved in autism pathogenesis, including genes involved in signal transduction (PGHD,

MNK2, CSNK1G2, CHD8, GPR44, TRAT1, FCGR1B, IFIT3, OAS1), transport (SORL1, ABCA7, VAMP2, KCNG1), and anti-apoptotic/pro-cell cycle genes (CABIN1, CHD8, UBA3, NAMPT, ARG1, IFI27). Of the 2312 differentially expressed genes, 67% and 23% were known to be expressed in the brain and fetal brain, respectively (eGenetics/SANBI EST database; http://biomart.org).

Differentially expressed genes were then subjected to enrichment analysis via MetaCore™ (GeneGO Inc, St. Joseph, MI, USA). The most significant GeneGo Pathway Maps (n = 68, p<0.001, FDR<0.005) (**Table 5-2A**) were heavily populated by development pathways (26/68), while GeneGO Process Networks (n = 17, p <0.001, FDR<0.01) (**Table 5-2B**) were most often related to cell cycle (4/17).

**Comparing lymphocyte gene expression to brain gene expression via differential expression and gene set enrichment analysis**

To test whether these findings are comparable to those from brain gene expression studies, we compared our results to those identified by Chow et al. (Chow et al., submitted) in which they identified 2017 genes to be differentially expressed in autism cases relative to controls. Comparing GeneGO Pathway Maps enrichment between the two tissues (**Table 5-3**), the same development pathway, A2A receptor signaling, was the most significant among differentially expressed brain and lymphocyte genes. In general, 9 of the top 15 pathways enriched among differentially expressed genes in lymphocytes were significantly enriched in the brain (**Table 5-3A**), while 12 of the top 15 pathways enriched among differentially expressed genes in brain were significantly enriched in lymphocytes (**Table 5-3B**).

We next looked at the individual genes differentially expressed in the same direction in both lymphocytes and brain (**Figure 5-1**); 20 genes were up-regulated (**Table 5-4**) while 56 genes were down-regulated (**Table 5-5**). Genome-wide association, copy-number variation, and/or gene expression analyses had previously identified many of these genes (33%) to be dysregulated or altered in autism cases. Among the genes up-regulated in both the brain and lymphocytes, apoptosis and survival pathways were among the most significant (**Table 5-6A**), while the most significant GeneGO Process Networks were heavily populated by immune response and inflammation processes (**Table 5-6B**). Down-regulated genes were heavily enriched for development pathways (**Table 5-7A**) and a variety of process networks, including several cell cycle networks (**Table 5-7B**).

**Diagnostic classification of autism using lymphocyte and brain gene expression**

Finally, we applied class prediction tools, as implemented in BRB-Array Tools, to assess the ability of differentially expressed genes to separate autism cases from controls using lymphocyte and brain gene expression (**Figure 5-2**). Using Receiver Operator Curve (ROC) analysis to test the significance of each model, a 67-gene model developed from lymphocyte gene expression yielded a cross-validated AUC equal to 0.668 (p=0.007) (**Figure 5-2A**, blue)(See Supplementary Material Table 5-S2). A 116-gene model developed from brain gene expression yielded a cross-validated AUC equal to 0.704 (p=0.024) (**Figure 5-2B**, blue)(See Supplementary Material Table 5-S3). These two models were not significantly different (p=0.757).

Testing the lymphocyte and brain-based models on the opposite tissue, the 67-gene lymphocyte model tested on brain gene expression yielded an AUC equal to 0.574 (p=0.240) (**Figure 5-2B**, green). The 116-gene brain model tested on lymphocyte gene expression yielded an AUC equal to 0.618 (p=0.042) (**Figure 5-2A**, green). These two models were not significantly different (p=0.732). Neither model, when tested on the opposite tissue, was significantly different compared to the original, cross-validated model (p=0.58152, **Figure 5-2A**; p=0.27665, **Figure 5-2B**).

The genes included in each model were enriched for apoptosis and survival, immune response, and development GeneGO Pathway Maps (**Table 5-8**).

## DISCUSSION

Understanding the strength of associations between gene expression patterns and a disease, but also their potential biological relevance to the disease, is important to the development of blood-based gene expression diagnostics. In the present study, we assessed differences in lymphocyte gene expression between young autistic cases and controls and compared them to the results of a previous brain gene expression study (Chow et al. 2011, submitted) and the current literature. Young autistic lymphocyte gene expression was found to display dysregulation among development, apoptosis and survival, cell cycle, and immune response pathways and networks, among others. Many of these pathways and networks were shown to be significantly dysregulated in the young autistic brain suggesting lymphocyte gene expression is capable of capturing biologically relevant genetic dysregulation. For instance, the top dysregulated pathway (A2A receptor signaling) in both lymphocytes and brain is a potent biological mediator that affects several cell types including

neuronal cells. In brain, A2AR in highly expressed in the dorso-ventral striatum and at lower levels in the cortex, cortico-striatal terminals and hippocampus (Svenningsson et al., 1997; Rebola et al., 2005) and is involved in neuronal excitability, neurotransmitters release, neuronal synaptic plasticity, cognition and neuro-protection/-inflammation (Wei et al., 2011). In vitro, stimulation of A2AR prevents apoptosis via PKA-cat activation (Huang et al., 2001) that in turn, upon stimulation, enhances PKC-zeta activity and cell survival (Qiu et al., 2000).

Similarly, GM-CSF signaling, ranked 2$^{nd}$ in lymphocytes and 17$^{th}$ in brain, is responsible for the proliferation, differentiation, survival and maturation of immune and neuronal cells via the transcription factors STAT3 and STAT5, that in turn activate proliferative proteins like cyclinD1/3, Pim-1 and anti-apoptotic proteins like Mcl-1 and BCL-2/-x as well as mitogen-activated proteins like ERK1/2 via SHC transforming protein 1 (Kolonics et al., 2001; Choi et al., 2007). A major pathway involved in apoptosis and survival (BAD phosphorylation) was also highly ranked in both lymphocytes and brain. BAD induces apoptosis by inhibiting anti-apoptotic BCL-2-family members BCL-x, Bcl-2 (Bergmann et al., 2002). Overall, development and apoptosis and survival pathways were significantly enriched in the analysis of the commonly dysregulated brain/lymphocytes genes as well as the gene expression classifiers, demonstrating the utility of lymphocyte gene expression in identifying biologically relevant disease profiles at a pathway or network level.

Lymphocytes were less successful at identifying individual genes shown to be significantly dysregulated in the autistic brain. Approximately 4% of the differentially expressed genes in the brain were dysregulated in the same direction in lymphocytes. Nonetheless, most of the genes are involved in developmental and

apoptotic functions, while one third of these common genes were involved or dysregulated in previous autism studies. DCUN1D1 is known to regulate cell growth, viability, and development (Kim et al. 2008), is a risk factor for frontotemporal lobar degeneration (Villa et al. 2009), and maps to an autism susceptibility locus (Mas et al. 2000). Other genes are involved in the dopamine and/or serotonin synthesis pathways (GCH1, YWHAZ) (Wang et al. 2009). YWHAZ, which has been nominally associated with autism (Anderson et al. 2009), has also been implicated in the regulation of neurite outgrowth (Ramser et al. 2010) and more generally in cell cycle regulation and cell growth and death (Mhawech et al. 2005). A previous study (Philippe et al. 1999) identified a candidate genomic region containing TCP1, a gene involved in cytoskeletal maintenance and neurotransmitter trafficking. VEZT is known to play a role in the establishment of adherens junctions thus regulating dendritic formation of hippocampal neurons (Sandra et al. 2010), a region of the brain enlarged in autism (Groen et al. 2010). SLC30A5 is believed to transport zinc and was shown to be down-regulated in both lymphocytes and brain (Chow et al. 2011, submitted, Gregg et al. 2008) as well as deleted among sporadic autism cases (O'Roak et al. 2011). Although it is not clear how several of these genes may play into neural development and the manifestation of autism, the converging evidence presented here suggests further study on the subject is warranted. Regardless, these findings demonstrate that peripheral blood, in particular lymphocytes, may be used to detect a proportion of the genetic dysregulation occurring in the brain of autistic subjects.

While we demonstrate the ability of lymphocyte gene expression to capture genes and pathways known to be dysregulated in neural tissue, it is necessary to show these profiles are capable of classifying autism cases from controls in both a

sensitive and specific manor. The evidence we provide suggests lymphocyte gene expression profiles are able to meet this requirement at a similar level as brain gene expression profiles, thus being a relevant RNA source for the development of gene expression-based biomarkers of autism. Nonetheless, future clinical studies specifically aimed at biomarker development will be needed to refine and test the brain and lymphocyte-based models built here as our study was not specifically designed to address the specific needs associated with development of a clinically relevant classifier and suffers from overall sample size, particularly among the brain. It also remains to be seen if gene expression will significantly add to the classification of autism using widely accepted clinical factors and screening tools such as The One-Year Well-Baby Check Up Approach (Pierce et al. 2011). Rather, gene expression may be more useful in tracking the success or failure of clinical interventions as well as differences in clinical progression or recession once a child is diagnosed with autism. Further studies equating gene expression with QTL and CNV analysis as well as clinical and imaging phenotypes will only enhance our ability to predict and classify autism in infants.

## MATERIALS AND METHODS

### Subject Identification

All procedures were performed according to protocols approved by the University of California, San Diego Institutional Review Board. All minor subjects assented to the study procedures, and one or both parents or legal guardians of each subject provided written informed consent for their child to participate.

Participants were obtained by: 1.) community referral (e.g., website or outside agency) or 2.) a general population-based screening method called the One-Year Well-Baby Check-Up Approach (Pierce et al. 2011) performed by the participant's pediatrician. Using the latter approach, toddlers as young as 12-months who were at-risk for an Autism Spectrum Disorder (ASD), Language Delay (LD), or Developmental Delay (DD) were recruited and tracked every six months until at least their third birthday, thus allowing for the prospective study of autism beginning at 12 months. Typically developing (TD) and type-1 error (TIE) control subjects were obtained from community referrals.

ASD subjects were diagnosed based on failure of the Autism Diagnostic Observation Schedule (ADOS)(Lord et al. 2001) as well as the clinical judgment of a PhD-level psychologist. While several ASD toddlers were only one year old at the time of blood sampling, all but one have been tracked and diagnosed with an ASD using the toddler module of the ADOS (Luyster et al. 2009) until at least age two, when the diagnosis of autism can be made reliably. Final diagnoses of an ASD for participants older than 30 months were confirmed with the Autism Diagnostic Interview–Revised (Luyster et al. 2009).

**Sample Collection and Processing**

From each subject, 4ml of venous blood was collected into EDTA-coated collection tubes and immediately transferred to an RNase-free laboratory, where all subsequent procedures took place. Total mRNA was extracted, stabilized, isolated, and stored from each blood sample in a manner as previously described (Glatt et al. 2009, Glatt et al. 2005, Tsuang et al. 2005). Briefly, each blood sample was passed

over a LeukoLOCK™ (Ambion, Austin, TX, USA) filter, which was flushed with PBS and then fully saturated with RNAlater® (Ambion, Austin, TX, USA). Each LeukoLOCK™ filter, containing bound, isolated, stabilized, and purified white blood cells, was sealed and stored in a sterile box at -20°C. Once all samples were collected, LeukoLOCK filters were processed by flushing the filter with TRI reagent® (Ambion, Austin, TX, USA) to lyse the cells and isolate mRNA. Eluted mRNA samples were stored at -20°C until transferred to Scripps Genomic Medicine (La Jolla, CA, USA) for quality assurance and microarray hybridization.

**Sample Quantification and Quality Control**

The concentration of mRNA in each sample was quantified by the absorption of ultraviolet light at 260 nm. The quantity of mRNA in each sample exceeded the minimally sufficient amount required for microarray hybridization. The purity of each mRNA sample was estimated by the 260:280 nm absorbance ratio, with an acceptable range designated a priori as 1.7-2.1. The quality of each mRNA sample was quantified by the RNA Integrity Number (RIN) and, according to convention, values of 6.0 or greater were deemed acceptable (Schroeder et al. 2006). A total of 339 samples selected for analysis in Wave I had acceptable levels of mRNA quantity, purity, and quality.

**Labeling, Hybridization, and Scanning**

Lymphocyte total RNA was assayed at Scripps Genomic Medicine (La Jolla, CA, USA) for labelling, hybridization, and scanning using Illumina HumanWG-6 v3.0 expression BeadChips (Illumina, San Diego, CA, USA) per the manufacturer’s

instruction. All arrays were scanned with the Illumina BeadArray Reader® and read into Illumina GenomeStudio® software (version 1.1.1). Raw data was exported from Illumina GenomeStudio® for data pre-processing and normalization.

### Data Processing

Data processing was performed using the lumi package (Du et al. 2008) for R (http://www.R-project.org) and Bioconductor (http://www.bioconductor.org) (Gentleman et al. 2004). Of the 347 arrays processed, 27 were identified as low-quality based on poor signal intensity (raw intensity box plots and average signal >2 standard deviations below the mean) and poor hierarchical clustering (Oldham et al. 2008) and were removed prior to log2 transformation and quantile normalization. The remaining 320 high-quality arrays were filtered for first time point, male, proband samples with a diagnosis of ASD, PDD-NOS, TD, or TIE (n=76).

### Data Analysis and Gene Set Enrichment Analysis

For differential expression analysis, normalized expression values were imported into BRB-Array Tools (http://linus.nci.nih.gov/BRBArrayTools.html). Gene filtering was performed as previously described (Chow et al. 2011, submitted) followed by differential expression analysis via Class comparison between groups of arrays using a random variance model and 10,000 univariate permutation tests. Differentially expressed genes ($p<0.05$) were then assessed for pathway enrichment using the MetaCore software suite (www.genego.com/metacore.php) (GeneGO, Inc., St. Joseph, MI, USA).

Genes imported into MetaCore were filtered for known expression specific to the brain or the fetal brain followed by enrichment analysis of GeneGO Pathway Maps and GeneGO Process Networks using the default background gene list. GeneGO Pathway Maps are defined as sets of linear consecutive signals, or metabolic transformations, that have been confirmed as a whole by inferred relationships or experimental data. GeneGO Process Networks are network models of main cellular processes that are created manually by GeneGO using information from GO processes and GeneGO Pathway Maps.

Multigene models for classifying autistic and control samples were constructed using BRB-Array Tools Class Prediction methods for both lymphocytes and brain gene expression profiles (Radmacher et al. 2002). Class Prediction creates a multivariate predictor for determining which of the two classes a given sample belongs. Leave-one-out cross-validation was used to determine misclassification rate. The Bayesian Compound Covariate Predictor results were exported from BRB-Array Tools and the reliability of this classification assessed via receiver-operator characteristic (ROC) curve analysis using the pROC package for R (Robin et al. 2011). ROC curve analysis was also used to compare different classification models as implemented in the pROC package. Two models were built: one using lymphocyte gene expression, the other using brain gene expression. Then both of these models were used to classify autism cases and controls using the other tissue source.

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Pierce, Eric Courchesne, Nicholas J Schork. The dissertation author was the primary investigator and author of this paper.

## TABLES

**Table 5-1.** Sample characteristics for lymphocyte gene expression**.**

| | **Cases** (n=45) | | | **Controls** (n=31) | | |
|---|---|---|---|---|---|---|
| | **All Cases** | **ASD** | **PDD-NOS** | **All Controls** | **TD** | **T1E** |
| Sample size (n) | 45 | 31 | 14 | 31 | 11 | 20 |
| Age (mean months ± s.d.) | 27.10±9.63 | 24.28±9.98 | 33.34±4.83 | 19.12±7.66 | 24.41±10.06 | 16.22±3.79 |
| Age (range in months) | 12.60-43.50 | 12.60-43.50 | 26.87-41.03 | 12.5-44.93 | 12.50-44.93 | 12.93-24.63 |

**Table 5-2.** Enriched GeneGo Pathway Maps and GeneGo Process Networks**.**

**A.** GeneGo Pathway Maps (FDR<0.01)

| GeneGo Pathway Map | p-value | Ratio | |
|---|---|---|---|
| Development_A2A receptor signaling | 4.672E-07 | 13 | 43 |
| Development_GM-CSF signaling | 4.841E-07 | 14 | 50 |
| Development_Flt3 signaling | 6.289E-07 | 13 | 44 |
| Development_PIP3 signaling in cardiac myocytes | 1.454E-06 | 13 | 47 |
| Translation_Insulin regulation of translation | 2.539E-06 | 12 | 42 |
| Apoptosis and survival_BAD phosphorylation | 2.539E-06 | 12 | 42 |
| Immune response_Function of MEF2 in T lymphocytes | 3.128E-06 | 13 | 50 |
| Development_Role of IL-8 in angiogenesis | 3.422E-06 | 14 | 58 |
| Transcription_CREB pathway | 4.358E-06 | 12 | 44 |
| Translation _Regulation of EIF4F activity | 6.329E-06 | 13 | 53 |
| G-protein signaling_Regulation of p38 and JNK signaling mediated by G-proteins | 7.680E-06 | 11 | 39 |
| Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis | 7.906E-06 | 13 | 54 |
| Chemotaxis_Leukocyte chemotaxis | 1.818E-05 | 15 | 75 |
| G-protein signaling_G-Protein alpha-12 signaling pathway | 3.020E-05 | 10 | 37 |
| Chemotaxis_CCR4-induced leukocyte adhesion | 3.037E-05 | 9 | 30 |
| Development_Thrombopoietin-regulated cell processes | 3.398E-05 | 11 | 45 |
| Regulation of lipid metabolism_Insulin signaling:generic cascades | 5.252E-05 | 11 | 47 |
| DNA damage_ATM/ATR regulation of G1/S checkpoint | 5.370E-05 | 9 | 32 |
| Cell cycle_Role of APC in cell cycle regulation | 5.370E-05 | 9 | 32 |
| Cytoskeleton remodeling_Cytoskeleton remodeling | 6.134E-05 | 17 | 102 |
| DNA damage_ATM / ATR regulation of G2 / M checkpoint | 6.926E-05 | 8 | 26 |
| Development_S1P1 receptor signaling via beta-arrestin | 7.013E-05 | 9 | 33 |
| Cell cycle_Spindle assembly and chromosome separation | 7.013E-05 | 9 | 33 |
| Chemotaxis_CXCR4 signaling pathway | 9.060E-05 | 9 | 34 |
| Regulation of degradation of deltaF508 CFTR in CF | 9.354E-05 | 8 | 27 |
| Development_EDNRB signaling | 9.630E-05 | 11 | 50 |
| Apoptosis and survival_HTR1A signaling | 9.630E-05 | 11 | 50 |
| Development_A2B receptor: action via G-protein alpha s | 9.630E-05 | 11 | 50 |
| Immune response _Immunological synapse formation | 1.051E-04 | 12 | 59 |
| Development_EPO-induced Jak-STAT pathway | 1.158E-04 | 9 | 35 |
| Development_Growth hormone signaling via STATs and PLC/IP3 | 1.158E-04 | 9 | 35 |
| Development_IGF-1 receptor signaling | 1.165E-04 | 11 | 51 |
| Development_ACM2 and ACM4 activation of ERK | 1.218E-04 | 10 | 43 |
| Development_SSTR2 in regulation of cell proliferation | 1.467E-04 | 9 | 36 |
| Immune response_IL-5 signalling | 1.496E-04 | 10 | 44 |
| Cell cycle_Sister chromatid cohesion | 1.575E-04 | 7 | 22 |
| Cell adhesion_Chemokines and adhesion | 1.651E-04 | 16 | 100 |
| Development_FGFR signaling pathway | 1.681E-04 | 11 | 53 |
| Development_A1 receptor signaling | 1.681E-04 | 11 | 53 |
| Cell cycle_Influence of Ras and Rho proteins on G1/S Transition | 1.681E-04 | 11 | 53 |
| Development_Delta- and kappa-type opioid receptors signaling via beta-arrestin | 2.153E-04 | 7 | 23 |
| Signal transduction_PTEN pathway | 2.217E-04 | 10 | 46 |
| Cytoskeleton remodeling_Fibronectin-binding integrins in cell motility | 2.712E-04 | 8 | 31 |
| Apoptosis and survival_Role of IAP-proteins in apoptosis | 2.712E-04 | 8 | 31 |
| Immune response_IL-3 activation and signaling pathway | 2.712E-04 | 8 | 31 |
| Development_GDNF signaling | 2.891E-04 | 7 | 24 |
| Cytoskeleton remodeling_FAK signaling | 3.303E-04 | 11 | 57 |
| Apoptosis and survival_Granzyme B signaling | 3.437E-04 | 8 | 32 |
| Development_PDGF signaling via STATs and NF-kB | 3.437E-04 | 8 | 32 |
| Immune response_TCR and CD28 co-stimulation in activation of NF-kB | 3.471E-04 | 9 | 40 |

**Table 5-2.** Continued.

**A.** Continued.

| GeneGo Pathway Map | p-value | Ratio | |
|---|---|---|---|
| Immune response_IL-22 signaling pathway | 4.312E-04 | 8 | 33 |
| G-protein signaling_Ras family GTPases in kinase cascades (scheme) | 4.970E-04 | 7 | 26 |
| Immune response_IL-10 signaling pathway | 4.970E-04 | 7 | 26 |
| Development_Growth hormone signaling via PI3K/AKT and MAPK cascades | 5.108E-04 | 9 | 42 |
| Cell adhesion_Alpha-4 integrins in cell migration and adhesion | 5.360E-04 | 8 | 34 |
| Development_NOTCH1-mediated pathway for NF-KB activity modulation | 5.360E-04 | 8 | 34 |
| Chemotaxis_Inhibitory action of lipoxins on IL-8- and Leukotriene B4-induced neutrophil migration | 5.366E-04 | 10 | 51 |
| Development_Membrane-bound ESR1: interaction with growth factors signaling | 6.138E-04 | 9 | 43 |
| G-protein signaling_G-Protein alpha-i signaling cascades | 6.382E-04 | 7 | 27 |
| Development_Angiopoietin - Tie2 signaling | 6.605E-04 | 8 | 35 |
| Development_Ligand-independent activation of ESR1 and ESR2 | 7.332E-04 | 9 | 44 |
| Immune response_Regulation of T cell function by CTLA-4 | 8.073E-04 | 8 | 36 |
| Immune response_IL-12-induced IFN-gamma production | 8.073E-04 | 8 | 36 |
| Signal transduction_Calcium signaling | 8.708E-04 | 9 | 45 |
| Atherosclerosis_Role of ZNF202 in regulation of expression of genes involved in Atherosclerosis | 8.972E-04 | 6 | 21 |
| Development_Beta-adrenergic receptors transactivation of EGFR | 9.792E-04 | 8 | 37 |
| Apoptosis and survival_nAChR in apoptosis inhibition and cell cycle progression | 1.015E-03 | 7 | 29 |
| NGF activation of NF-kB | 1.015E-03 | 7 | 29 |
| Chemotaxis_Lipoxin inhibitory action on fMLP-induced neutrophil chemotaxis | 1.029E-03 | 9 | 46 |
| Immune response_MIF - the neuroendocrine-macrophage connector | 1.029E-03 | 9 | 46 |
| Apoptosis and survival_DNA-damage-induced apoptosis | 1.133E-03 | 5 | 15 |
| Development_S1P4 receptor signaling pathway | 1.175E-03 | 6 | 22 |
| Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing | 1.175E-03 | 6 | 22 |
| Cell cycle_Role of 14-3-3 proteins in cell cycle regulation | 1.175E-03 | 6 | 22 |
| Transcription_Sin3 and NuRD in transcription regulation | 1.179E-03 | 8 | 38 |
| Transport_Alpha-2 adrenergic receptor regulation of ion channels | 1.209E-03 | 9 | 47 |

**B.** GeneGo Process Networks (FDR<0.01)

| GeneGo Process Networks | p-value | Ratio | |
|---|---|---|---|
| Cell cycle_G2-M | 3.704E-08 | 46 | 205 |
| Signal Transduction_Cholecystokinin signaling | 2.000E-06 | 27 | 106 |
| Proteolysis_Ubiquitin-proteasomal proteolysis | 7.311E-06 | 35 | 166 |
| Transcription_Chromatin modification | 1.047E-05 | 29 | 128 |
| Cell cycle_G1-S | 1.259E-05 | 34 | 163 |
| Cell cycle_S phase | 3.194E-05 | 31 | 149 |
| Inflammation_IL-10 anti-inflammatory response | 6.473E-05 | 21 | 87 |
| Development_Hemopoiesis, Erythropoietin pathway | 8.098E-05 | 28 | 135 |
| Immune response_TCR signaling | 1.238E-04 | 33 | 174 |
| Cytoskeleton_Regulation of cytoskeleton rearrangement | 1.492E-04 | 34 | 183 |
| Cytoskeleton_Cytoplasmic microtubules | 2.333E-04 | 24 | 115 |
| Development_Regulation of angiogenesis | 3.947E-04 | 38 | 223 |
| Cell cycle_Mitosis | 4.751E-04 | 32 | 179 |
| Apoptosis_Apoptotic nucleus | 6.167E-04 | 29 | 159 |
| Cell adhesion_Leucocyte chemotaxis | 6.389E-04 | 35 | 205 |
| Apoptosis_Anti-Apoptosis mediated by external signals via MAPK and JAK/STAT | 1.006E-03 | 31 | 179 |
| Translation_Regulation of initiation | 1.064E-03 | 24 | 127 |

**Table 5-3.** Overlap of the top 15 significantly enriched GeneGO Pathway Maps among differentially expressed genes in lymphocytes and brain.

**A.** Top 15 GeneGo Pathway Maps among differentially expressed genes in lymphocytes

| GeneGO Pathway Map | Lymphocytes | | | | Brain | | | |
|---|---|---|---|---|---|---|---|---|
| | Rank | Ratio | | p-value | Rank | Ratio | | p-value |
| Development_A2A receptor signaling | 1 | 13 | 43 | 4.67E-07 | 1 | 11 | 43 | 1.92E-07 |
| Development_GM-CSF signaling | 2 | 14 | 50 | 4.84E-07 | 17 | 7 | 50 | 1.76E-03 |
| Development_Flt3 signaling | 3 | 13 | 44 | 6.29E-07 | 153 | 4 | 44 | 6.93E-02 |
| Development_PIP3 signaling in cardiac myocytes | 4 | 13 | 47 | 1.45E-06 | 6 | 8 | 47 | 2.13E-04 |
| Translation_Insulin regulation of translation | 5 | 12 | 42 | 2.54E-06 | 73 | 5 | 42 | 1.57E-02 |
| Apoptosis and survival_BAD phosphorylation | 6 | 12 | 42 | 2.54E-06 | 25 | 6 | 42 | 3.37E-03 |
| Immune response_Function of MEF2 in T lymphocytes | 7 | 13 | 50 | 3.13E-06 | 183 | 4 | 50 | 1.00E-01 |
| Development_Role of IL-8 in angiogenesis | 8 | 14 | 58 | 3.42E-06 | 9 | 8 | 58 | 9.27E-04 |
| Transcription_CREB pathway | 9 | 12 | 44 | 4.36E-06 | 4 | 8 | 44 | 1.32E-04 |
| Translation _Regulation of EIF4F activity | 10 | 13 | 53 | 6.33E-06 | 56 | 6 | 53 | 1.07E-02 |
| G-protein signaling_Regulation of p38 and JNK signaling mediated by G-proteins | 11 | 11 | 39 | 7.68E-06 | 237 | 3 | 39 | 1.59E-01 |
| Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis | 12 | 13 | 54 | 7.91E-06 | 120 | 5 | 54 | 4.15E-02 |
| Chemotaxis_Leukocyte chemotaxis | 13 | 15 | 75 | 1.82E-05 | 77 | 7 | 75 | 1.66E-02 |
| G-protein signaling_G-Protein alpha-12 signaling pathway | 14 | 10 | 37 | 3.02E-05 | 219 | 3 | 37 | 1.42E-01 |
| Chemotaxis_CCR4-induced leukocyte adhesion | 15 | 9 | 30 | 3.04E-05 | 314 | 2 | 30 | 2.88E-01 |

**B.** Top 15 GeneGO Pathway Maps among differentially expressed genes in brain

| GeneGO Pathway Map | Brain | | | | Lymphocytes | | | |
|---|---|---|---|---|---|---|---|---|
| | Rank | Ratio | | p-value | Rank | Ratio | | p-value |
| Development_A2A receptor signaling | 1 | 11 | 43 | 1.92E-07 | 1 | 13 | 43 | 4.67E-07 |
| Development_A2B receptor: action via G-protein alpha s | 2 | 10 | 50 | 7.83E-06 | 28 | 11 | 50 | 9.63E-05 |
| Transcription_P53 signaling pathway | 3 | 8 | 39 | 5.34E-05 | 431 | 3 | 39 | 3.90E-01 |
| Transcription_CREB pathway | 4 | 8 | 44 | 1.32E-04 | 9 | 12 | 44 | 4.36E-06 |
| Development_Thrombopoietin-regulated cell processes | 5 | 8 | 45 | 1.55E-04 | 16 | 11 | 45 | 3.40E-05 |
| Development_PIP3 signaling in cardiac myocytes | 6 | 8 | 47 | 2.13E-04 | 4 | 13 | 47 | 1.45E-06 |
| Cell adhesion_ECM remodeling | 7 | 8 | 52 | 4.37E-04 | 504 | 2 | 52 | 8.08E-01 |
| Cell cycle_Role of 14-3-3 proteins in cell cycle regulation | 8 | 5 | 22 | 8.65E-04 | 74 | 6 | 22 | 1.18E-03 |
| Development_Role of IL-8 in angiogenesis | 9 | 8 | 58 | 9.27E-04 | 8 | 14 | 58 | 3.42E-06 |
| Cytoskeleton remodeling_Cytoskeleton remodeling | 10 | 11 | 102 | 9.27E-04 | 20 | 17 | 102 | 6.13E-05 |
| Reproduction_GnRH signaling | 11 | 9 | 72 | 9.33E-04 | 109 | 11 | 72 | 2.49E-03 |
| Immune response_MIF - the neuroendocrine-macrophage connector | 12 | 7 | 46 | 1.07E-03 | 70 | 9 | 46 | 1.03E-03 |
| Signal transduction_PTEN pathway | 13 | 7 | 46 | 1.07E-03 | 42 | 10 | 46 | 2.22E-04 |
| Development_HGF signaling pathway | 14 | 7 | 47 | 1.22E-03 | 218 | 7 | 47 | 1.68E-02 |
| Development_Melanocyte development and pigmentation | 15 | 7 | 49 | 1.56E-03 | 358 | 5 | 49 | 1.48E-01 |

**Table 5-4.** Genes up-regulated in both lymphocytes and brain.

| Gene Symbol | Chr. | Lymphocytes | | Brain | | Reference |
|---|---|---|---|---|---|---|
| | | Fold Change | p-value | Fold Changes | p-value | |
| C17orf65 | 17q21.31 | 1.11 | 0.0183 | 1.29 | 0.0229 | |
| CYP2S1 | 19q13.1 | 1.12 | 0.0267 | 1.28 | 0.0352 | |
| CYP4F12 | 19p13.1 | 1.23 | 0.0059 | 1.61 | 0.0493 | |
| DCLRE1C | 10p13 | 1.12 | 0.0497 | 1.33 | 0.0482 | Pinto et al. (2010) |
| IL5RA | 3p26-p24 | 1.19 | 0.0395 | 1.62 | 0.0264 | Pinto et al. (2010) |
| KIAA0664 | 17p13.3 | 1.18 | 0.0180 | 1.35 | 0.0493 | |
| LAT2 | 7q11.23 | 1.18 | 0.0050 | 1.34 | 0.0063 | Jacquemont et al. (2006) |
| LILRB1 | 19q13.4 | 1.15 | 0.0126 | 1.86 | 0.0047 | |
| MYBPH | 1q32.1 | 1.22 | 0.0233 | 1.46 | 0.0297 | |
| NFKB2 | 10q24 | 1.13 | 0.0403 | 1.56<br>1.43 | 0.0032<br>0.0420 | |
| P2RY8 | Yp11.3 | 1.11 | 0.0475 | 1.40 | 0.0224 | Marshall et al. (2008) |
| PIM1 | 6p21.2 | 1.13 | 0.0201 | 1.40 | 0.0109 | |
| RAPGEF1 | 9q34.3 | 1.13 | 0.0120 | 1.68 | 0.0097 | |
| RUNX1 | 21q22.3 | 1.15 | 0.0267 | 1.83 | 0.0004 | |
| SEPN1 | 1p36.13 | 1.19 | 0.0437 | 1.37 | 0.0229 | |
| SIGLEC7 | 19q13.3 | 1.11 | 0.0136 | 1.60 | 0.0080 | |
| SLC44A4 | 6p21.3 | 1.14 | 0.0255 | 1.46 | 0.0379 | |
| SSH1 | 6p24 | 1.13 | 0.0302 | 1.31 | 0.0252 | |
| TFAP2A | 6p24 | 1.18 | 0.0258 | 2.13 | 0.0131 | |
| UNC13D | 17q25.1 | 1.21 | 0.0066 | 1.32 | 0.0377 | |

**Table 5-5.** Genes down-regulated in both lymphocytes and brain.

| Gene Symbol | Chr. | Lymphocytes | | Brain | | Reference |
|---|---|---|---|---|---|---|
| | | Fold Change | p-value | Fold Change | p-value | |
| ABCE1 | 4q31 | -1.14<br>-1.14 | 0.015<br>0.017 | -1.51 | 0.029 | |
| ACAT2 | 6q25.3 | -1.11 | 0.022 | -1.49 | 0.005 | |
| ACN9 | 7q21.3 | -1.20 | 0.023 | -1.27 | 0.018 | |
| AIFM1 | Xq25-q26 | -1.14 | 0.010 | -1.36 | 0.008 | |
| ALG13 | Xq23 | -1.23 | 0.008 | -1.42 | 0.030 | |
| APIP | 11p13 | -1.15<br>-1.27 | 0.004<br>0.009 | -1.29 | 0.036 | |
| ARL4A | 7p21.3 | -1.11 | 0.025 | -1.56 | 0.025 | AGPC et al. (2007) |
| ATG4C | 1p31.3 | -1.16 | 0.013 | -1.31 | 0.016 | |
| C18orf10 | 18q12.2 | -1.20 | 0.003 | -1.35 | 0.014 | Marshall et al. (2008) |
| C1orf124 | 1q42.12-q43 | -1.18 | 0.007 | -1.30 | 0.017 | |
| CSNK1A1 | 5q32 | -1.18 | 0.028 | -1.30 | 0.014 | Sarachana et al. (2010) |
| CYSLTR1 | Xq13.2-q21.1 | -1.18 | 0.014 | -1.42 | 0.016 | |
| DCTN6 | 8p12-p11 | -1.16 | 0.005 | -1.48 | 0.005 | |
| DCUN1D1 | 3q26.3 | -1.20 | 0.007 | -1.51 | 0.024 | Villa et al. (2009) |
| EEF1B2 | 2q33-q34 | -1.18<br>-1.14 | 0.032<br>0.038 | -1.30 | 0.024 | |
| FAM18B | 17p11.2 | -1.27<br>-1.32 | 0.019<br>0.021 | -1.35 | 0.031 | |
| FBXO3 | 11p13 | -1.16 | 0.022 | -1.39 | 0.003 | |
| GCC2 | 2q12.3 | -1.14 | 0.045 | -1.31<br>-1.26 | 0.038<br>0.039 | |
| GNPDA2 | 4p12 | -1.20 | 0.007 | -1.50 | 0.041 | Gregg et al. (2008) |
| GTPBP8 | 3q13.2 | -1.11<br>-1.12 | 0.014<br>0.045 | -1.28 | 0.015 | |
| GTSF1 | 12q13.13 | -1.16 | 0.009 | -1.40 | 0.026 | |
| HAT1 | 2q31.2-q33.1 | -1.14<br>-1.20 | 0.019<br>0.022 | -1.39 | 0.031 | |
| HDAC9 | 7p21.1 | -1.10 | 0.036 | -1.26 | 0.040 | Berkel et al. (2010) |
| HIF1A | 14q21-q24 | -1.23 | 0.039 | -1.33 | 0.027 | |
| HINT3 | 6q22.32 | -1.12 | 0.010 | -1.51 | 0.009 | AGPC et al. (2007) |
| ISCA1P1 | 5q12.1 | -1.12 | 0.036 | -1.56 | 0.000 | |
| KLHL5 | 4p14 | -1.10 | 0.039 | -1.34<br>-1.37 | 0.021<br>0.026 | Gregg et al. (2008) |
| MIER1 | 1p31.3 | -1.14 | 0.018 | -1.41 | 0.022 | |
| MTRR | 5p15.3-p15.2 | -1.15 | 0.019 | -1.26 | 0.013 | Mohammad et al. (2009)<br>AGPC et al. (2007) |
| NDUFS4 | 5q11.1 | -1.15 | 0.013 | -1.32 | 0.021 | Pinto et al. (2010) |
| NET1 | 10p15 | -1.10 | 0.042 | -1.38 | 0.013 | |
| OSGEPL1 | 2q32.2 | -1.12 | 0.019 | -1.27 | 0.032 | |
| PDCD10 | 3q26.1 | -1.20<br>-1.25<br>-1.23 | 0.002<br>0.003<br>0.006 | -1.41 | 0.014 | |
| PIK3CA | 3q26.3 | -1.11 | 0.042 | -1.39 | 0.043 | |
| POT1 | 7q31.33 | -1.16 | 0.019 | -1.36 | 0.038 | Marshall et al. (2008) |
| PRKACB | 1p36.1 | -1.33 | 0.010 | -1.47 | 0.005 | |
| PRPF4B | 6p25.2 | -1.22 | 0.046 | -1.55 | 0.009 | Gregg et al. (2008) |

**Table 5-5.** Continued.

| Gene Symbol | Chr. | Lymphocytes | | Brain | | Reference |
|---|---|---|---|---|---|---|
| | | Fold Change | p-value | Fold Change | p-value | |
| PSMD10 | Xq22.3 | -1.22<br>-1.16<br>-1.28 | 0.004<br>0.015<br>0.024 | -1.33 | 0.005 | Piton et al. (2011) |
| RAD51AP1 | 12p13.2-p13.1 | -1.18 | 0.000 | -1.32 | 0.037 | |
| RANBP6 | 9p24.1 | -1.15 | 0.028 | -1.33 | 0.009 | |
| RBM41 | Xq22.3 | -1.11 | 0.007 | -1.36 | 0.012 | |
| SC4MOL | 4q32-q34 | -1.23 | 0.011 | -1.45 | 0.007 | AGPC et al. (2007) |
| SEC22C | 3p22.1 | -1.14 | 0.020 | -1.29 | 0.045 | |
| SFRS13A | 1p36.11 | -1.18 | 0.017 | -1.56 | 0.030 | |
| SLC25A40 | 7q21.12 | -1.18<br>-1.14 | 0.026<br>0.027 | -1.32 | 0.041 | |
| SLC30A5 | 5q12.1 | -1.16 | 0.016 | -1.42 | 0.033 | O'Roak et al. (2011)<br>Gregg et al. (2008) |
| SYF2 | 1p36.11 | -1.22 | 0.030 | -1.23 | 0.048 | |
| TAF9 | 5q11.2-q13.1 | -1.16 | 0.025 | -1.29 | 0.019 | |
| TCP1 | 6q25.3-q26 | -1.33 | 0.011 | -1.31 | 0.046 | Philippe et al. (2009) |
| TMPO | 12q22 | -1.23 | 0.004 | -1.30<br>-1.47 | 0.023<br>0.016 | Gregg et al. (2008) |
| TPM4 | 19p13.1 | -1.10 | 0.023 | -1.21 | 0.041 | Baron et al. (2006) |
| TRMT11 | 6q11.1-q22.33 | -1.20 | 0.007 | -1.40 | 0.026 | AGPC et al. (2007) |
| TWF1 | 12q12 | -1.14 | 0.006 | -1.48 | 0.037 | Wang et al. (2010) |
| USP16 | 21q22.11 | -1.16 | 0.008 | -1.63 | 0.012 | |
| VEZT | 12q22 | -1.11 | 0.017 | -1.29 | 0.030 | |
| YWHAZ | 8q23.1 | -1.30 | 0.010 | -1.45 | 0.003 | Anderson et al. (2009) |

**Table 5-6.** GeneGO Pathway Maps and Process Networks enriched among genes up-regulated in both lymphocytes and brain.

**A.** GeneGO Pathway Maps (FDR < 0.05)

| GeneGO Pathway Map | p-value | Ratio | |
|---|---|---|---|
| Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway | 4.606E-05 | 3 | 41 |
| Immune response_IL-27 signaling pathway | 7.906E-04 | 2 | 24 |
| Apoptosis and survival_APRIL and BAFF signaling | 1.985E-03 | 2 | 38 |
| Transcription_NF-kB signaling pathway | 2.090E-03 | 2 | 39 |
| Apoptosis and survival_Lymphotoxin-beta receptor signaling | 2.308E-03 | 2 | 41 |

**B.** GeneGO Process Networks (FDR < 0.05)

| GeneGO Process Network | pValue | Ratio | |
|---|---|---|---|
| Inflammation_IL-10 anti-inflammatory response | 2.595E-05 | 4 | 87 |
| Immune response_BCR pathway | 1.538E-04 | 4 | 137 |
| Immune response_Phagocytosis | 9.858E-04 | 4 | 223 |
| Inflammation_IL-2 signaling | 1.224E-03 | 3 | 104 |
| Inflammation_Protein C signaling | 1.365E-03 | 3 | 108 |
| Inflammation_Inflammasome | 1.516E-03 | 3 | 112 |
| Inflammation_Amphoterin signaling | 1.762E-03 | 3 | 118 |
| Inflammation_IL-6 signaling | 1.805E-03 | 3 | 119 |
| Cell cycle_G1-S Interleukin regulation | 2.225E-03 | 3 | 128 |
| Development_Hemopoiesis, Erythropoietin pathway | 2.590E-03 | 3 | 135 |
| Inflammation_IgE signaling | 2.758E-03 | 3 | 138 |
| Inflammation_MIF signaling | 2.873E-03 | 3 | 140 |
| Immune response_IL-5 signalling | 3.748E-03 | 2 | 44 |
| Immune response_TCR signaling | 5.304E-03 | 3 | 174 |
| Inflammation_Innate inflammatory response | 6.013E-03 | 3 | 182 |
| Cell cycle_G1-S Growth factor regulation | 7.282E-03 | 3 | 195 |
| Immune response_Antigen presentation | 7.491E-03 | 3 | 197 |
| Reproduction_Feeding and Neurohormone signaling | 8.933E-03 | 3 | 210 |
| Inflammation_Histamine signaling | 9.528E-03 | 3 | 215 |
| Inflammation_Neutrophil activation | 1.053E-02 | 3 | 223 |
| Immune response_Phagosome in antigen presentation | 1.420E-02 | 3 | 249 |

**Table 5-7.** GeneGO Pathway Maps and Process Networks enriched among genes down-regulated in both lymphocytes and brain.

**A.** GeneGO Pathway Maps (FDR < 0.065) *1st three pathways significant with a FDR < 0.025

| GeneGO Pathway Map | p-value | Ratio | |
|---|---|---|---|
| Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional silencing | 1.965E-05 | 3 | 22 |
| Apoptosis and survival_BAD phosphorylation | 1.419E-04 | 3 | 42 |
| Development_Role of HDAC and calcium/calmodulin-dependent kinase (CaMK) in control of skeletal myogenesis | 3.007E-04 | 3 | 54 |
| Apoptosis and survival_Beta-2 adrenergic receptor anti-apoptotic action | 1.437E-03 | 2 | 23 |
| Cytoskeleton remodeling_Role of PDGFs in cell migration | 1.565E-03 | 2 | 24 |
| Cell adhesion_Alpha-4 integrins in cell migration and adhesion | 3.133E-03 | 2 | 34 |
| Inhibitory action of Lipoxin A4 on PDGF, EGF and LTD4 signaling | 3.133E-03 | 2 | 34 |
| Development_Regulation of telomere length and cellular immortalization | 3.318E-03 | 2 | 35 |
| Development_Lipoxin inhibitory action on PDGF, EGF and LTD4 signaling | 3.318E-03 | 2 | 35 |
| G-protein signaling_G-Protein alpha-12 signaling pathway | 3.703E-03 | 2 | 37 |
| Transcription_Receptor-mediated HIF regulation | 4.108E-03 | 2 | 39 |
| Translation _Regulation of EIF2 activity | 4.108E-03 | 2 | 39 |
| Development_A2A receptor signaling | 4.976E-03 | 2 | 43 |
| Transcription_CREB pathway | 5.206E-03 | 2 | 44 |
| Development_Ligand-independent activation of ESR1 and ESR2 | 5.206E-03 | 2 | 44 |
| Immune response_Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling | 5.440E-03 | 2 | 45 |
| Development_Hedgehog signaling | 5.678E-03 | 2 | 46 |
| Regulation of lipid metabolism_Insulin signaling:generic cascades | 5.922E-03 | 2 | 47 |
| Development_Leptin signaling via PI3K-dependent pathway | 5.922E-03 | 2 | 47 |
| Development_PIP3 signaling in cardiac myocytes | 5.922E-03 | 2 | 47 |
| Regulation of metabolism_Triiodothyronine and Thyroxine signaling | 6.170E-03 | 2 | 48 |
| Development_Melanocyte development and pigmentation | 6.423E-03 | 2 | 49 |
| Development_GM-CSF signaling | 6.681E-03 | 2 | 50 |
| Immune response_Function of MEF2 in T lymphocytes | 6.681E-03 | 2 | 50 |
| Development_A2B receptor: action via G-protein alpha s | 6.681E-03 | 2 | 50 |
| Development_IGF-1 receptor signaling | 6.943E-03 | 2 | 51 |
| ENaC regulation in airways (normal and CF) | 7.210E-03 | 2 | 52 |
| PGE2 pathways in cancer | 8.039E-03 | 2 | 55 |
| Regulation of lipid metabolism_Insulin regulation of glycogen metabolism | 8.324E-03 | 2 | 56 |

**B.** GeneGO Process Networks (FDR < 0.20) *No significant networks with a FDR < 0.05

| GeneGO Process Network | p-value | Ratio | |
|---|---|---|---|
| Inflammation_IL-6 signaling | 2.363E-03 | 4 | 119 |
| Development_Skeletal muscle development | 4.698E-03 | 4 | 144 |
| Inflammation_TREM1 signaling | 4.815E-03 | 4 | 145 |

**Table 5-7.** Continued.

| GeneGO Process Network | p-value | Ratio | |
|---|---|---|---|
| Apoptosis_Apoptotic mitochondria | 5.771E-03 | 3 | 77 |
| Cytoskeleton_Intermediate filaments | 6.644E-03 | 3 | 81 |
| Cell adhesion_Platelet aggregation | 9.119E-03 | 4 | 174 |
| Signal transduction_Leptin signaling | 1.385E-02 | 3 | 106 |
| Cell cycle_Meiosis | 1.385E-02 | 3 | 106 |
| Cell cycle_G2-M | 1.591E-02 | 4 | 205 |
| Reproduction_Feeding and Neurohormone signaling | 1.724E-02 | 4 | 210 |
| Muscle contraction_Nitric oxide signaling in the cardiovascular system | 1.762E-02 | 3 | 116 |
| Transcription_Chromatin modification | 2.283E-02 | 3 | 128 |
| Development_Melanocyte development and pigmentation | 2.364E-02 | 2 | 50 |

**Table 5-8.** GeneGO Pathway Maps enriched among genes included in the lymphocyte-based class prediction model (n=67) and brain-based class prediction model (n=116).

| GeneGO Pathway Map | Lymphocytes | | | Brain | | |
|---|---|---|---|---|---|---|
| | p-value | Ratio | | p-value | Ratio | |
| Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway | 6.05E-02 | 1 | 41 | 5.85E-05 | 4 | 41 |
| Immune response_CXCR4 signaling via second messenger | 5.04E-02 | 1 | 34 | 7.24E-04 | 3 | 34 |
| Immune response_Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling | 2.03E-03 | 2 | 45 | 1.65E-03 | 3 | 45 |
| Development_PEDF signaling | 2.40E-03 | 2 | 49 | 2.28E-01 | 1 | 49 |
| Apoptosis and survival_HTR1A signaling | 2.50E-03 | 2 | 50 | 2.32E-01 | 1 | 50 |
| Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/IAP pathway | 4.03E-02 | 2 | 27 | 8.72E-03 | 1 | 27 |
| Immune response_IL-4 - antiapoptotic action | 4.46E-02 | 2 | 30 | 1.07E-02 | 1 | 30 |
| Transcription_NF-kB signaling pathway | 5.77E-02 | 2 | 39 | 1.77E-02 | 1 | 39 |
| Immune response_TCR and CD28 co-stimulation in activation of NF-kB | 5.91E-02 | 2 | 40 | 1.86E-02 | 1 | 40 |
| Development_A2A receptor signaling | 6.34E-02 | 2 | 43 | 2.13E-02 | 1 | 43 |

**FIGURES**

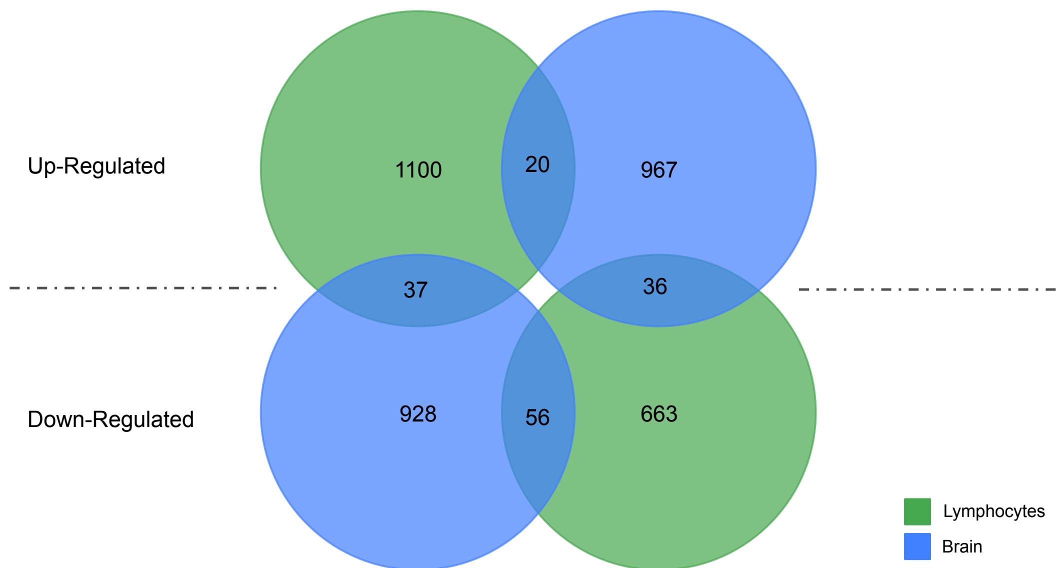


**Figure 5-1.** Overlap between differentially expressed genes in lymphocytes and brain. Green = lymphocytes (n=2321); Purple = brain (n=2017).

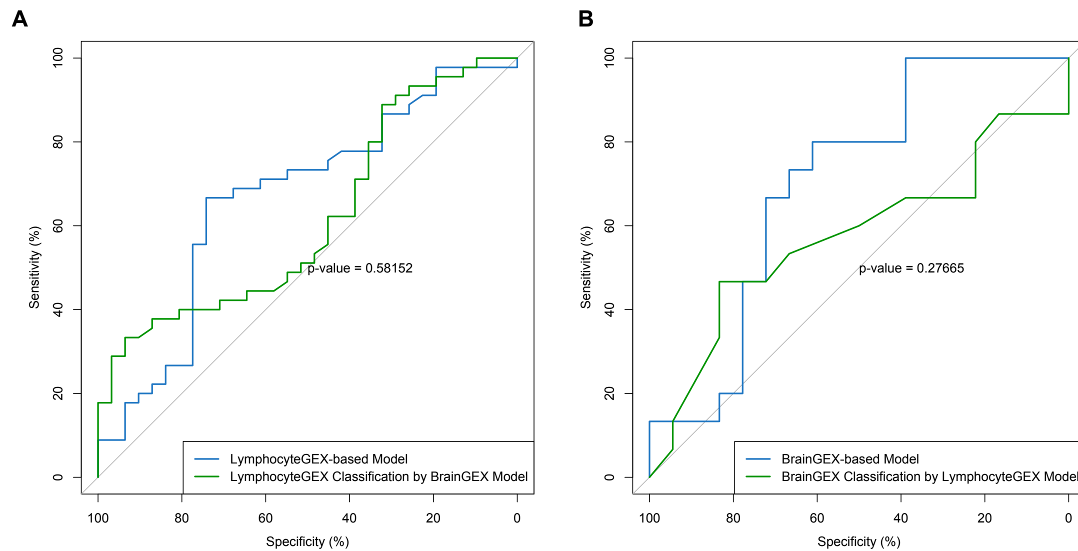


**Figure 5-2.** Receiver Operating Characteristic (ROC) curves for classification of autism cases and controls using A.) lymphocyte and B.) brain gene expression. A.) Comparison of a classification model built and tested via cross validation with lymphocyte gene expression (blue) (AUC: 0.668, 95% CI: 0.540-0.796) and a classification model built with brain gene expression and tested with lymphocyte gene expression (green) (AUC: 0.618, 95% CI: 0.746-0.489 B.) Comparison of a classification model built and tested via cross validation with brain gene expression (blue) (AUC: 0.704, 95% CI: 0.520-0.888) and a classification model built with lymphocyte gene expression and tested with brain gene expression (green) (AUC: 0.574, 95% CI: 0.785-0.363).

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# CHAPTER 6

## Conclusions and Future Directions

The aim of the studies described in this dissertation was to explore the technical aspects and biological relevance of microarray gene expression profiling of peripheral whole blood in regards to neural-based diseases. The studies were motivated by the technical difficulties associated with assaying peripheral whole blood via microarray, the difficulties associated with directly comparing blood and brain gene expression profiles, and the general lack of understanding in regards to the ability of blood-based gene expression profiling to capture biologically relevant neural-based disease profiles. The study of blood gene expression profiles is likely to be important to the development of expression-based biomarkers for neuropsychiatric, neurodegenerative, and neurodevelopmental diseases given the difficulties associated with collecting the large number of human brains necessary for clinical validation of such biomarkers. The chapters in this dissertation tackle various aspects associated with the processing and evaluation of microarray gene expression profiles of peripheral whole blood in mouse and humans. Here I provide an overview of the main findings as well as discuss limitations and future directions.

**MAIN FINDINGS**

The evaluation of a globin reduction method using microarray-based blood gene expression profiling in mouse highlighted the need for and the importance of removing or reducing globin transcripts in peripheral whole blood prior to microarray analysis (Chapter 2). Comparing blood gene expression profiles from before and after globin reduction, the removal of globin transcripts was found to improve detection sensitivity of low abundance transcripts thus significantly improving the ability to evaluate biological pathways and disease networks via whole blood. The need to

reduce globin transcripts in the analysis of anxiety-related phenotypes in particular was emphasized by the significant increase in the number of Schizophrenia network objects identified following globin reduction.

Given the positive effect of globin reduction on microarray-based gene expression profiling of mouse whole blood, globin reduced blood gene expression profiles were utilized in conjunction with neural tissue gene expression profiles in the evaluation of anxiety-related behavioral phenotypes in mice (Chapter 3). Several recent studies have compared blood and brain gene expression profiles with conflicting results as to the potential of blood as a surrogate tissue for blood (Cai et al. 2010, Davies et al. 2009, Jasinska et al. 2009). However, these studies only assess natural variation and heritability. Although naturally occurring variation and patterns may fail to be well correlated between blood and brain such as seen by Cai et al., it remains unclear whether genes and pathways associated with disease in blood will reflect those genes and pathways associated with disease in brain. Here we went beyond strain- and tissue-specific variation to evaluate genes and pathways associated with anxiety-related phenotypes in mice. We concluded blood gene expression profiles were able to capture only a small portion of the total trait-associated genes and enriched pathways identified in brain. Despite this finding, the genes and pathways associated with behavioral phenotypes in blood were highly enriched for biologically relevant genes and pathways suggesting blood is a viable surrogate tissue.

Next, blood gene expression profiles were assessed in the context of human samples. Human, peripheral whole blood is known to benefit from the reduction of globin transcripts similar to the effected reported here in the context of mice (Tian et

al. 2009, Field et al. 2007). The evaluation of a microarray target preparation method (WG-DASL) with the potential to eliminate the need for globin reduction confirmed the benefits of reducing globin transcripts (Chapter 4) suggesting the elimination of globin-specific probes from sample amplification is not sufficient to improve detection sensitivity.

The correlation between human lymphocyte and brain gene expression profiles associated with autism was assessed in a similar manner as behavioral traits in mice (Chapter 5). Approximately 4% of the genes dysregulated in the brain were dysregulated in lymphocytes and many of the same pathways dysregulated in brain were also significantly enriched in lymphocytes. We also found many of these genes (33%) were identified in previous genetic studies of autism. These results confirm the ability to identify neurobiologically relevant genes using blood-derived cells. Furthermore, lymphocyte gene expression profiles were able to classify disease state at a level similar to brain gene expression.

## CONCLUSIONS AND FUTURE DIRECTIONS

In this work, I assessed the potential of blood as a surrogate tissue for the analysis of neural-based diseases. The correlation between blood and brain was ultimately assessed comparing significantly associated behavioral trait/disease genes and significantly enriched pathways and disease networks identified in blood and brain. We conclude whole blood or blood-derived cells reflect biologically relevant disease profiles despite capturing only a small fraction of the whole picture. However, these studies are not without their limitations, particularly in regards to sample size and microarray processing.

Microarray studies are especially prone to batch effects (i.e. time of sample collection, time of microarray processing, array manufacture date, etc.) and other technically-induced variation (i.e. RNA processing, scanning intensity, location of the probe on the array, etc.). This becomes exceptionally relevant when batch effects are correlated with the phenotype of interest. In the mouse gene expression profiles studied here, tissue type was highly correlated with the time of sample collection and microarray processing. Due to this correlation, correcting for batch (Johnson et al. 2007) would remove variation associated with both time of microarray processing as well as tissue type. The batch effects associated with tissue type may have also played a part in the difference in the number of significantly associated genes in blood as compared to brain (7%-35%) and hence the number of genes that overlap or the number of pathways capable of being significantly enriched in blood. The small biological differences associated with natural variations in inbred mouse strain behavioral phenotypes may largely have been overshadowed by differences associated with technical processing (Bryant et al. 2011).

Genome-wide transcriptional profiling of whole blood is also hampered by its heterogeneous nature. The effects of this heterogeneity maybe reflected in the larger overlap between blood and brain using lymphocytes as compared to globin-reduced whole blood. While brain tissues can be finely dissected to ensure a rather homogeneous cell population from the start, blood cells must first be collected and immediately fractionated or stored and subjected to further downstream processing at a later date or immortalized as cell lines. The effects of fractionation and storage on gene expression profiles have been well studied (Debey-Pascher et al. 2011, Debey et al. 2004). At the same time, various computational methods have been developed

in an attempt to identify cell populations (Bolen et al. 2011, Grigoryev et al. 2010). However, the most effective approach to dealing with different cell populations in whole blood maybe running a Complete Blood Count (CBC) test for each sample collected.

Sample size is also a problem given the variability associated with genome-wide transcriptional profiling and behavioral phenotype testing. Increasing the number of mice per tissue, behavioral phenotype, and strain should result in decreased variability due to sampling differences and technical variation thus improving the ability to detect smaller changes in gene expression and behavioral phenotypes across the different mouse strains. Our analyses may also be improved by limiting the study to mice on the extreme ends of the phenotype spectrum or by increasing the number of strains thus expanding the range of the behavioral phenotypes studied.

In the end, genome-wide transcriptome analyses will benefit from the development of more sensitive assays such as RNA sequencing (Ozsolak et al. 2011). RNA sequencing overcomes the limited dynamic range of microarray platforms and hence the contributing factor to the need for reducing globin transcripts in whole blood. As RNA sequencing continues to advance, whole blood gene expression studies will benefit from the ability to assess not only mRNA levels but also the ability to more accurately predict alternative splicing events. Alternative splicing is widespread in the brain (Lin et al. 2011, Boutz et al. 2007) and may be one of the reason blood gene expression profiles do not significantly overlap with brain gene expression profiles.

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