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Gene expression abnormalities in the autistic brain

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

Doctor of Philosophy

in

Neurosciences

by

Maggie Lok Mun Chow

Committee in charge:

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2011

The Dissertation of Maggie Lok Mun Chow is approved, and it is acceptable in quality and form for publication on microfilm:

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2011

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ABSTRACT OF THE DISSERTATION

Gene expression abnormalities in the autistic brain

By

Maggie Lok Mun Chow

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2011

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Professor Nicholas J Schork, Co-Chair

Although it is accepted that autism is a highly heritable neurodevelopmental disorderthat exhibits brain overgrowth in early years of life, underlying genetic mechanismsof this abnormal developmentremain unknown. It has been long hypothesized that autism begins prenatally due to abnormal cell proliferation, cell number regulation, and cell migration functions as the cortex is formed. These possibilities have never been systematically examined in the young developing autistic cerebral cortex, in part due to the limitations of tissue preservation quality. We aimed to test these hypotheses through genome-wide expression profiling and RTPCRon mRNA from postmortem brain tissue of young autistic and control cases. To yield the most reliable dataset with the largest sample size possible for this analysis, we first compared *in vitro* transcription (IVT)- and cDNA-mediated Annealing Selection and Ligation (DASL)-based expression platforms on

partially degraded RNA from reference pools, and frozen and formalin fixed postmortem brain tissue. Results suggested that the DASL-based platform produced more reliable expression data than the IVT-based platform with partially degraded RNA. Next, data preprocessing procedures for the DASL-based gene expression results from postmortem frozen brain samples were systematically tested. We developed a data preprocessing pipeline to prepare this dataset for downstream analyses between autistic and control samples. Differential expression and enrichment analyses revealed that genes regulating cell cycle, apoptosis, proliferation, cellular differentiation, and neural patterning functions were dysregulated in young autistic cases. Similar results were found in an expanded analysis of autism at young and adult ages. Our observations suggest that molecular aberrancies in prenatal and early postnatal neurodevelopment may be responsible for brain overgrowth, cortical asymmetry, cortical disconnectivity, aberrant neural function, and ultimately the behavioral phenotype of autism.

Chapter 1 – Introduction

Autism Spectrum Disorders

The child psychiatrist Leo Kanner was the first to formally document the incidence of autism in his paper "Autistic Disturbances of Affective Contact" in the Journal *Nervous Child* in 1943 (Kanner, 1943). Eleven children were described as exhibiting irregular autistic behavior in this publication.

Currently, due to a striking increase in the prevalence of autism, the Centers for Disease Control have declared autism a national public health crisis (CDC ASD Common, 2007). This declaration underscores the pressing urgency of finding the etiological basis of autism, as well as performing translational studies for earlier diagnostic tools and biological treatments.

This introduction will briefly describe the clinical characteristics of autism, epidemiology, comorbid disorders, and current diagnostic and therapeutic strategies. Then, literature on the genetic bases of autism will be explored. Finally, current theories of autism pathogenesis, limitations of autism research, remaining questions, and aims and hypotheses for our studies will be presented.

Epidemiology and hereditability.

Autism research continues have increasing impact due to the increasing prevalence of autistic disorder since the 1960s in the United States and Europe. Autism spectrum disorders occur in an estimated sixty cases per ten thousand children (Sponheim and Skjeldal, 1998; Kadesjö et al., 1999; Fombonne, 2005). The more strictly-defined Autistic Disorder is estimated to have a prevalence of ten to twenty per ten thousand children (Newschaffer et al., 2007). The Center for Disease Control estimates one in one hundred fifty children to have an ASD.

Due to a heritability of greater than 90% (Bailey et al., 1996), genetics are hypothesized to play an important role in causing the aberrant brain growth that result in the autistic behavioral phenotype. The heritability of autism is greater than many other diseases and disorders known to have a genetic component, including breast cancer, colon cancer, Alzheimer's disease, and schizophrenia (Courchesne et al., 2007).

A 50- to 100- fold risk for ASD is found in children born to a family already with an ASD child, with a 5-8% recurrence rate (Szatmari et al., 1998). Furthermore, ASD is diagnosed in about four times as many boys as girls (Constantino and Todd, 2003). Female individuals with autistic features differ in the severity and clustering of behavioral and cognitive symptomology than male individuals with autism, as well as in morphological features (Bloss and Courchesne, 2007; Schumann et al, unpublished).

These findings, in addition to twin studies that will be discussed in a later section, suggest that autism has a heritable component.

Clinical Characteristics.

Autistic disorder lies on the Autism Spectrum Disorders (ASD) with pervasive developmental disorder-not otherwise specified (PDD-NOS) and Asperger's syndrome, presenting with varying severity of behavioral and cognitive effects. Three domains of deficits are observed in individuals with autism. They include social interaction, communication, and restricted interests, stereotyped, and repetitive behaviors (American Psychiatric Association, 1994). Autism is usually diagnosed by 3 years, but recent studies have shown that earlier detection may be possible (Pierce et al., 2009). At 1 or 2 years of age, autistic children already exhibit aberrant behaviors in language development, social attention, and emotional reactivity (Baron-Cohen et al., 1992; Wetherby et al., 2004; Zwaigenbaum et al., 2005; Landa and Garrett-Mayer, 2006).

Thethree core domains abnormal in autism have also been subdivided into behavioral features (Levy et al., 2009). The deficits in socialization include: 1.Little or no social reciprocity and absence of social judgment; 2.Absence of looking to share enjoyment and interests with others; 3. Delayed and infrequency of peer interactions; 4. impaired usage of nonverbal behavior to regulate social interactions; and 5. delayed initiation of interactions.

In the communication domain, autistic individuals exhibit: 1. Delayed imaginative and socially imitative play; 2. Stereotyped, repetitive, or idiosyncratic language; 3.Impairment in expressive language and conversation, and disturbance in pragmatic language use; 4. Delay in verbal language without nonverbal compensation.

Lastly, in the domain of restricted, stereotyped, and repetitive patterns of behavior, autistic individuals show: 1. Stereotyped, repetitive motor mannerisms and self-stimulating behaviors; 2. Preoccupation or fascination with parts of objects and unusual visual exploration; 3. Preoccupation with stereotyped or restricted interests or topics; and 4. Adherence to perseverative, rigid, and routine behaviors.

Diagnostic methods.

Autism is currently typically diagnosed at 3 years of age or later. However, earlier identification of autism through behavioral and, in the future, biological markers will be necessary for early referral to intervention services and family support. Improved outcome is hypothesized to result from early detection (Howlin et al., 2009).

Currently, three major categories of methods are employed to diagnose Autism Spectrum Disorders: checklist or inventory, structured interview, or observational measures (Levy et al., 2009). The differences between the methods include age at which the measures are valid and administration time of the measures.

Diagnostic inventories such as the DSM-IV-TR, the autism diagnostic observation schedule (ADOS), and the revised autism diagnostic interview (ADI-R) are used most frequently for research purposes. Other checklist or inventory screening methods include the Autism Behavior Checklist, and the Pervasive Developmental Disorder Rating Scale. For dimensional assessment, diagnosticians utilize the Aberrant Behavior Checklist and the Social Communications Disorders Checklist to diagnose autism. Structured interviews include the Parent Interview for Autism; the ADI-R; the Developmental, Dimensional, and Diagnostic interview; and the Diagnosis of Social and Communication Disorder Schedule. Lastly, observational measures include the Childhood Autism Rating Scale (CARS), the ADOS, the Checklist for Autism in Toddlers, and the Autism Observation Schedule for Infants (Levy et al., 2009).

Comorbid disorders and abnormalities.

Comorbid disorders of autism are hypothesized to have a greater effect on function and outcome than do core symptoms, and are common in individuals with ASD(Levy et al., 2009).

About 25% of individuals with autistic disorder will experience seizures over their lifespan. Approximately 10% of individuals with Asperger's or PDD-NOS will experience seizures. Greater than 60% individuals with autism will experience intellectual disability, whereas individuals with Asperger's will experience mild to no cognitive impairment, and individuals with PDD-NOS will have mild to severe

intellectual disability (Minshew and Williams, 2007; Levy et al., 2009). Mental retardation and seizures are thought of as occurring frequently with autism.

Many other comorbid developmental, psychiatric, behavioral, sensory, neurological, and gastrointestinal abnormalities are also observed in individuals with autism. Language deficits, attention problems, impulsivity or hyperactivity, motor delay, and hypotonia are all observed in greater than 10% of autistic individuals. Anxiety is the psychiatric disorder with highest prevalence (42-84%) amongst autistic individuals, followed by depression (2-30%). Other psychiatric disorders often found to be comorbid with autism include obsessive-compulsive disorder, interfering repetitive behavior, oppositional defiant disorder, and behavioral problems. These behavioral problems consist of disruptive, irritable or aggressive behavior, and self-injurious behaviors. Tactile and auditory sensitivities, as well as sleep disruption are also common in autistic individuals, in addition to tics of neurologic origin. Finally, many autistic individuals also experience gastrointestinal disorders such as food selectivity, gastro-esophageal reflux, and constipation.

Disorders with increased risk for autism.

Autistic symptoms have been shown to be more frequent in several neurological disorders, which have the potential to point to important common genetic and epigenetic mechanisms.

Epileptiform abnormalities are present in as many as 60% of children with ASD (reviewed by Spence and Schneider 2009). Prevalence estimates range from 5 to 46 percent. 30% of children with epilepsy also reached diagnostic criteria for ASD on the Social Communication Questionnaire. Children with seizures starting in the first year of life have an estimated 6-7% chance of developing an ASD with intellectual disability (Saemundsen et al., 2007). Children with seizures from 2 years of earlier and/or low cognitive levels were more likely to reach ASD criteria (Clarke et al., 2005).

It has been historically acknowledged that an overlap between intellectual disability and ASD incidence exists (Bartak & Rutter, 1976). One study found 50-70% of individuals with ASD to also have intellectual disability (Stomme and Diseth, 2000). Another concluded that 70% of individuals with ASD also have intellectual disability, and 40% of individuals with intellectual disability have some form of ASD. The more severe an individual's intellectual disability was, the greater the likelihood that they also had ASD (Vig and Jedrysek 1999). More recently, however, the distinction between individuals with intellectual disability, individuals with ASD, and individuals with both ASD and intellectual disability has been made, with novel diagnostic tools to help define these distinctions (Noterdaeme, 2009).

Tuberous sclerosis complex is another such disorder showing clinical overlaps with autism. In one study by Bolton and Griffiths (2001), 8 of 9 patients showing autistic symptomology also exhibited tubers in the temporal lobes. Other studies have estimated rates of autism in patients with tuberous sclerosis at 17-68% (Smalley and Petri 2005),

and the number of autistic patients with tuberous sclerosis range from 0.4-4% (Filipek 2005). It has been shown that susceptibility to developing an ASD increases when tubers present in this disorder are densely localized to frontotemporal regions of the brain (Gillberg 2005).

Since there is a putative role of the environment in the pathogenesis of autism, many have hypothesized that epigenetic regulation of the genome may be a mechanism underlying aberrant genetic function and brain development. The following section will examine the evidence of epigenetic mechanisms in autism through exploring evidence of syndromes of epigenetic origin associated with an increased risk of ASD symptomology (Grafodatskaya et al., 2010).

Rett's Syndrome, in which 80% of patients have a mutation in the MECP2 gene, is one such disorder that is often misdiagnosed as autism (Abdul-Rahman and Hudgins 2006). The MECP2 gene encodes a methyl CpG-binding protein that regulates transcription, facilitation of histone modifications, inhibition of transcription factor binding, alternative RNA splicing, and chromatin looping. It is expressed in mature neurons (Shahbazian et al., 2002) and is important for maturation of hippocampal neurons (Smrt et al., 2007). 11 of 14 cases with autism had lower levels of MECP2 than controls in frontal cortex, which was associated with a MECP2 promoter variant in a female case and increased MECP2 promoter DNA methylation in male cases (Nagarajan et al., 2006). Furthermore, a microsatellite marker in intron 2 and a SNP in the 3' UTR of MECP2 was identified as being associated with autism (Loat et al., 2008).

Fragile X is a disorder in which the risk of showing autistic behaviors is 18-30% for strict diagnosis and 60-67% for broad diagnosis for full mutation males, and 10% for strict diagnosis and 23% for broad diagnosis for full mutation females. From the opposite perspective, 2-8% of individuals with autism have the fragile X mutation (Harris et al., 2008). FMR1, the gene that is mutated in fragile X, is an RNA-binding protein involved in RNA transport, translation, and stability (Pfeiffer and Huber 2009; Tan et al., 2009).

15-50% of individuals with CHARGE (Coloboma of the eye, Heart defects, Atresia of the nasal choanae, Retardation of growth and/or development, Genital and/or urinary abnormalities, and Ear abnormalities/deafness) also show symptoms of ASD (Hartshorne et al., 2005; Johansson et al., 2006; Smith et al., 2005). CHARGE is attributed to abnormalities in chromodomain helicase DNA-binding protein 7, which is involved in chromatin remodeling (Kramer and Bokhoven 2007).

Interestingly, individuals that suffer from aberrant imprinting mechanisms such as Angelman's syndrome and Prader-Willi syndrome also have increased rates of ASD symptoms. 42-100% of individuals with Angelman's syndrome show autistic features (Bonati et al., 2007; Trillingsgaard 2004; Peters et al., 2004; Steffenburg et al., 1996). About 19-36.5% of individuals with Prader-Willi Syndrome exhibit ASD characteristics (Descheemaeker et al., 2006; Veltman et al., 2004; Milner et al., 2005). This syndrome results from abnormalities in genomic imprinting, particularly from a functional loss of

paternally expressed genes (Horsthemke and Wagstaff 2008; Nicholls and Knepper 2001).

Other rare disorders associated with increased risk for ASD include deletions of 22q11, which causes Velo-cardio-facial Syndrome, duplications of 7q11.23, which causes Williams-Beuren-Syndrome, and Klinefelter Syndrome (XXY; Freitag et al., 2009). Similarities between these disorders and ASD must be assessed to identify common underlying themes, especially to connect the genetic level to the functional behavioral level.

Current Treatments and Outcomes.

Many factors determine the clinical outcome of a child with autism. Poor outcomes are correlated with early communication deficits, intelligence quotient of less than 70, and continued ritualistic and stereotyped behavior (Howlin et al., 2004). Better outcomes are associated with intensive treatment and early detection (Volkmar and Pauls, 2003), though long-term data is not yet available.

Two main treatment strategies are utilized for autistic children: psychosocial treatments and pharmacological/medical approaches. Several methods are applied in the psychosocial realm. These therapies often utilize positive reinforcement to increase imitation and attention in children with autism. Speech and language therapy is

considered to be important in the treatment of autism due to the centrality of communication impairment in the disorder (Paul, 2008). Many developmental models have shown auspicious results in treatment by assuming an association betweenthe development of communication and social relationships. Such models include: the Denver model (Rogers and DiLalla, 1990), the Social Communication, Emotional Regulation and Transactional Support Model (SCERTS; Prizant and Wetherby, 1998; Prizant et al., 2003), and Developmental Individual Difference, and Relationship-based Floortime Models (Wieder and Greenspan, 2003).

Applied behavior analysis (ABA; Ospina et al., 2008) represents a variation of the aforementioned early intervention strategies for autistic children designed to modify dysfunctional behaviors. One example of ABA is discrete trial learning, which has been very effective in half of participants in ten randomized clinical trials(Rogers and Vismara, 2008). ABA-based therapies alone tend to be highly structured,however, and often the autistic child is not able to generalize the skills learned in therapy sessions. A treatment component that increases the effectiveness of these structured therapies is parent-mediated: Sherer and Schreibman(2005) showed that when parents were trained in highly structured behavioral methods, generalization and maintenance of behavioral modifications of their child with autism were improved.

Naturalistic psychosocial therapies have also been developed, encouraging generalization of psychosocial therapy to everyday life. Such programs as pivotal response training (Koegel et al., 2001) and incidental teaching (McGee et al., 1985) have

also had positive outcomes in about half of treated children (Sherer and Schreibman, 2005). The goals of these therapies have shifted from teaching one behavior at a time to increasing generalized responsiveness and motivation (Koegel et al., 1987).

These widely used sources of treatment are usually employed by the autistic child's family members or educational system (O'Roak and State, 2008). The psychosocial treatments aim to improve functionality of the individual through teaching skills lacking in the core deficits, and also to decrease negative effects of comorbid disorders. The components of comprehensive therapy include developmental therapies, behaviorally based treatments, specialized educational curricula, and parent training in the home, community, or school (Rogers and Vismara, 2008; National Research Counsel, 2001; Parr, 2008). Family support is a vital part of any comprehensive therapy because parental stress may impair the effectiveness of treatments (Osborne et al., 2008).

Additionally, though no pharmacotherapeutic agent currently exists to ameliorate the core deficits of ASDs, drugs may be able to treat comorbid disorders to complement the psychosocial developmental, behavioral and educational treatments (Levy et al., 2009). As described above, the most debilitating comorbid conditions in children with autism include hyperactivity, attentional difficulties, interfering repetitive activity, affective difficulties, self-injurious behavior, irritability, aggression, and sleep disruption. For example, a double-blind, placebo-control crossover study of children with ASD and comorbid attention deficit hyperactivity disorder found a positive effect of methylphenidate on joint attention (Jahromi et al., 2009). Until drugs able to specifically

treat the core symptoms of ASD are developed, however, their usage in the treatment of autism is limited.

The current treatments described above focus mainly on behavioral or developmental manifestations of the core autistic symptoms. New advances in genetic technology, affective developmental and cognitive neuroscience, and neurobiology have enabled researchers to identify novel biological methods for autism therapy. Primary goals of this research include improving early detection, treatment effectiveness, and improved biological targeting. However, these methods have yet to be employed, and no biological treatment that treats all the symptoms of ASD are yet available (Levy et al., 2009). Given the debilitating nature and increasing prevalence of these disorders, there is a pressing need for this type of research.

Autism Neuropathology

Early Brain Overgrowth Trajectory.

Among the most well-replicated finding in the autism literature is evidence of an aberrant brain growth trajectory in which the brain experiences overgrowth in the early years of life followed by arrested growth and possible neurodegeneration later on in childhood (Courchesne et al., 2001; Sparks et al., 2002; Courchesne, 2004; Hazlett et al., 2005; Courchesne et al., 2007; Amaral et al., 2008; Schumann et al., 2010). Previously

thought as a static brain abnormality, brain development in ASD is now seen as being age-dependent and dynamic.

Head circumference and Magnetic Resonance Imaging (MRI) volumetric investigations have shown that autistic individuals have larger brains than normal at the earliest ages (Filipek, 1995; Courchesne et al., 2001; Carper et al., 2002; Sparks et al., 2002; Herbert et al., 2003;Courchesne et al., 2007). In the first year of life, but not before, ~20% of subjects with autism have a head circumference above the 97th percentile. This size is indicative of macrocephaly (Bailey et al., 1993; Davidovitch et al., 1996; Woodhouse et al., 1996; Stevenson et al., 1997; Miles et al., 2000; Aylward et al., 2002; Courchesne et al., 2003). These findings of enlarged brain size in autism are independent of height, gender, and incidence of epilepsy and other medical disorders (Piven et al., 1995; Fombonne et al., 1999).

This overgrowth abnormality is sometimes corrected or overcorrected later on in childhood, resulting ultimately in normal or smaller brain sizes in individuals with autism (Redcay and Courchesne, 2005). In addition to a 5-12% increase in overall MRI brain volume (Courchesne et al., 2001; Sparks et al., 2002; Hazlett et al., 2005; Redcay and Courchesne, 2005), the size of the amygdala in 4-year-old autistic children is also increased (Sparks et al., 2002). This increase in amygdala size correlates positively with the severity of social and communication deficits (Munson et al., 2006).

One computerized tomography (CT) study showed progressive, age-related degeneration in the autistic brain (Hoshino et al., 1984). These results are consistent with qualitative findings of cortical thinning, sulcal widening, and ventricular enlargement in frontal and superior parietal areas (Courchesne et al., 1993), as well as thinning cortices in superior parietal, temporal, and frontal regions in autistic adolescents (Hadjikhani et al., 2006). Frontal lobe and corpus callosum subregion volumes, furthermore, are reduced in autistic adults (Egaas et al., 1995; Manes et al., 1999; Hardan et al., 2000; Chung et al., 2004; Alexander et al., 2007; Just et al., 2007; Schmitz et al., 2007). Across ages studied, furthermore, the cross sectional area of the corpus callosum is decreased (Egaas et al., 1995; Piven et al., 1997; Manes et al., 1999; Tsatsanis et al., 2003; Rice et al., 2005; Boger-Megiddo et al., 2006; Vidal et al., 2006). Similarly, the amygdala has been shown to be abnormally small in autism cases (Pierce et al., 2004; Nacewicz et al., 2006), or similar to controls in others (Schumann et al., 2004). The cerebellar vermis, moreover, is reported to be smaller in autistic than control cases (Courchesne et al., 1988; Courchesne et al., 1994; Ciesielski et al., 1997; Levitt et al., 1999; Courchesne et al., 2001; Kaufmann et al., 2003), or similar to controls in others (Piven et al., 1992).

Using analyses with greater resolution, other studies have found cortical gray matter enlargement in adolescent and adult autistic individuals (Lotspeich et al., 2004; Hazlett et al., 2006) or no difference in cortical thickness of autistic individuals (Hardan et al., 2006). Due to the differences in gray matter observed in autistic individuals, it is logical that the underlying white matter is also affected. Indeed, white matter abnormalities have been found in dorsal and medial prefrontal cortices, superior temporal

cortex, the temporoparietal junction, and the corpus callosum (Barnea-Goraly et al., 2004). In young autistic children, furthermore, evidence of premature myelination in frontal, but not posterior, white matter regions has been reported (Ben Bashat et al., 2007). Some have hypothesized as a unifying theory that short distance connectivity is increased in individuals with autism based on these pieces of evidence at the expense of long distance connectivity (Belmonte et al., 2004; Just et al., 2004; Courchesne and Pierce, 2005).

Interestingly, in a recent imaging study of monozygotic twins discordant for autism (Mitchell et al., 2009), no differences in the volume or area of frontal lobe, amygdala, hippocampus, subregions of the prefrontal cortex, corpus callosum, and cerebellar vermis were identified. However, the volume of the dorsolateral prefrontal cortex and anterior area of the corpus callosum were different between autistic twins and typically developing children. Volumes of the posterior vermis were different in both autistic and nonautistic twins compared with typically developing children. In subjects with strict diagnosis of autism, ADOS-G scores correlated significantly with volumes of the dorsolateral prefrontal cortex, amygdala, and posterior vermis. This study points to the important variability among individuals with autism.

Structural abnormalities are accompanied by increases in brain weight of autistic cases compared with controls (Kemper 1988; Bailey et al., 1998; Kemper and Bauman, 1998; Courchesne et al., 1999; Casanova et al., 2002). Interestingly, in this series of studies, 8 of 11 ASD cases under 12 years of age showed increased brain weights. 6 of 8

brains from cases 18 years and older, however, showed reduced brain weight. Though postmortem tissue treatment is a confounding factor of brain weight measurements, this series of studies validates the structural imaging data. 5% of adolescent and adult autistic individuals have abnormally heavy brains (Courchesne et al., 1999; Redcay and Courchesne, 2005).

It also is important to note that the timing of brain overgrowth in autism coincides with the onset of clinical symptoms, suggesting that the aberrant growth of the brain is a pathological process that disrupts the formation of normal brain structure and function of some autistic individuals (Minshew and Keller, 2010). Interestingly, a greater than 75th percentile head circumference correlates with more impaired adaptive behaviors and less impairment in IQ, motor, and verbal language development (Sacco et al., 2007). The degree of brain enlargement is not, however, predictive of performance IQ (Lainhart et al., 1997; Piven et al., 1995; Stevenson et al., 1997).

These results suggest that abnormal brain overgrowth in autism is a widely-documented phenomenon but also that there is great heterogeneity among individuals with autism. The degree and location of brain overgrowth, further, may determine clinical presentation in these individuals. Gross morphological abnormalities observed in the developing brain are not all consistent with observations in the adolescent or adult autistic brain. This suggests that age-related changes in brain morphology are a pattern among individuals with autism. Thus, when drawing conclusions from data of

individuals with autism, it is important to consider the ages of subjects and cases, or important effects may be missed (Courchesne et al., 2007).

Discontinuous brain phenotype.

An anterior-posterior gradient of neuropathology is also evident in the autistic brain as suggested by the evidence presented above. Gray matter volumes of the frontal and temporal lobes are enlarged the most, and the occipital lobe is enlarged the least (Carper et al., 2002; Kates et al., 2004; Hazlett et al., 2005; Palmen et al., 2005; Bloss and Courchesne, 2007). This gradient of enlargement abnormality is hypothesized to correlate with severity of cognitive symptoms (Courchesne et al., 2007). It is suspected that these developmental abnormalities lead to the behavioral differences observed in autistic individuals, since the regions of concentrated brain volume overgrowth include structures associated with cognitive, language, emotional, social functions, such as the amygdala, and frontal and temporal lobes (Hashimoto et al., 1995; Courchesne et al., 2001; Aylward et al., 2002; Carper et al., 2002; Sparks et al., 2002; Herbert et al., 2003; Schumann et al., 2004; Carper and Courchesne, 2005; Hazlett et al., 2005).

Size is not the only aberrant measure in the autistic brain that exhibits regional differences. Other MRI studies have found anterior and superior displacement of superior and inferior frontal sulci and superior temporal sulcus in the autistic brain (Levitt et al., 2003). These studies suggest that homogeneity of neuropathology in the autistic brain cannot be assumed.

Autism Genetics

Genetic studiesin the autism literature include predominantly genotype-phenotype association studies and also severalstudies of gene expression. Thus far, multiple methods have inconsistently detected genes that may be related to autism pathogenesis: several factors have been hypothesized to explain the inconsistency of candidate genes and loci identified through multiple methods.

Firstly, the aforementioned heterogeneity of the autistic behavioral phenotype may create inconsistencies; secondly, individuals with autism rarely reproduce, failing to pass on inherited mutations. Further, autism is likely a polygenic disorder: it may be caused by concerted actions of different genes, each giving rise to different phenotypes that together cause the broad behavioral aberrancies (Pickles et al., 1995, Risch et al., 1999, Szatmari et al., 1999, Losh et al., 2008). These studies, however, have been criticized as having sample sizes inadequate for detecting true effects (Losh et al., 2008). Others caution that linkage analysis has inherent limitations for detecting pathogenic alleles in oligogenic disorders (Risch et al., 1996). Other theories that attempt to consolidate these observations, such as autism being a fragile genome syndrome (Sebat et al., 2007), have also been put forth. This section will review the rationale underlying studying genetics in autism and discuss landmark results in the literature from genotypic, expression, and protein analyses of autism.

The Hereditability of Autism

Twin studies have formed the basis for hypothesizing that ASDs are strongly influenced by genetic factors. A ~60% concordance rate for autism has been identified in monozygotic twins, while dizygotic twins only show a 3-5% concordance rate (Steffenburg et al., 1989; Folstein and Rutter 1977; Bailey et al., 1995; Le Couteur et al., 1996). With a heritability estimate of ~90% and a 5-8% recurrence rate within families (Szatmari et al., 1998) for strict autism diagnosis, relatives of affected individuals often show milder phenotypes of autism-related language, cognitive, and repetitive behavioral symptoms. There is a 80% concordance rate for the broader definition of ASD in monozygotic twins, compared with a 10% difference in dizygotic twins (Steffenburg et al., 1989; Folstein and Rutter 1977; Bailey et al., 1995; Le Couteur et al., 1996). These results convincingly suggest that autism has at least a heritable basis.

Genome-wide Linkage and Association studies.

Despite these strong hypotheses, analyses of the autistic genome have not yielded consistent results. Chromosome 7q loci have been implicated as a linkage site most consistently. While a meta-analysis by Trikalinos et al., (2006) showed that 7q22-q32 was the most strongly linked locus on chromosome 7, other studies with even greater sample sizes have not replicated this finding (Szatmari et al., 2007).Loci on 2q21–33, 3q25–27, 3p25, 4q32, 6q14–21, 7q22, 7q31–36, 11p12–13, and 17q11–21have also been identified in two or more independent studies (Duvall et al., 2007; Freitag et al., 2007; Schellenberg et al., 2006; Szatmari et al., 2007;Alarcon et al., 2008; Freitag et al., 2009).

Studies have identified such genes as NRXN1, CNTN4, AUTS2, NLGN3, MET and SHANK3 as having copy number variations associated with autism, in regions such as 15q11-13, 22q11.21, and 16p11.2 (Glessner et al., 2009).

After performing linkage analyses of combined datasets with added subjects, it became quickly evident that previously described loci did not exhibit increased linkage to autism. Freitag et al. (2009) hypothesized that this may be because a) false positive or negative results may occur due to different linkage patterns in different populations, b) susceptibility genes are encoded by different loci in different populations, and c) rare risk alleles cannot be detected by linkage studies. These were obvious challenges to performing linkage analyses.

In 1996, Risch and Merikangas showed that association analysis to screen for susceptibility loci conferring weak effects has greater statistical power than linkage analysis. Genome-wide association analyses (GWAS) have the advantage of unbiased distribution of markers throughout the genome to compare between risk factors in autistic and control individuals. Since then, efforts have gone to accumulating large samples of DNA genotypes for this type of analysis. These studies have identified a multitude of candidate genes for autism.

In a recent study leveraging homozygosity mapping, Morrow et al. (2008) mapped several loci in families in which parents share ancestors. PCDH10 and DIA1

contained the largest deletions in these families. This study showed that these genes modulated levels of expression in response to neuronal activity, and the authors postulated that these genes are involved in the synaptic changes responsible for learning.

More recently, using 500K SNP markers in 1031 multiplex autism families with 1553 autistic children, Weiss et al (2009) identified suggestive linkage of chromsome 6q27 and significant linkage on 20p13. This group found association of a SNP on chromsome 5p15 between SEMA5A and TAS2R1, and a decrease in expression of SEMA5A in postmortem brain from autistic cases.

Other genes identified by association studies have fallen into three categories: developmental genes, synaptic genes, and signaling-related genes.

Developmental Genes. The MET gene is encoded in 7q31, a region identified by linkage studies (Campbell et al., 2006). This gene is shown to be involved in neuronal growth and organization, and immunological and gastrointestinal functioning, all domains of function that are associated with autism (Losh et al., 2008). A C allele in the promoter region of the MET receptor tyrosine kinase gene was found to be significant associated with autism, especially in families with more than one affected child. A 2-fold decrease in MET promoter activity and binding of transcription factor complexes in addition to a 2.27 relative risk of being diagnosed with autism was conferred with having 2 copies of the C allele (CC; Campbell et al., 2006). Compared with the promoter activity associated with this C allele, mouse cells transfected with human MET promoter

variants exhibited a 2-fold decrease in promoter activity. Through expression analysis, Campbell et al., (2007) also found that MET protein levels in the postmortem autistic brain were significantly decreased compared with controls.

EN2 is a homeobox gene that plays important roles in posterior segmentation, in particular embryonic and postnatal development of mouse cerebellum (Millen et al., 1995; Kuemerle et al., 1997; Baader et al., 1998). A marker in the EN2 locus was linked to autism (Auranen et al., 2002). Moreover, association of this gene was tested with the autistic phenotype, and significant association was found for some markers within EN2 (Petit et al., 1995; Gharani et al., 2004) but not others (Zhong et al., 2003).

PTEN encodes a tumor suppressing protein. In mice, this gene regulates arborization of neuron processes and mutations in this gene cause autistic-like behaviors. PTEN has been associated with autistic individuals with macrocephaly: 3 of 18 individuals showed mutations in this gene in an original study (Butler et al., 2005). Since then, other studies have replicated this finding (Boconne et al., 2006; Buxbaum et al., 2007; Herman et al., 2007).

Reelin is a gene associated with neuron migration and positioning that is active during neurodevelopment (Rice et al., 2001). Reeler mice, which have a deletion in the RELN locus, exhibit aberrant cortical organization similar to that of the autistic cortex (Bailey et al., 1998). The 7q22 chromosomal region where the reelin gene resides exhibits linkage disequilibrium with autism (reviewed by Losh et al., 2008). A

trinucleotide repeat in the 5' UTR of Reelin has also been associated with autism (Ashley-Koch et al., 2007; Zhang et al., 2002). These repeat polymorphisms preferentially transmit to autistic siblings (Persico et al., 2001; Dutta et al., 2007), and suggest that this gene may be regulated epigenetically as the long allele causes suppression of gene expression *in vitro* (Persico et al., 2006). Other studies, however, did not identify this gene (Zhang et al., 2002; Krebs et al., 2002; Bonora et al., 2003; Devlin et al., 2004; Li et al., 2004).

TSC1 and TSC2, which are involved in tuberous sclerosis that sometimes presents with autistic clinical features, has also been associated with autism in 15-60% of cases (Wiznitzer 2004).

Synaptic genes. Neurexins encode proteins that promote synaptic functioning in neurons (Ushkaryov et al., 1992). The Autism Genome Project Consortium found a demizygous deletion of coding exons in a pair of affected siblings (Szatmari et al., 2007). In a second study, rare coding variants of NRXN1 coding exons were identified in 57 individuals with autism (Kim et al., 2008). Finally, 4 individuals with autism of 72 had missense mutations in the exon 1 of NRXN1beta. 9 cases additionally had in frame deletions and insertions in this exon, and these abnormalities were not detected in control individuals (Feng et al., 2006).

Interestingly, NRXN1 deletions do not only predispose individuals to ASDs. Ching et al. (2010) tested 3540 individuals clinically referred for CGH testing. 12 of

these cases had exonic deletions in NRXN1, which was statistically significantly higher than the incidence in control populations. In addition to ASD, NRXN1 deletion was found to predispose individuals to mental retardation, language delays, and hypotonia.

Neuroligins, which are cell adhesion molecules that interact with neurexin proteins, are also involved in synaptic function and maturation (Zoghbi 2003). Though three studies have reported insignificant association of autism with neuroligins (Vincent et al., 2004; Gauthier et al., 2005; Blasi et al., 2006), others have found mutations in NLGN3 and NLGN4 of 2 sibling pairs (Jamain et al., 2003). In a further study, 4 of 148 individuals with autism showed missense mutations in the NLGN4 gene (Yan et al., 2005). In a family with multiple affected individuals, a 2 base pair deletion in the NLGN4 gene segregated with the phenotype. Exonic deletions were also identified in a family with autism and other learning and psychiatric disorders (Lawson-Yuen et al., 2008).

CNTNAP2 is another adhesion molecule that forms CASPR2 with the neurexin superfamily (Poliak et al., 2003). It was associated with autism with cortical dysplasia-focal epilepsy and showed a frameshift mutation in exon 22 of the gene in 9 affected individuals (Strauss et al., 2006). In 72 multiplex families, moreover, 7q35 showed significant linkage in autistic cases and the T allele of the intron between exons 2 and 3 on the gene was overtransmitted (Arking et al., 2008). CNTNAP2 was also significantly associated with an index of language delay in autistic children (Alarcon et al., 2008). In this same study, a microdeletion in this gene was identified in an autistic individual and

his father. Furthermore, several rare variants of CNTNAP2 were discovered in 635 individuals with autism that were not present in 942 controls (Bakkaloglu et al., 2008). Patients homozygous for a frameshift mutation in CNTNAP2 exhibit cortical dysplasia, thickened cortex, and migratory abnormalities including ectopic neurons in subcortical white matter (Abrahams et al., 2007).

SHANK3, a synapse and neuronal scaffolding protein that binds to neuroligins and regulates dendritic spine morphology (Kreienkamp 2008), is a gene that is hypothesized to be involved in autism pathogenesis. In a first study, 3 of 226 families with autistic individuals showed SHANK3 mutations, with one of the autistic children showing a *de novo* deletion (Durand et al., 2007). Similarly, a *de novo* mutation and 2 deletions in SHANK3 were identified in 400 subjects (Moessner et al., 2007).

LAMB1, which encodes the Beta-1 chain of the laminin protein, has been found to be associated with autism (Hutcheson et al., 2004; Bonora et al., 2005). This gene promotes neuronal migration and outgrowth during neurodevelopment (Freitag et al., 2009).

Signaling Genes. The SLC6A4 locus has been implicated in autism by several association analyses (McCauley et al., 2004; Sutcliffe et al., 2005; Mulder et al., 2005; Tordjman et al., 2001; Wassink et al., 2007). Similarly, the SLC25A12 gene, which encodes a mitochondrial aspartate/glutamate carrier, contained several variants significantly associated with autism (Freitag et al., 2007; Silverman et al., 2008).

5HTTLPR, the promoter region of SLC6A4, has also shown transmission disequilibrium in autism (Kim et al., 2002; Cook et al., 1997; Yirmiya et al., 2001; Klauck et al., 1997; Maestrini et al., 1999).

Furthermore, SNPs and haplotypes of the oxytocin receptor, which plays important roles in social cognition and behavior (Donaldson et al., 2008), were associated with autism (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2007). It also falls under a linkage peak on 3p24-26.

The heterogeneity of the findings described in these candidate gene analyses underscores that autism is a polygenic disorder. No single gene can explain the entire autistic phenotype, and hypothesizing to find a candidate gene or one candidate locus may be overly reductionistic.

Copy Number Variation.

At a global scale, 3-5% of autistic individuals have cytogenetic abnormalities (Reddy et al., 2005). Approximately 1% of autistic cases have abnormalities on chromosome 15q11-13 (Depienne et al., 2009; Freitag et al., 2007). With novel technology, the field has been able to identify submicroscopic karyotypic abnormalities in the autistic genome.In a small percentage of simplex autistic individuals, *de novo* copy number variations (CNV) have been identified, typically within one gene only (Sebat et

al., 2007). It remains unclear whether increased CNV incidence is caused as a byproduct of the event that causes autism, or if the increase causes autism.

The association between *de novo* CNVs was identified in a study done by Jacquemont et al., (2006). 27.5% of 29 individuals with autism in the study were found to have *de novo* CNVs that were clinically relevant. Of these were six deletions and two duplications. The authors suggested that array CGH should be part of a diagnostic study for autistic individuals.

In Sebat et al., (2007) comparative genomic hybridization (CGH) was performed on genomic DNA of patients and controls, with candidates validated using higher resolution CGH, paternity testing cytogenetics, fluorescence in situ hybridization, and microsatellite genotyping. 12 of 118 (10%) patients with sporadic autism, 2 of 77 (3%) patients with an affected first-degree relative, and 2 of 196 (1%) controls showed confirmed de novo CNVs. These CNVs, though highly heterogeneous, were significantly associated with autism. The authors suggested that *de novo* germline mutations may introduce significant susceptibility to autism. They also hypothesized that autism may be a 'fragile genome disorder'.

Szatmari et al., (2007) analyzed linkage and CNVs in a large sample of 1181 families with at least two affected individuals. Linkage analysis identified 11p12-p13 as a putative candidate locus. 254 CNVs were identified in 196 ASD cases from 173 families. 10 families had *de novo* CNVs: 3 of these families had CNVs in both affected

siblings. 18 of the identified CNVs were coincident with chromosome arrangements already in the literature. 126 of the identified CNVs had recurrent (47) or overlapping (79) borders. 7 cases had maternally-transmitted chromosome 15q gains. This study also gave additional evidence for NRXN1 being a susceptibility gene for ASD.

To examine the etiologic role of structural variation, or CNVs including deletions and duplications, translocations, and inversions in autism, Marshall et al., (2008) used SNP microarrays and karyotyping to analyze 427 ASD cases. The group identified a CNV with 1% frequency at 16p11.2 with characteristics of a genomic disorder. 44% of ASD families showed evidence of 277 unbalanced CNVs. These CNVs were not present in 500 control cases or a replicate control set of 1152 individuals. 27 ASD cases had *de novo* variants, and 3 of these individuals had two or more new variants. The authors provide supporting evidence of postsynaptic genes (SHANK3, NLGN4, NRXN1), synapse complex (DPP6, DPP10, PCDH9), and other genes contributing to ASD susceptibility. The authors suggested that the frequency of the identified structural variants warranted screening in routine clinical examinations.

The gene NRXN1 was again implicated in ASD susceptibility in a study by Kim et al., (2008). Karyotypes of two individuals with ASD and balanced chromosomal abnormality involving 2p16.3 were examined in detail. The first subject had a karyotype of 46,XX,ins(16;2)(q22.1;p16.1p16.3)pat and a direct disruption of the fifth intron of NRXN1. The father of this subject also had this abnormality but did not exhibit symptoms of ASD. The second subject had a karyotype of 46,XY,t(1;2)(q31.3;p16.3)dn.

The deletion occurs within a 2.6 Mb unannotated genomic segment about 750 kb 5' to NRXN1. Furthermore, the study showed that rare sequence variants in the alpha-neurexin 1 leader sequence and EGF-like domain were present in ASD subjects. However, amino acid alterations in NRXN1 do not occur at high frequency in ASD subjects compared with control subjects.

In a similarlarge-scale genome screen, Weiss et al., (2008) identified 16p11.2 to be a site of duplications and deletions in 1% of autistic cases that were not identified in 2 other psychiatric control groups but in 0.01% of an Icelandic populations. 5 of 512 children with developmental delay, mental retardation or suspected ASD and 3 of 299 individuals with autism had an identical deletion. 7 individuals in AGRE and 4 of 512 autistic children had duplications in this region. No other locus in the genome showed this effect.

Glessner et al., (2009) also performed CNV analysis in 859 ASD and 1409 healthy children of European ancestry through the use of SNP microarrays, with a replication group of 1336 ASD and 1110 controls of European history. The group replicated earlier findings of CNV enrichment in NRXN1 (Kim et al., 2008) and CNTN4 (Fernandez et al., 2008). Additional genes involved in neuronal cell adhesion were also enriched with CNVs in ASD cases, including NLGN1 and ASTN2. Furthermore, CNVs in genes regulating Ubiquitin pathways such as UBE3A, APRK2, RFWD2, and FBXO40, were also overrepresented in ASD cases. These results were interpreted as

being evidence supporting the hypothesis that abnormalities converging on cell adhesion and Ubiquitin pathways played a role in ASD pathogenesis.

In a recent CNV study of a Chinese Han family with ASD (Yan et al., 2010), the 15q11 locus of three autistic siblings out of six showed a de novo CNV. Within this region, olfactory genes OR11K1P, OR4Q1P, OR4H6P, and OR4M2 were hypothesized to contribute to the ASD phenotype in this family.

In a recent study by Bremer et al., (2010), 223 ASD patients were screened using array CGH for gene dose alterations. 8% of the individuals were found to have clinically significant copy number variations. 4% of these cases had *de novo* events. Interestingly, different CNV patterns emerged if patients were divided into phenotypic and inheritance pattern groups. Patients with first- or second-degree relatives with an ASD-related neuropsychiatric phenotype showed an increased rate of rare inherited CNVs, and syndromic ASD cases had a higher frequency of clinically relevant CNVs than nonsyndromic ASD cases. The authors hypothesized that rare CNVs may contribute to susceptibility for autism.

In a first look at the global functional impact that copy number variants may have on individuals with autism, Pinto et al. (2010) performed CNV detection and enrichment analysis on these CNVs identified in 996 ASD individuals in comparison to 1287 controls. These ASD subjects overall had a increased incidence, 1.19-fold, of rare genic CNVs. The authors identified additional candidate genes for autism susceptibility,

including DLGAP2, DDX53-PTCHD1, SYNGAP1, and SHANK2. Proliferation, projection and motility, GTPase/Ras signaling functional gene sets were particularly enriched in ASD CNVs. This method was a novel approach to addressing the possible effects of interaction between variants in the autistic genome that may have downstream functional consequences.

These studies have contributed to our thinking of the abnormalities in autism: loci that may hold pathogenic potential do not appear consistently across subjects and cases with autism. In fact, others have emphasized that rare variants identified within patients with autism should not always be interpreted as being meaningful (Belloso et al., 2007; Abrahams and Geschwind, 2008). Much work remains to be done to distinguish between the pathogenic variants for autism, pathogenic variants for disorders other than autism that may be comorbid within an individual, and nonpathogenic variants. Functional genomic studies can provide clues to the downstream consequences of genotypic differences.

Gene Expression studies.

While investigations of DNA from patients with autism provide information on the basic mutations that may have occurred to produce the behavioral and cognitive phenotypes, it is the investigations of RNA expression that provide functional genetic information on the brain. The few published of brain gene expression studies are riddled with confounds. Due to scarcity of well-preserved tissue, studies have focused on a few genes hypothesized to be related to autism, and sample sizes are typically small. Due to

the small sample sizes, processing steps following the biological experiments are unable to statistically account for known variables, which are sure to affect gene expression in important ways. Furthermore, the ages of the subjects investigated are relatively old compared to the ages of ongoing neurodevelopment, RNA integrity is reportedly poor in autistic tissue, and genetic findings are seldom replicated by subsequent investigations. Preserving the postmortem brain for gene expression analysis has indeed proven challenging, since high RNA quality is required for reliable and precise quantitative experiments. Someinvestigators have approached this issue by assessing gene expression in immortalized lymphoblastoid cell lines (LCLs) derived from individuals with ASDs.

Gene expression in the postmortem brain

The earliest study of genome-wide expression in the postmortem autistic brain identified abnormalities of glutamate receptors and transporters in the autistic cerebellum (Purcell et al., 2001). Samples of (mostly) cerebellum from 10 autistic and 23 matched control cases were processed on cDNA microarrays. Results from these microarrays were validated using RTPCR and western blot. Furthermore, receptor autoradiography was applied to examine the density of AMPA and NMDA-type receptors in the cerebellum, caudate-putamen, and prefrontal cortex in autism. AMPA1 receptor and excitatory amino acid transporter 1 mRNA levels, including GluR1, GluR2, GluR3, SLC1A2, EAAT2 were significantly increased in autistic cases. However, AMPA glutamate receptor density was significantly decreased in the granule cells and molecular layer of autistic cerebellum. Purcell and colleagues interpreted this disparity as a possible

signature of excitotoxicity in this region of the brain. They argued that extracellular glutamate concentrations in the cerebellar cortex are maintained below a neurotoxic threshold exclusively by glial glutamate transport, despite the fact that the cerebellar cortex is enriched with glutamatergic neurons.

The only other genome-wide expression study on brain gene expressionwas conducted by Garbett et al. (2008). This studyfound important abnormalities in antigen-specific immune responses, inflammation, cell death, autoimmune disorders, and migration and targeting of the immune response to specific cells (NKT pathway) in the superior temporal gyrus of six ASD cases and six age, sex, and PMI-matched controls.They identified strong upregulation of innate immune genes in IL2RB, TH1TH2, and FAS pathways. Neuronal differentiation and outgrowth gene sets, including genes such as GFG12, MYT1L, and GAS7, however, were downregulated. The authors strongly suggested that transcription induction outnumber transcription repression processes in autism; immunological, cell communication, differentiation, and cell cycle regulation aberrancies characterize the autistic brain; and that transcriptome variability is increased in autism.

Other smaller-scale studies have been conducted to examine possible candidate genes in the autistic brain, including genes regulating neurodevelopment. Reelin is one such protein that has been studied in a variety of brain areas. One such study identified in frontal and cerebellar cortex reduced Reelin 410, 330, and 180 kDa values using QPCR. mRNA of both reelin and Dab-1 were decreased in superior frontal and cerebellar regions

of autistic brains, but that of the reelin receptor VLDL-R was increased (Fatemi et al., 2005). SEMA5A, which regulates axon guidance during neurodevelopment, was found to be decreased in autistic brains (Weiss et al., 2009).

Furthermore, qPCR for nicotinic receptor subunit α4, α7 and β2 and protein expression immunochemistry and radioligand receptor binding of α3, α4, α7 and β2 analysis of adult autistic cortex and cerebellum was performed by Martin-Ruiz et al., (2004). The α4 and β2 nicotinic receptor subunits had reduced expression levels in the parietal cortex. A7 receptor binding and α4 transcript expression was increased in the cerebellum. α4 protein and receptor levels were decreased in the cerebellum. These scattered and variable results call for a large-scale genome-wide expression study of the postmortem autistic cortex.

LCL gene expression studies in autism

As mentioned above, due to the scarcity of postmortem brain tissue from autistic donors, investigators have circumvented the issue by profiling gene expression in LCLs derived from living subjects.

LCLs derived from (1) three monozygotic twin pairs discordantfor severity of autistic symptoms and (2) two monozygotic twin pairs concordant for autism diagnosis but discordant for severity of language impairment were analyzed for genome-wide expression profiles in Hu et al. (2006). Immune pathways involving TNFα and

proinflammatory cytokines overlapped between the differential expression of twin sets (1) and (2). Neurodevelopmental and neurofunctioning genes NAGLU, ASS, FLAP/ALOX5AP, DAPK1, IL6ST, CHL1, and ROBO1 were found to be different between twin sets (1) and (2). The authors suggested that these findings support the idea that lymphoid cell lines from autistic individuals may be examined to find candidate genes, and that expressed biomarker screens from peripheral blood lymphocytes may be possible in the future.

Similarly, LCLs from 8 male autistic patients with a fragile X mutation (FMR1-FM), 7 males with autism due to a 15q11-q13 duplication (dup(15q)), and 15 control subjects were profiled for genome-wide expression (Nishimura et al., 2007). Cell communication and signal transduction, immune response and defense response, chaperone and protein folding, and RNA binding and mRNA metabolism were found to be enriched in the differentially expressed genes between these groups. Cell communication and immunological aberrancies were observed across autistic samples regardless of genetic etiology. The genes dysregulated in the FMR1-FM cells, however, were selectively enriched in chaperone and RNA binding protein functions.

In a similar study, Gregg and colleagues (2008) conducted a gene expression assay in LCLs from 12 controls, 14 patients on the autism spectrum but not reaching diagnostic thresholds for Autistic Disorder, and 35 patients with Autistic Disorder. Among the 35 patients with Autistic Disorder, samples were again divided into a group with early onset (n=17) and a group with late onset (n=18) and/or history of regression.

The KEGG pathway, which regulates natural killer cell-mediated cytotoxicity, was dysregulated across ASD groups. The IL2 pathway was upregulated in all patients on the spectrum and those in the early onset group. SEMA4C and GLUD1, which are involved in neurodevelopment and glutamatergic neurotransmission, were upregulated in the autistic group.

Expression from LCLs of 52 children on the autism spectrum and 27 control subjects (Enstrom et al., 2009) showed an upregulation of natural killer cell-related pathways, killer cell immunoglobulin receptors, and inhibitory killer cell immunoglobulin receptors. Enstrom and colleagues also found a downregulation of genes involved in cell proliferation and differentiation, ribosomal processing, cell metabolism, intracellular organelles, and cellular biosynthesis.

The final LCL expression study was performed on cells derived from 86 male autistic patients and 30 age-matched controls (Hu et al., 2009). The majority of dysregulated transcripts identified as differentially expressed between autistic and control groups were in pathways related to androgen sensitivity and the steroid hormone biosynthetic pathway. The autistic patients were separated according to ADI-R scores into three categories. Group 1 consisted of patients with severe language impairment and high overall severity scores. Group 2 consisted of patients with mild severity scores, including patients with Asperger's Syndrome or PDDNOS. Group 3 consisted of patients displaying high scores in savant skills. Group 1, the most severely affected patients, was characterized by aberrance of circadian rhythm regulatory or responsive genes.

Apoptosis, inflammation and oxidative stress, synaptogenesis and axon guidance, epigenetic regulation and protein ubiquitination functions were enriched in the overlapping differentially expressed genes of groups 1-3. The authors suggested that genes associated with androgen sensitivity may underlie the strong gender bias in autism.

Protein expression studies in the autistic brain.

Lastly, several protein expression studies have been performed using postmortem tissue to understand molecular mechanisms in the autistic brain. 2-dimensional gel electrophoresis identified increased polarity of glyoxalase I in autistic brains. This study also identified a higher frequency of the A419 allele in the GLO1 gene in autistic cases, which causes an Ala111Glu change in the protein (Junaid et al., 2004). Abnormalities in the cholinergic and GABAergic systems in the autistic brain have also been characterized (Blatt et al., 2001; Perry et al., 2001; Fatemi et al., 2002; Lee et al., 2002).

Furthermore, quantitative immunoblotting found myelin basic protein to be different between brain extracts of autistic and control cases (Silva et al., 2004). Other studies using ELISA, immunocytochemistry, and cytokine protein assays on ceberbral cortex, white matter, cerebellum and cerebral spinal fluid of autistic patients and postmortem cases have found evidence of active and ongoing neuroinflammatory processes (Vargas et al., 2005). The authors identified microglial and astroglial activation in the autistic brain. Moreover, MCP1 and TGF1 were upregulated in the autistic tissue. MCP1 was also increased in the CSF of autistic cases. Finally, Laurence

and Fatemi (2005) identified elevated levels of glial fibrillary acidic protein (GFAP) in three areas of the autistic brain, including superior frontal, parietal, and cerebellar cortices. These results support the immune activation hypothesis of autism pathogenesis

In addition, Araghi-Niknam and Fatemi (2003) found that anti-apoptotic Bcl2 protein was decreased in autistic superior frontal and cerebellar cortices. P53, on the other hand, was increased in these areas. Though the sample size was small (N=5 autism and N=5 control), the authors found no correlation between age and expression levels of either protein. This study pointed to aberrant apoptotic functions in the autistic brain.

The brief review of the genetics literature here captures the myriad of genotypic, expression, and protein abnormalities that have been identified in autism. To date, no overarching theory can explain the presence of these highly variable results. They show the basis for the copious current competing theories of autism pathogenesis.

Current theories of autism pathogenesis

The etiology of ASD is still unclear: decades of research have resulted in inconclusive and conflicting pieces of evidence that are often attributed to the heterogeneity of the disorder at all levels of study. This disorder, however, is agreed

upon as one of prenatal and postnatal brain development. As reviewed in the preceding sections, scientists have investigated this neurodevelopmental disorder by examining functional neural networks, cognition, brain growth, neuropathology, electrophysiology, and neurochemistry (Levy et al., 2009).

Many theories have been put forth based on these studies to explain the deficits observed in children with autism: synapse pathology(Garber, 2007; Bourgeron, 2009), white matter pathology (Courchesne et al., 2001; Herbert et al., 2004), hyperconnectivity, hypoconnectivity or disconnectivity (Belmonte and Yurgelun-Todd, 2003; Courchesne and Pierce, 2005; Geschwind, 2007; Just et al., 2007), abnormal minicolumns (Casanova et al., 2002), excess excitation(Rubenstein and Merzenich, 2003; Courchesne, 2004; Courchesne et al., 2007), and neuroinflammation (Vargas et al., 2005).

The hypothesis of synapse pathology (Garber, 2007) arose from genetic association studies implicating Neurologin, Neurexin, and other cell adhesion molecules that may affect synapse formation during development (Jamain et al., 2003; Chubykin et al., 2005). There is, however, a complete lack of quantitative studies of cortical synapses in the autism brain. Thus, despite evidence of genetic abnormalities in the genome, functional, or morphological studies need to be performed in order to support this hypothesis (Courchesne et al., 2007).

Another leading theory of autism pathogenesis is immune activation or neuroinflammation in the brain during development(Licinio et al., 2002; Vargas et al.,

2005). Garbett et al. (Garbett et al., 2008) performed a whole genome brain gene expression study and found a constellation of immune processes dysregulated in autistic subjects. The lack of phenotypic information, the small number of postmortem cases, and a constellation of agesmade the results difficult to definitively interpret. However, active immune processes in the cerebral cortex, white matter, and cerebella of autism cases have been identified in another study, as well as activation of microglia and astroglia (Vargas et al., 2005). Vargas and colleagues suggest that neuroimmune responses play a role in the pathogenesis in a certain portion of autistic individuals.

Abnormalities in neurogenesis, cell proliferation, cell cycle, and neuronal migration have also been hypothesized to underlie brain overgrowth in autism (Courchesne et al., 2007; Courchesne et al., 2001). Recent neuropathological studies of the autistic brain have shown that this may indeed be the case (Wegiel et al., 2010). For example, thickening of the subependymal cell layer in two cases with autism as well as subependymal nodular dysplasia in one case with autism suggests that ongoing active neurogenesis occurred antemortem. Additional neuropathological analyses need to be performed to support this hypothesis.

Finally, the notion of a triple hit hypothesis has also been put forth to explain and unify the data on individuals with autism. Three factors reaching a certain threshold tip an infant onto the ASD spectrum. These factors include: 1) a critical period of brain development, 2) underlying genetic vulnerability, and 3) environmental stressors (Casanova 2007).

These theories are not necessarily mutually exclusive, and may continue to bear relevance for diagnosis and treatment in autism as more evidence is collected. None of these models, however, fully explain all of the results reported above; the field is in need of a unifying theory. We attempted to address these theories in our studies of the postmortem autistic brain.

Constraints and Considerations in autism research, and Novel Experimental Methods

Limitations of Tissue Availability.

As with any neuropsychiatric disorder that is unique to humans, examination of the tissue that is abnormal in the disorder is vital to understanding of fundamental biological mechanisms that cannot be modeled through other organisms or cell lines (Mirnics et al., 2001; Mirnics et al., 2004). With the limitations of tissue donation, however, neural tissue from autistic and control cases is scarce (Kretzschmar 2009).

It is especially difficult to obtain brain samples from young autistic children since autism is not a fatal disease. Autistic individuals often live past childhood into adulthood. Though the functional changes in the adult brain may still contain remnants of the causative factors initiating the neurodevelopmental pathway of autism, it is

impossible to discern from changes that are related to age and not autism. Each sample must be utilized to yield as much information as possible.

Technological constraints and considerations.

In addition to the availability of tissue, the quality of tissue preservation is an important challenge for research examining autism pathogenesis. The study of mRNA transcripts and proteins are especially vulnerable since these molecules degrade rapidly after death. Unfortunately, these issues make measures like postmortem interval necessary to record so that it can be taken into account in downstream analyses and during data interpretation. In order to study the remaining genetic questions in the field, especially genetic functional studies, novel techniques must be employed to work with the limited and often poor-quality postmortem brain samples collected from autistic individuals.

In addition to these issues of preservation, a multitude of case factors that have no bearing on disease pathogenesis may produce confounds in analyses of genetics and neuropathology. Agonal factors and postmortem conditions have long been known to confound expression-profiling studies (Tomita et al., 2004). Furthermore, many antemortem conditions could modify the results ofgene expression studies, protein assays, and epigenetic analyses of postmortem tissue. These conditions include but are not limited to comorbid conditions, such as seizures in autism, and medication use.

Statistical procedures have been developed to account for these factors and general systemic error in datasets, which can prepare the data for downstream analyses if used correctly. For gene expression profiling studies in particular, data preprocessing steps have been shown to change downstream differential expression results (Lim et al., 2007; Schmid et al., 2010). They must therefore be tested systematically to ensure that bias is not introduced to the dataset in combination with each type of expression profiling platform.

DNA-mediated Annealing, Selection, and Ligation (DASL) for genome-wide expression profiling.

Whole genome expression microarrays are a logical and powerful technique for assaying the time course and changes of gene expression in specific areas of the human brain. They are especially useful in the identification of candidate genes of heterogenous disorders. The scarcity of postmortem human brain tissue is the bottleneck for these types of studies, and the availability of tissue that has high enough RNA quality to perform reliable microarray analyses make whole-genome human brain gene expression studies rare. Those that are able to collect samples of postmortem brain tissue rarely have adequate numbers for statistical comparison.

One method with great clinical promise to address these issues that has been developed is the DNA-mediated annealing, selection and ligation (DASL) procedure. This techniquehas been developed to contend with RNA degradation in human tissue

samples. DASL is a newly commercialized method from Illumina Inc, capable of assaying reliable quantitative gene expression results from even formalin fixed, paraffin embedded tissue with degraded RNA (Fan et al., 2004; Hoshida et al., 2007; April et al., 2009).

At baseline, this assay produces a correlation of 0.99 with traditional IVT-based approaches. DASL, however, has greater sensitivity to even degraded RNA due to both the random priming step in addition to using probes of only 25 nucleotides in solution. Preservation by deep freezing is less damaging to RNA than formalin fixation and paraffin embedding, so in theory this technique should be suitable for profiling gene expression in the brain at least for frozen tissue. If indeed this were the case, then even brains that were not preserved adequately for high quality microarray array results using conventional methods could be used for gene expression profiling. The fields of autism and many other neuropsychiatric disorders would highly benefit from the DASL technique. Using such a method would enable us to characterize changes in gene expression through time using increased sample sizes of human postmortem tissue for characterizing pathological changes that cannot be modeled in cell lines or animals.

Remaining Questions

Despite the progress that the field has made elucidating possible mechanisms underlying aberrant neurodevelopment in autism, many questions open to the field remains. Is autism of a prenatal or postnatal origin, and what are genetic, environmental, or combination of influences that generate the autistic behavioral phenotype? What biological changes throughout development cause the changes in brain overgrowth that is observed in autism subjects, and what causes the different regions of the brain to be affected differently? How may we utilize cognitive and behavioral characteristics to help identify the biological variants of autism in order to more effectively examine the biological underpinnings of the autism brain? Why are children with autism so prone to other comorbid disorders, and what can the occurrence of these comorbid disorders tell us about the autistic brain? What are the aftereffects of brain overgrowth, and how do we distinguish the causative features (e.g. neurogenesis, neuronal migration) of autism in the brain with the reactive ones (e.g. neuroinflammation, neuroimmune activation)?

There is also the question of age-related changes in the autistic brain. What are the triggers in the brain that are going awry as a child with autism develops? At what level (DNA, RNA, or protein) are these changes occurring, and at what level may we target biotherapeutics to change the trajectory of brain growth? Now that copious association and CNV studies in autism have identified multiple candidate loci and genes (Bucan et al., 2009; Glessner et al., 2009; Wang et al., 2009; Weiss et al., 2009), what are the downstream functional consequences of these mutations? Are these mutated genes causative, or are they a downstream consequence of the initial insult of autism, such as DNA damage, or part of a fragile genome disorder(Sebat et al., 2007)? It is difficult to

interpret results of differing levels of single genes, without the context of changes in other genes: thus, whole genome analyses would allow us to examine entire pathways and processes that may be dysregulated. In our studies, we will try to answer some of these questions.

Aims and Hypotheses

Hypotheses.

The observation that the frontal cortex is among the specific regions selectively affected by aberrant overgrowth (Courchesne et al., 2001; Carper et al., 2002; Sparks et al., 2002; Carper and Courchesne, 2005; Hazlett et al., 2005) has important implications due to the role of the frontal cortex in higher order human cognitive functions such as language and complex social interaction, the domains most severely affected in autism. Thus, this area is an obvious choice for exploratory investigations of genetic defects in postmortem autistic brain tissue.

Since the developmental mechanisms that are potentially producing the neuropathological abnormalities and autistic behaviors may occur early in life, it is of critical importance to examine subjects whose brains are still developing. Brain size and development are hypothesized to be at least in part genetically programmed, but few

investigations have directly linked genetic abnormalities to brain size in autism (Courchesne et al., 2007).

We hypothesized that the brain overgrowth and these other signatures of neuropathology in autism are at least in part due to abnormalities in neuronal migration and proliferation, which are predominantly genetically determined phenomena. Under the assumption that there is more than one neurodevelopmental defect that underlies autism, we searched for consistent neuropathological and genetic signatures in the postmortem autistic brain. To understand the molecular mechanisms underlying aberrant brain overgrowth in autism, we used a novel genome-wide expression profiling approach to assess expression abnormalities in the autistic brain.

Overview of study.

1) We assessed the efficacy and reliability of DASL for elucidating differences in gene expression in degraded RNA samples from reference RNA

2) We assessed the efficacy and reliability of DASL for elucidating differences in gene expression in degraded RNA samples from frozen and formalin fixed postmortem brain tissue samples.

3) We systematically developed a statistical preprocessing pipeline specific for DASL-generated brain tissue expression data that would adequately prepare the raw data for downstream differential expression and enrichment analyses.

4) We characterized the aberrations in genome-wide expression of autistic dorsolateral prefrontal cortex, and statistically testeddifference of transcript levels in autistic and control dorsolateral prefrontal cortex. We examined the pathways of abnormality in autism that are independent of age, and those that are dysregulated in the youngest autistic cases.

Chapter 2 –Genome-Wide Expression Assay Comparison Across Frozen and Fixed Postmortem Brain Tissue Samples.

Introduction

Gene expression profilinginvestigations involving postmortem brain tissue of cases with neuropsychiatric disorders have been limited due to tissue availability, tissue quality, and antemortem conditions of donors (Mirnics et al., 2004; Mirnics and Pevsner, 2004; and Mirnics et al., 2006). Such investigations, however, are critical for understanding uniquely human disorders (Horvath et al., 2011). While experimenters cannot control tissue availability, novel technologies can be employed to utilize the precious and scarce tissue resources available from brain banks, even if preservation quality is not ideal (Fan et al., 2004).Antemortem conditions of donors may also be accounted for in various ways, including via sophisticated statistical methods, but remain problematic.

The cDNA-mediated annealing, selection, and ligation (DASL) gene expression assay has been shown to produce highly reliable results when applied toformalin-fixed, paraffin-embedded tissues (Hoshida et al., 2008). This methodology has already been applied in the study ofhuman liver, esophagus, breast, prostate, ovarian, and other biopsy and autopsy tissue (Hoshida et al., 2008; Reinholz et al., 2010; Sboner et al., 2010; Chien et al., 2009; Hammoud et al., 2009). This DASL-based approach can be extended for use in a genome-wide format, which may be of value in the elucidation of genes

mediating neuropsychiatric diseases(Reinholz et al., 2010; Horvath et al., 2011). However, it has not yet been applied to postmortem frozen and formalin-fixed brain tissue, despite its potential benefits when assaying samples with low RNA quality.

As relevant and sophisticated as the DASL and other assays may be for exploring expression profiles of brain tissue from psychiatric cases, the application of those assays also requires important considerations in handling and preprocessing of the generateddata. While many transformation and normalization procedures are easily implemented through available and widely used software packages such as Bioconductor (Gentleman et al., 2004), most procedures differ in the way they remove systemic variance and preparing datasets for downstream processing (Lim et al., 2007; Schmid et al., 2010).For example, batch effects and issues of antemortem conditions documented by medical records mustbe addressed viastatistical analyses (Johnson et al., 2007), but can be dealt with in a variety of ways.Therefore, the efficacy of these methods should be compared and assessed. This can be achieved by considering resulting tests of associations between the expression dataand other variables of interest using analysis of variance (ANOVA)-based techniques such as Multivariate Distance Matrix Regression (MDMR; Zapala and Schork 2006).

In this study, we investigated the utility of the DASL- and standard IVT-based genome-wide expression profiling assays and related data preparation and preprocessing techniques involving postmortem frozen and formalin-fixed brain tissue in the context of

a clinically importantneuropsychiatric disorder, autism. Our objective was to: 1) compare the quality of microarray data from DASL-based and IVT-based platforms on artificially degraded reference RNA; 2) compare the quality of microarray data from these two RNA preparation platforms on postmortem frozen and formalin-fixed brain tissue; and 3) investigate the potential of different transformation, normalization, and other preprocessing strategieson postmortem brain gene expression data.

Materials and Methods

Artificial Degradation of Reference RNA

Ambion® human reference brain RNA and Stratagene® reference pooled RNA samples were heated at 95°C for 0, 10, 30, and 60 minutes to artificially degrade them. Samples were analyzed using BioAnalyzer® (Agilent Technologies) to visualize levels of degradation (Supplementary Figure S1). Combinations of 75% brain reference RNA and 25% pooled reference RNA, and 75% pooled reference RNA and 25% brain reference RNA were also mixed for comparison with pure samples. Samples were then prepared for profiling on microarray platforms as described below.

Frozen and formalin-fixed postmortem human brain samples for gene expression profiling

57 frozen blocks of fresh frozen brain tissue and 4 blocks of formalin-fixed brain tissue from the prefrontal cortex of normal controls and autistic, male and female cases were obtained from the Harvard Brain and Tissue Resource Center (United States Public Health Service) and from the University of Miami/University ofMaryland Brain and Tissue Bank (National Institute of Child Health and Human Development; Supplementary Table S1). The formalin-fixed tissue was processed by different protocols, and samples were labeled by processing method 'M' or 'EC'.

All autistic cases met criteria for autistic disorder on the Autism Diagnostic Interview-Revised (Lord et al., 1994; ADI-R), the Autism Diagnostic Observation Schedule (Lord et al., 2000; ADOS), or were diagnosed by medical records (Supplementary Table S1). Seizure incidence of autistic cases was assessed by a clinical psychologist (CCB).

Brain sample collection

Due to documented variability of gene expression in neighboring brain areas (Rehen et al., 2005; Lein et al., 2007), care was taken to identify comparable regions between cases for expression profiling. Anatomical landmarks were identified as consistently as possible for dissection across cases with the goal of obtaining a set of highly controlled, comparable tissue for brain gene expression profiling. When available, tissue from the superior frontal gyrus of the dorsal lateral prefrontal cortex (DLPFC) was dissected in each case. When this area was not available, we sampled from the middle frontal gyrus. The formalin-fixed samples were obtained from larger areas of frontal cortex. Cytoarchitecture and anatomical landmarks were also used to determine the area of DLPFC similar to that of the frozen tissue for dissection.

RNA Extraction from Tissues

Extraction of total RNA from 5-10 mg of frozen tissue from both grey and white matter, with as many layers of cortex as possible, was performed using MELT® kit from Ambion according to manufacturer's instructions (www.ambion.com). Extraction of total RNA from 5-10 mg of formalin-fixed tissue sections was performed using the Roche® High Pure FFPE RNA Micro Kit. Since a visual distinction between grey and white matter could be clearly made in the 80 micron thick formalin-fixed sections sampled, some samples were exclusively taken from either the grey or white matter and designatedas such. Select RNA samples were analyzed with Bioanalyzer® (Agilent)

according to the manufacturer's protocol for quality control and quantification, and available RNA Integrity Numbers (RIN values) are reported in Supplementary Table S1. Whole RNA from remaining samples was quantified using a NanoDrop® spectrophotometer.

DASL Labeling, Hybridization, and Scanning

450 ng of total RNA from reference samples, frozen, and formalin-fixed cases was submitted to Illumina, Inc. (San Diego, CA). These samples underwentDNA annealing, selection, and ligation (DASL)-based labeling, hybridization to Illumina Human 12K and HumanRef8 v3 microarrays, and scanning on two separate occasions. Both biological and technical replicates were included for quality control. Using biotinylated random primers and oligo-dT, 200 ng RNA was converted to cDNA. The biotinylated cDNA was then immobilized to a streptavidin-coated solid support, and annealed. Following extension and ligation, the ligatedoligonucleotides were PCR amplified with biotinylated or fluorophore-labeled universal primers, and captured using streptavidin paramagnetic beads. Finally, the products were washed and denatured before hybridization to the BeadChips at 58˚C for 16 hours. A BeadArray Reader was used to extract images and read fluorescence intensities, and all data was uploaded into BeadStudio software without normalization or background subtraction for quality control and processing.

IVT Labeling, Hybridization, and Scanning

Gene expression profiling was also performed on RNA from reference samples, frozen, and fixed cases using the Illumina Human Ref-8 v3 Expression BeadChip platform (Illumina Inc., San Diego, CA, USAaccording to manufacturer's protocols. Following RNA extraction, an IVT reaction for biotinylatedcRNA was performed overnight (~16 h). 750 ngcRNA were hybridized on the beadchip at 58°C overnight and detected with Cyanine3-streptavidin. Arrays were again scanned with the IlluminaBeadArray Reader and read into IlluminaBeadStudio software without normalization or background subtraction.

Exclusion Criteria for DASL-processed frozen samples

Only 4 autistic and 4 control female cases passed all quality control checks and had high quality microarray results. 5 female cases were excluded following quality control procedures according to criteria outlined below. Due the large discrepancy in ages between useable autistic and control female cases, however, these cases were excluded from final expression profiling analyses. 11 of the remaining male cases were excluded due to poor quality gene expression hybridization based on quality control using visualizations from the *lumi*package in Bioconductor (Du et al., 2008).

Several criteria were considered to identify possible outliers. Samples were eliminated due to low median distribution amplitudes and because the range or interquartile range of intensity values were visually different from the remainder of the arrays. Samples were also eliminated due to poor correlations with other samples: all samples with average clustering more disparate than the distance between the two batches were removed. We confirmed these choices with analysis of pairwise correlations between biological and technical replicates using several visualization techniques. Further, according to outlier removal procedures from Oldham et al (2008), the mean normalized inter-array correlation (IAC) of the remaining borderline outlying samples was maximized (Oldham et al., 2008). Finally, we performed all preprocessing steps, differential expression, and enrichment analysis including and excluding borderline outliers to ensure that inclusion of single cases could not account for the reported results. The final dataset consists of high quality arrays of 33 male ASD and control cases. All outliers were removed before transformation, normalization, and batch correction procedures.

Data Preprocessing

For data pre-processing and normalization, we aimed to identify a workflow that would: 1) Maximize mean interarray correlation across the dataset (Oldham et al., 2008);

2) Remove known confounds from the dataset; and 3)Prepare the data for downstream processing (e.g. differential expression and enrichment analysis).

Average clustering with Euclidean distances, scatterplots, distribution histograms, correlation measures, and boxplots were used to visualize the data before and after processing steps. For details on the implementation of the following transformation and normalization techniques, see and Du et al., (2007; 2008). Mean inter-array correlations (IAC; Oldham et al., 2008) were used as a basis to identify reasonable processing candidates at each step for further investigation.

Transformation Procedures.

Transformation methods involving the log2, variance stabilizing transformation (VST; Lin et al., 2008), and cubic root were implemented using the *lumi*package (Du et al., 2007; 2008) before data normalization.

Normalization Procedures.

Robust spline normalization (RSN), simple scaling normalization (SSN), Quantile normalization, Variance Stabilizing Normalization (VSN), Loess, and Rank Invariant (RI) normalization methods were tested in conjunction with the above transformation procedures (Du et al., 2008; Lin et al., 2008).

Batch Correction Procedures.

The software suiteCombat (Johnson et al., 2007) was used to remove the variance attributed to batch effect, since our frozen samples were processed at different time points on the DASL platform. In addition, we also attempted to remove the confounding effects of seizures in our dataset, since many individuals with autism have comorbid seizure incidence. Multivariate Distance Matrix Regression was used to assess the efficacy of the correction methods as described below.

Multivariate Distance Matrix Regression.

To assessthe variance within the dataset attributable to a set of variables before and after manipulating and pre-processing the expression assay results (e.g. batch correction), multivariate distance matrix regression (MDMR; Zapala and Schork, 2006) with 10000 permutations was applied to the Euclidean distance matrices constructed from the expression values between each sample (http://polymorphism.scripps.edu/~cabney/cgi-bin/mmr.cgi). Variables of interest that were related to the expression profiles reflected in the distance matrices included batch, RNA source (reference RNA, frozen tissue, formalin-fixed tissue), assay type (DASL or IVT), diagnosis, age, and seizure incidence of cases from which we sampled. We leveraged both single independent variable and multiple independent MDMR results.

Independent qPCR Validation of Microarray Results

Genes and Cases.

RNA from 1 male autistic and 1 male control case of 31 years were analyzed using SYBR green RT-PCR to validate the intensity values detected by microarray. 19 genes were chosen with a wide range of fold change values (positive and negative), and are listed in Supplementary Table S3. Using Primer3 software, (Rozen et al., 2003) primers of these genes were designed across splice junctions to avoid artifacts by DNA contamination and to produce amplicons of ~200 bp. RPL13A, B2M and ACTB, three genes highly expressed in the brain at stable levels (Vandesompele et al., 2002) were chosen as reference genes for each experiment. Expression values for the remainder of the genes were normalized to these reference gene controls.

cDNA Synthesis and qPCR.

One microgram of total RNA was used for cDNA synthesis using random hexamers and AMV reverse transcriptase. An equivalent of 50 ng of RNA was processed by qPCR using Roche's LightCycler rapid thermal cycler system (Roche Diagnostics Ltd, Lewes, UK) according to the manufacturer's instructions, in a 96-well, 10-uL format using standard PCR conditions. 1 μL of cDNA template, 250 nM of forward and reverse primer, and 5 μL ofqPCR Master Mix(Roche) were mixed for each reaction.

Statistical analysis.

According to Vandesompele et al (2002), we took the geometric mean of all reference genes and the difference between this mean and the average intensity of experimental genes to find the delta Ct for each experimental gene. Subsequently, log2 fold change was assessed using -(T-C) where T = delta Ct of gene of the autistic case, and C = delta Ct of gene of the control case. Spearman's rank correlation was then applied to the results from the qPCR and microarray assays.

Results

DASL-based and IVT-based platforms on artificially degraded reference RNA.

Based on the strengths of the correlations between log2 expression fold changes across increasingly degraded brain reference RNA samples (Supplementary Figure S1), we found that the DASL-based approach yielded more reliable microarray data than the IVT-based approach. The intensity values for calculating the correlations in this initial analysis were not normalized. Correlation between the fold change of the brain reference and pooled reference RNA heated at 95 degrees for 10-minutes ($\log_2$(B10/P10)) using the DASL approach yielded an R^2 of 0.826 (Figure 1A). By comparison, that of the IVT

approach yielded an R^2 of 0.717. The differences inreliability between the two platforms were more disparate at 30 and 60 minutes of artificial degradation (Figure 1A). At 30 minutes, the correlation between the fold changes for the DASL-approach dropped to R^2=0.558, while that of the IVT-approach dropped to R^2=0.154. At 60 minutes, the correlation between the fold changes for the DASL-approach dropped to R^2=0.272, while that of the IVT-approach dropped to R^2=0.039.

We also directly correlated the intensity data of the intact RNA with that of the subsequent degradation experiments on the two assays (Figure 1B, and Supplementary Figure S2). Partially degraded RNA assayed by the DASL-based approach had higher correlation to intact RNA of the same category (brain or pooled) than IVT. For example, the Pearson's correlation between intact brain reference RNA (B0) and brain reference RNA heated for 60 minutes (B60) using the DASL-based approach was 0.706, in comparison to 0.561 for the IVT-based approach. Despite these observations, the correlation between intact RNA to different combinations (i.e., those involving B25P75 and B75P25) of RNA was comparable in the two platforms (Figure 1B).In general, the IVT-based assay was affected more severely than the DASL-based assay with increasing RNA degradation (Supplementary Figures S3, S4, and S5). Correlations within the same sample processed by the two different assays were low at baseline and decreased with RNA degradation (Supplementary Figure S6).

DASL-based and IVT-based platforms on postmortem frozen and formalin-fixed brain tissue.

Although we could not control degradation levels of the tissue-extracted RNA, we repeated the above comparison between IVT- and DASL-based approaches on postmortem frozen and formalin-fixed brain tissue. While samples processed with the same platform (IVT or DASL) and tissue preservation method (frozen or formalin-fixed) tended to correlate, the distance between samples with the same preservation method across assay types was variable (Figure 2A), suggesting that the assays and preservation method impact expression level values. The fixed tissue, regardless of assay type, clustered together. Frozen tissue profiled by the DASL-based approach and the IVT-based approach, however, clustered separately. MDMR analysis (Figure 2B) showed that tissue preservation method accounted for 66% of the variance in the distance between expression profiles across the samples, followed, in degree of percentage variation explained, by assay type (IVT or DASL) which accounted for 15%, batch accounting for 2%, diagnosis which accounted for 0.3%, gender which accounted for 0.3%, and age of the case which accounted for 0.3% of the variance.

One sample,UMB 1796, with both fixed and frozen tissue samplesavailable in DLPFC of contralateral brain hemispheres,was tested using IVT- and DASL-based platforms (Supplementary Figure S7). Correlations between assay types and preservation methods were low: using DASL, the correlation of resulting gene expression levels between frozen and fixed tissue was 0.44. It appeared that the correlation between

frozen and fixed tissue using the IVT-based assay was higher than that of the DASL-based assay (0.74). However, distribution plots in Supplementary Figure S7 show that the range of intensity values is severely truncated for both frozen and fixed samples using the IVT-based approach. In general, correlations of the same case on two platforms even after within-assay normalization were low (Supplementary Figure S8).

Fixed tissue-extracted RNA from 2 of 4 (50%) of cases was potentially useable on the DASL-based platform (Supplementary Figure S9), but 0 of 4 (0%) were useable on the IVT-based platform (Supplementary Figures S10). Frozen tissue-extracted RNA from 41 of 57 (72%) cases passed quality control measures outlined in section 2.7 on the DASL-based platform. In comparison, frozen tissue-extracted RNA from 6 of 9 (67%) cases passed quality control measures on the IVT-based platform. 1 of the 3 frozen samples that the IVT platform failed to sufficiently assay was successfully recovered by DASL. Correlations of technical replicates and between samples in these frozen tissue-DASL-based assays were comparable with those of IVT-based assays (Supplementary Figure S11).

RIN value was the primary factor that explained variability in the gene expression distances between the samples with available RNA integrity numbers and both DASL- and IVT- based processing (N=4). However, the influence of RIN on the IVT-processed samples (Percentage of variance explained (PVE) = 0.8245, p=0.21) was greater than that on the DASL-processed samples (PVE = 0.6452, p=0.1178).

Transformation, normalization, and other preprocessing techniques on high quality DASL frozen tissue-RNA microarray data.

We systematically tested data preprocessing methods on the frozen tissue-extracted RNA samples from 33 male autistic and control cases (Supplementary Figure S12). From the 57 originally assayed samples, outliers were removed according to the criteria outlined in Methods. We chose not to include the DASL-based fixed tissue profiling, IVT-based frozen and IVT-based fixed tissue profiling results in this analysis due to small sample size.

We carefully examined the effect of different transformation and normalization method combinations on our dataset in a pipeline depicted in Figure 3A. Log2 transformation of the immediate resulting assay data was chosen for further investigation based on convention, and VST transformation was chosen based on high mean IAC. RSN and Quantile normalization for each of these transformation methodsyielded the highest mean IACs and so were further investigated. Slight differences in data distribution were evident between these four techniques (Figure 3B, 3C, and 3D; Supplementary Figure S13). These differences are important due to assumptions of normality that must be met for downstream analyses (Giles and Kipling, 2003; Zapala and Schork 2006; Johnson et al., 2007).

Qualitatively, Log2-transformed datasets showed a more normal distribution, while VST-transformed datasets showed a distribution skewed to lower intensity values. Correlations between arrays were slightly higher in the log2/quantile combination, but more genes were detected as differentially up/down regulated between technical replicates. Based on this combination of factors, including the intensity distribution and inter array correlations, we chose the log2/quantile method for our DASL brain gene expression dataset to assay expression differences in autistic and control brains (Chow, Pramparo, Boyle et al. *in review*).

In addition to standard transformation and normalization procedures, it was necessary to consider batch and variable correction procedures because the frozen tissue samples were processed in two separate batches. This created substantial batch effects (Supplementary Figure S12A and S12B). Furthermore, since epileptiform abnormalities are present in as many as 60% of children with autism (Spence and Schneider 2009), it was important to account for the variance attributable to seizures noted by medical records in cases assayed (Supplementary Table S1).

Batch correction and adjustment for seizures as a covariate was performed using ComBat, which applies an empirical Bayes method (Johnson et al., 2007) for the four pipeline pathways. MDMR and mean IAC were used to gauge the effectiveness of this stage of processing (Supplementary TableS2) by determining if batch explained differences in gene expression profile similarity across these before and after batch

correction. Batch correction by ComBat decreased the percentage of variation that could be attributable to batch to less than 1% in each of the four pipeline pathways. Scatterplots and IAC calculations indeed showed higher correlation between samples in two separate batches after correction (Supplemental Table S2 andFigure S14). The effect of seizures was also similarly decreased after correction. This suggests that the use of Combat and appropriate preprocessing can effectively eliminate the potential for artifactual associations between gene expression levels and important covariates.

Validation of microarray results by qPCR.

We compared the log2 fold changes of the qPCR data with the log2 transformed, quantile normalized data. Using a Spearman's rank correlation, the log2 fold changes of these 19 genes across qPCR and microarray platforms were found to be correlated at R=0.78 (p=0.000075, DF=17; Supplementary Figure S15).

Discussion

We have shown that IVT- and DASL-based genome-wide expression-profiling approaches applied to brain-specific reference RNA and postmortem brain tissue produce different results, raising questions about the reliability of the assays and the need for methods to account for potential confounding factors that could impact interpretation of

association analyses involving gene expression levels. Not surprisingly, the greater the degradation of reference RNA, the less reliable results from both platforms become. Therefore, although the DASL-approach appeared to recover more reliable gene expression values from partially degraded RNA samples, reliable results may still not be gleaned from the most severely degraded RNA samples. The RNA extracted from fixed brain tissue samples we assayed may be past this threshold of utility, as few samples passed quality control measures using either platform. Unfortunately, our sample sizes were too small to make conclusive arguments about the relative efficacy of DASL- and IVT-based platforms on RNA extracted from tissue.

Through the examination of the expression datasets in the tissue-based RNA samples, we explored important factors to consider in expression profiling of frozen and fixed postmortem brain tissue. The most important explanatory variableof gene expression profile variance across samples in ourdataset was in fact not the platform on which the RNA was assayed, but how the tissue was preserved and the quality of tissue preservation. This result has important implications for brain banks in the methods used for preserving tissue (Kretzschmar 2009) and for comparing across-brain gene expression studies, especially in neuropsychiatric disorders with a spectrum of phenotypes likeautism (Mirnics and Pevsner 2004).

In order to assess the effect of case-specific factors such as diagnosis, gender, and age, it is necessary to remove the systematic variance introduced by experimental and quantifiable data handling factors. Furthermore, if disease-specific mechanisms are to be uncovered, the effect of medications, lifestyle, comorbid conditions, and other confounding variables on brain gene expression must be elucidated (Horvath et al., 2010). Additionally, our assessment of the RIN as a predictor of dataset variance was not consistent with that found in Abramovitz et al. (2008). Though the number of samples that we tested in this context was extremely small (n=4), RIN accounted for a large percentage of variance in the dataset. Two of the samples with RINs below 3 were excluded as outliers from the final dataset of 33 male autistic and control cases; the remaining two had RINs of 4.9 and 6.3.

We also found that not all bioinformatics and biostatistical pre- and post-processing techniques will generate reliable results from brain gene expression datasets, in accordance with previous reports (Lim et al., 2007; Schmid et al., 2010). The transformation methods resulting in the highest inter-array gene expression profile correlations and that are historically used for expression microarray preprocessing all yielded varying results. Our decision to transform and normalize our dataset by the log2/quantile method, followed by batch and covariate correction, were based on careful consideration of the downstream statistics that would be applied on our dataset, as detailed in Chow, Pramparo, Boyle et al. (*in review)*. Although the choice of

preprocessing techniques remains an art, we hope to have brought up issues and a procedure worth considering for brain gene expression microarray studies.

We have shown that the DASL-based genome-wide expression microarray assaymay be a solution to some of the challenges of postmortem human brain research. We also propose methods to prepare the microarray data for downstream analyses. In addition to basic preprocessing steps, correction for covariates may be especially important for disorders like autism, where patients have many comorbid conditions. These methods will be vital in helping make use of scarce and precious brain tissue to elucidate uniquely human genetic pathogenic mechanisms.

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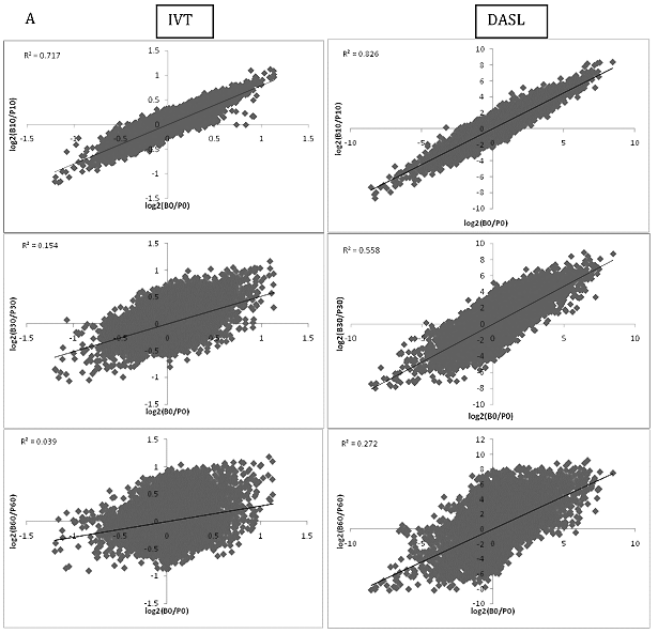
A



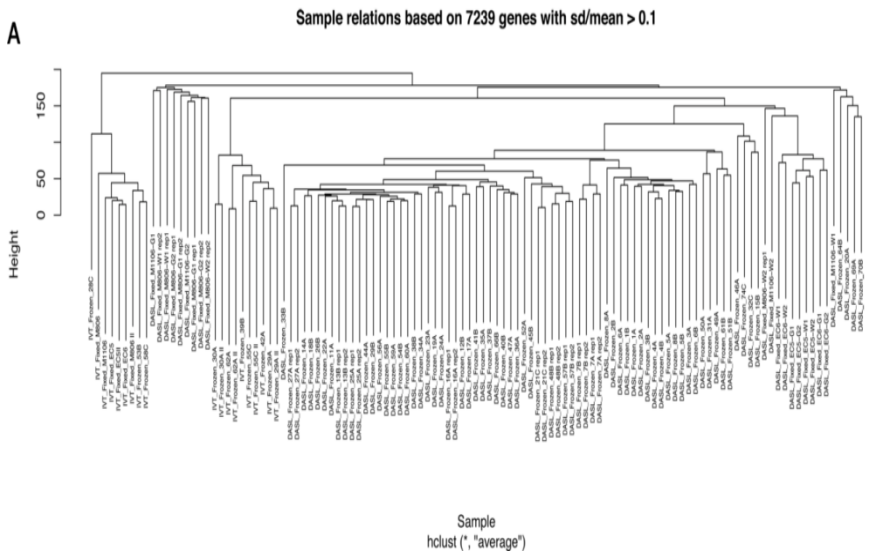
B

	IVT		DASL	
	Correlation B0	Correlation P0	Correlation B0	Correlation P0
B0	1	0.79214	1	0.74079338
B10	0.939746	0.750981	0.96454523	0.72067615
B30	0.802383	0.635742	0.84377519	0.62910865
B60	0.560765	0.439058	0.70603451	0.51851733
P0	0.79214	1	0.74079338	1
P10	0.723885	0.867639	0.67402006	0.89181599
P30	0.453686	0.512249	0.46300584	0.63873448
P60	0.239121	0.246651	0.28860867	0.45960031
B25P75	0.902288	0.964832	0.87821646	0.95620062
B75P25	0.976753	0.879765	0.97542304	0.84591328

Ch2 Figure 1 -- Comparison of correlations (R^2) between log2(fold changes) of intact and artificially degraded reference RNA using IVT and DASL-based approaches and Pearson's correlations between unnormalized reference RNA samples, probes detected (p<0.05), and percentage of genes detected (p<0.05) using IVT and DASL-based approaches.

(A) Log2(fold change) of detected intensity values between intactbrain and pooled reference RNA samples were correlated with increasingly degraded brain and pooled reference RNA samples.B, brain reference RNA; P, pooled reference RNA; 0, intact; 10, heated at 95C for 10 min; 30, heated at 95C for 30 min; 60, heated at 95C for 60 min. (B) Table listing Pearson's R between genes assayed using IVT- and DASL-based approaches in increasing degradation levels of reference RNA samples, and probes and % genes detected by each assay at each degradation level. IVT- and DASL- based assays were processed on 12K and 24K chips, respectively. B, brain reference RNA; P, pooled referenceRNA; 0, intact; 10, heated at 95C for 10 min; 30, heated at 95C for 30 min; 60, heated at 95C for 60 min.

A

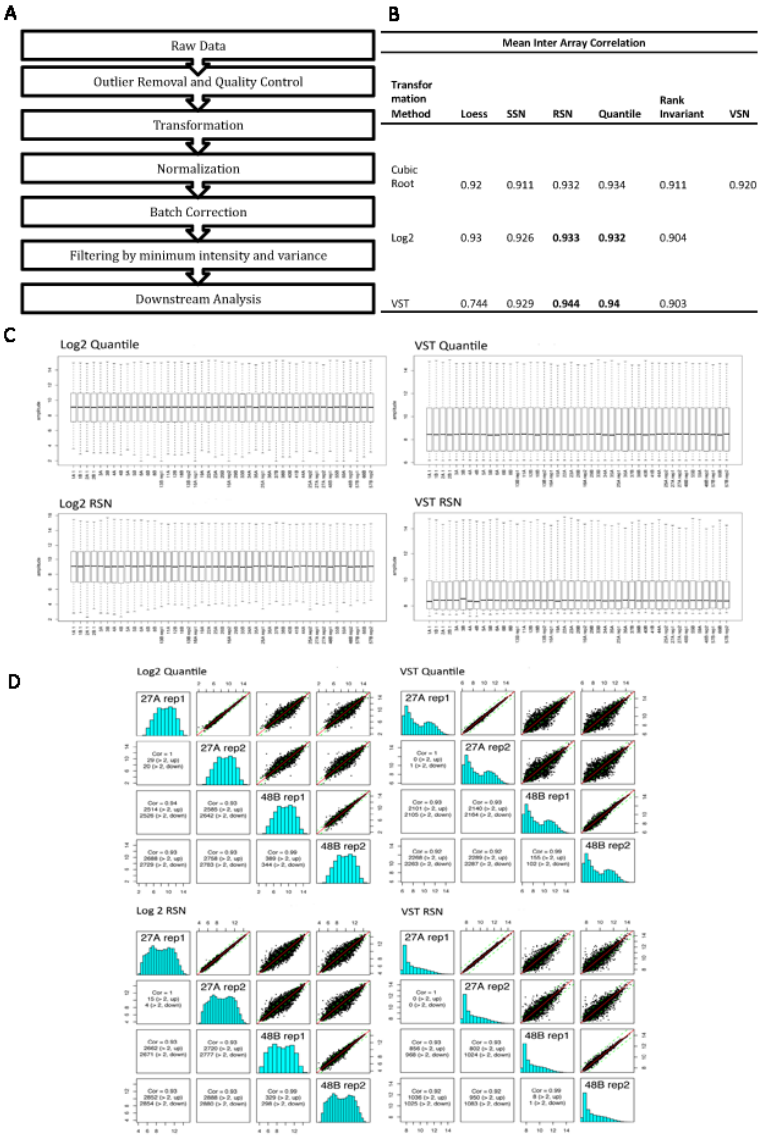


B

Single Regression	F statistic	PVE	p-Value
Assay: IVT/DASL	23.20696	0.188358	0
Source: Frozen/Fixed	198.1341	0.66458	0
Batch	1.381838	0.01363	0.227
Diagnosis	3.607305	0.034817	0.05
Age	0.35639	0.003551	0.623
Gender	6.040014	0.05696	0.006
Multiple Regression			
Source: Frozen/Fixed	198.1341	0.66458	0
Assay: IVT/DASL	77.18393	0.811523	0
Batch	11.49014	0.831303	0
Diagnosis	2.060068	0.834811	0.09
Gender	1.854291	0.837941	0.092
Age	1.67718	0.840753	0.113

Ch2 Figure 2-- Cluster dendrogram of microarray intensity data of tissue-extracted RNA samples using IVT- and DASL-based approaches and Variables contributing to variance within unnormalized tissue-only dataset.

(A)Dendrogram of average clustering based on Euclidean distance between tissue-extracted RNA samples. Each sample is labeled according to IVT/DASL-based approach, and frozen/fixed method of tissue preservation. Labels indicate assay type (DASL/IVT), preservation method (Frozen/Fixed), sample code, and replicate number (if applicable). (B) Contribution of each listed variable calculated by MDMR analysis (Zapala et al., 2006) to the variance in the dataset, F-statistic, percentage variance, cumulative variance explained, and p-value are presented. Distance matrices were calculated using Euclidean distance. PVE, percentage of variance explained.

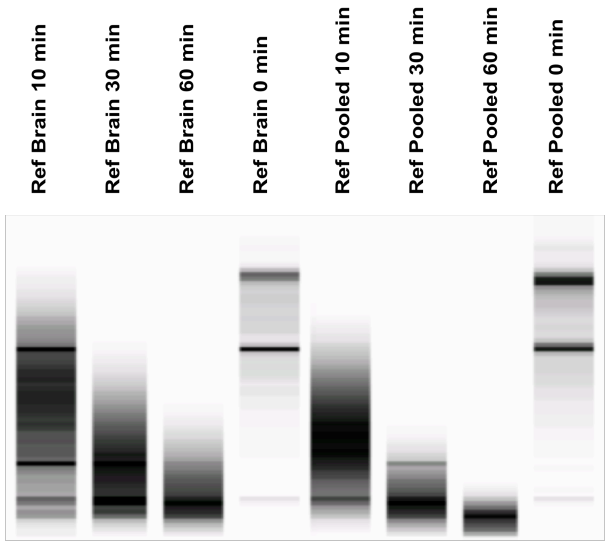


Ch2 Figure 3 -- Preprocessing procedureand Mean inter array correlation for 33 frozen brain tissue samples assessed by DASL before batch correction, and boxplotsand scatterplotsof four highlighted transformation and normalization methods.

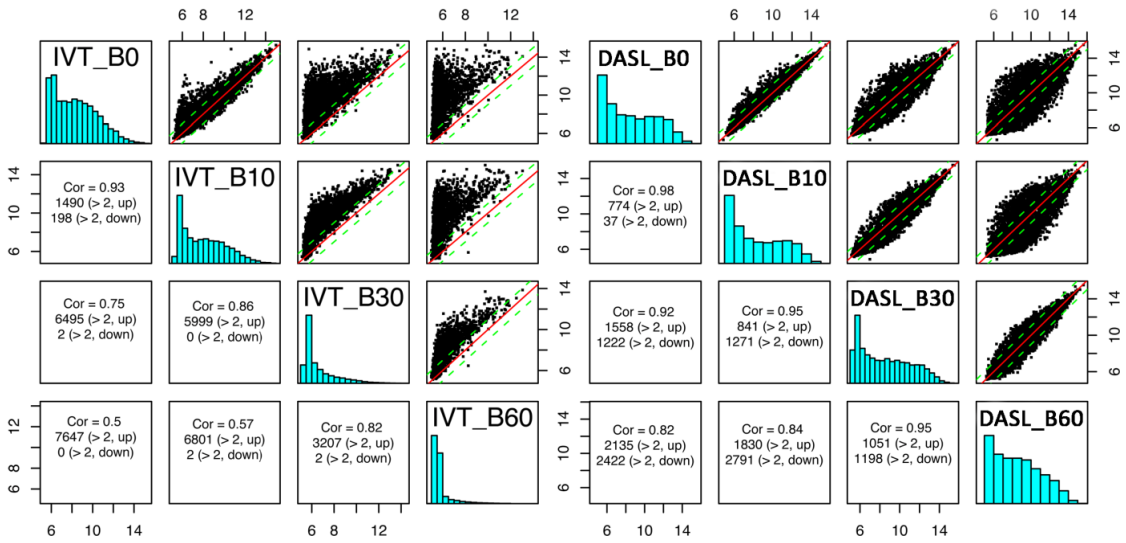
(A) Flowchart depicting preprocessing steps taken for microarray data generated by DASL-based profiling of frozen tissue samples in Chow,Pramparo, Boyle et al., (*in review*). Downstream analyses include differential expression, coexpression, and gene set enrichment analyses. (B)The mean inter array correlation for the different combinations of transformation and normalization techniques implemented in lumi (Du et al., 2008) before batch correction of the 33 frozen brain tissue samples. Columns denote six normalization methods (SSN, RSN, Rank Invariant, Loess, Quantile, and VSN); rows denote three transformation methods (Cubic Root, Log2, and VST). VSN was not applied with any transformation method. (C) Boxplots of four highlighted transformation and normalization methods (Log2 with quantile; log2 with RSN; VST with quantile; VST with RSN). X-axis, sample name; Y-axis, normalized and transformed intensity amplitude. (D) Scatterplots of 2 samples and respective replicates, histogram of data distribution, correlation, and number of up and downregulated genes differing between comparison samples using each of 4 highlighted transformation and normalization methods. 27 and 48 are two postmortem frozen samples. Cor = correlation;Rep = replicate number

Supplementary Figures

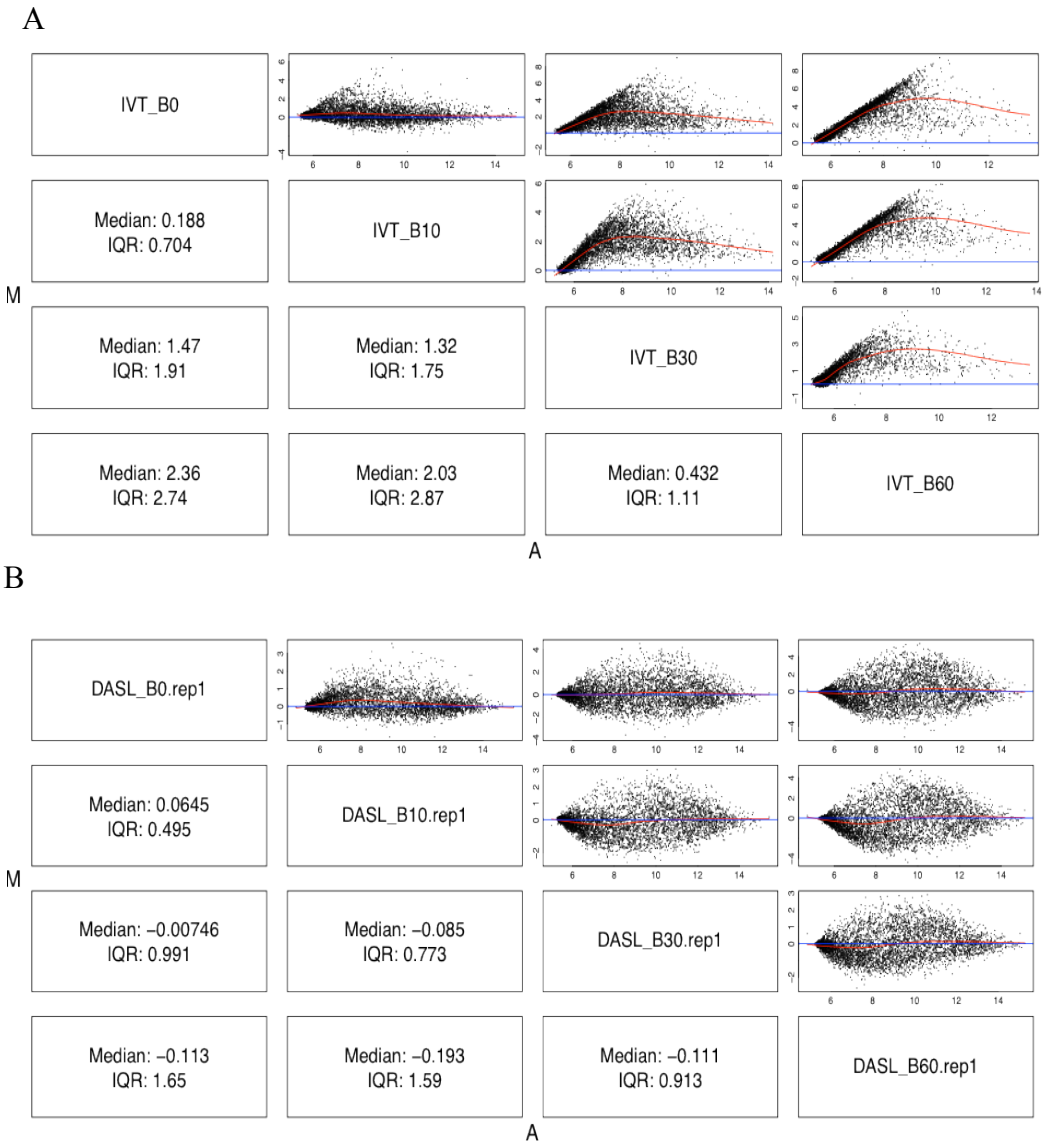
1. DASL-based and IVT-based platforms on artificially degraded reference RNA



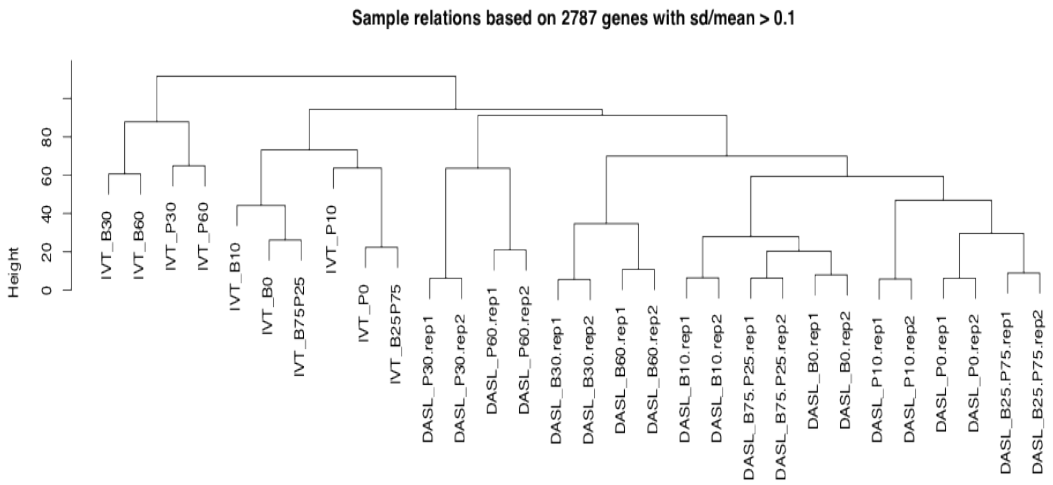
Ch2 Figure S 1-- Artificial degradation of brain and pooled reference RNA.
Visualization and RINs of varying levels of brain and pooled reference RNA degradation correlating with duration of heating at 95°C by BioAnalyzer®.



Ch2 Figure S 2-- Comparison of IVT- and DASL-based platforms on reference brain RNA.
Scatterplots of reference RNA samples with increasing levels of artificial degradation, histogram of data distribution, correlation, and number of up- and downregulated genes differing between samples in IVT- (left) and DASL- (right) based platforms. B0 = intact reference brain RNA; B10 = 10 minute heating at 95°C; B30 = 30 minute heating at 95°C; B60 = 60 minute heating at 95°C.

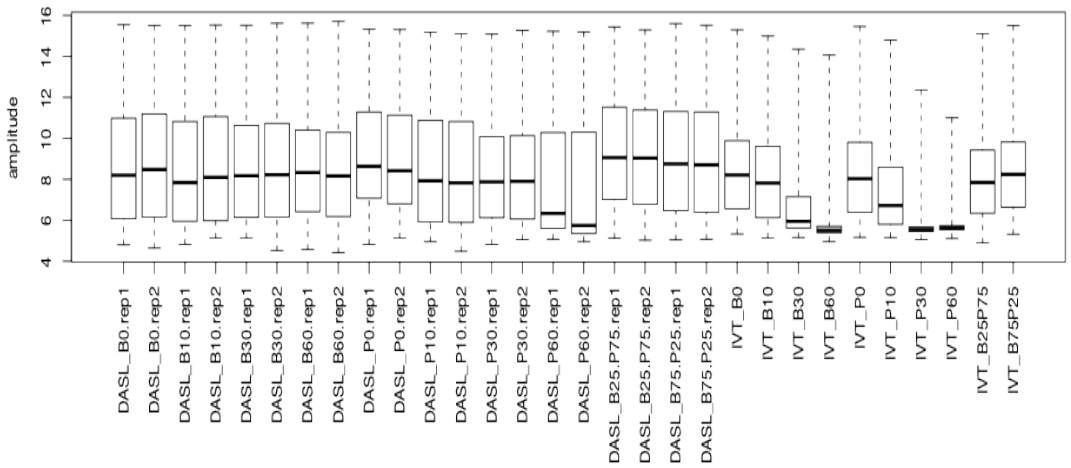


Ch2 Figure S 3-- MA plots comparing IVT- and DASL-based platforms on reference brain RNA. Pairwise MA plots showing differences between medians and interquartile ranges of intact and artificially degraded brain RNA samples on (A) IVT- and (B) DASL- based platforms. IQR = interquartile; B0 = intact reference brain RNA; B10 = 10 minute heating at 95°C; B30 = 30 minute heating at 95°C; B60 = 60 minute heating at 95°C.



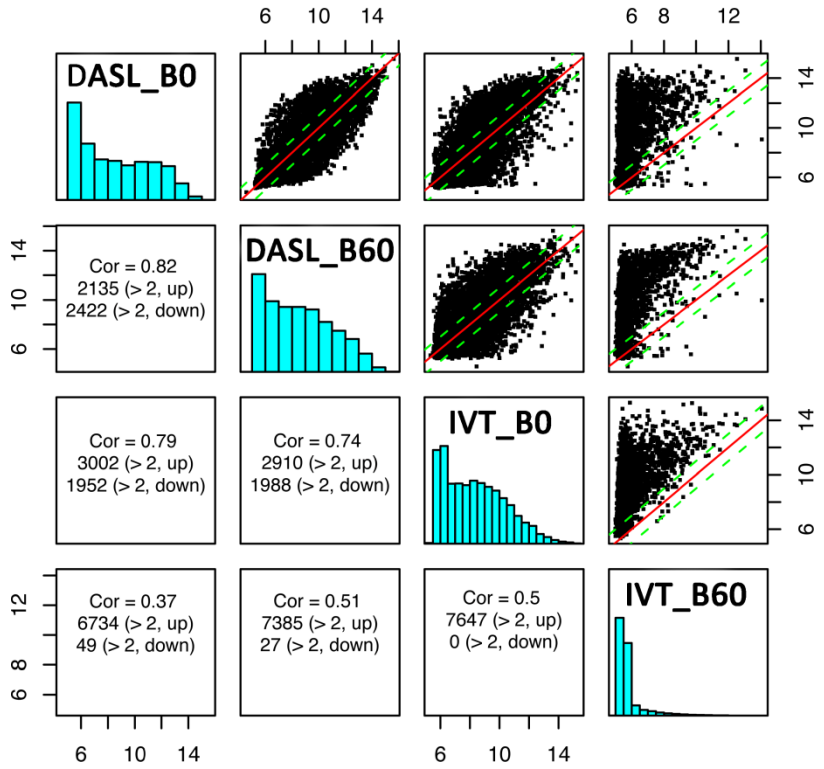
Ch2 Figure S 4-- Average clustering between intact and artificially degraded reference RNA samples on IVT- and DASL-based assays.

Average hierarchical clustering was applied to the Euclidean distance between intact and artificially degraded brain and pooled reference RNA samples. Labels indicate assay type and time of degradation. B0 = intact reference brain RNA; B10 = 10 minute heating at 95°C; B30 = 30 minute heating at 95°C; B60 = 60 minute heating at 95°C; P0 = intact reference pooled RNA; P10 = 10 minute heating at 95°C; P30 = 30 minute heating at 95°C; P60 = 60 minute heating at 95°C; B75P25 = 75% brain reference RNA and 25% pooled reference RNA combination; B25P75 = 25% brain reference RNA and 75% pooled reference RNA combination; rep = technical replicate.

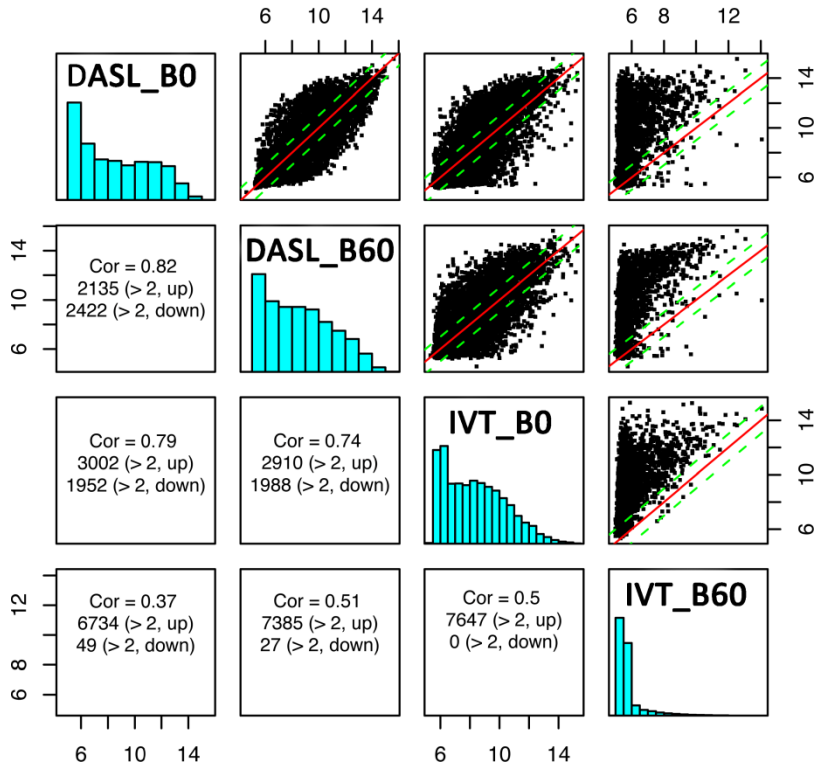


Ch2 Figure S 5- Intensity distributions comparing IVT- and DASL-based platforms on reference brain RNA.

Boxplot showing the amplitude of intensity distribution for intact and artificially degraded reference brain and pooled RNA samples. B0 = intact reference brain RNA; B10 = 10 minute heating at 95°C; B30 = 30 minute heating at 95°C; B60 = 60 minute heating at 95°C; P0 = intact reference pooled RNA; P10 = 10 minute heating at 95°C; P30 = 30 minute heating at 95°C; P60 = 60 minute heating at 95°C; B75P25 = 75% brain reference RNA and 25% pooled reference RNA combination; B25P75 = 25% brain reference RNA and 75% pooled reference RNA combination; rep = technical replicate.



Ch2 Figure S 6-- Correlation of brain reference RNA results across IVT- and DASL-based assays. Direct comparisons between intact brain reference RNA and brain reference RNA degraded for 60 minutes at 95°C on IVT- and DASL-based assays. Scatterplots of reference RNA samples with increasing levels of artificial degradation, histogram of data distribution, correlation, and number of up- and downregulated genes differing between samples are shown. B0 = intact reference brain RNA; B60 = 60 minute heating at 95°C.



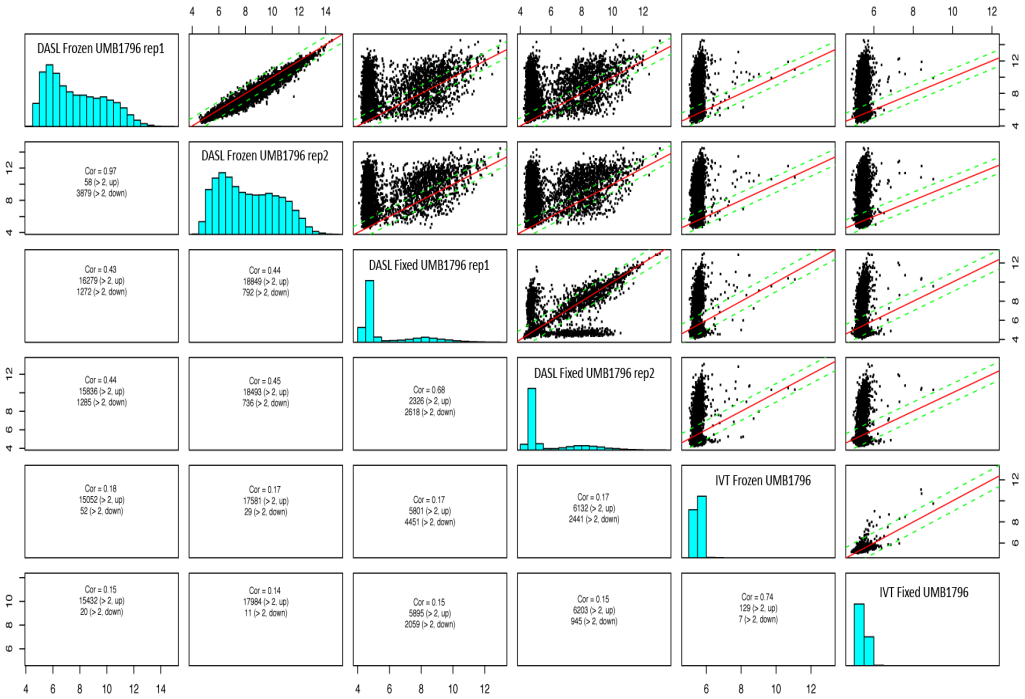
Ch2 Figure S 7-- Correlation of brain reference RNA results across IVT- and DASL-based assays. Direct comparisons between intact brain reference RNA and brain reference RNA degraded for 60 minutes at 95°C on IVT- and DASL-based assays. Scatterplots of reference RNA samples with increasing levels of artificial degradation, histogram of data distribution, correlation, and number of up- and downregulated genes differing between samples are shown. B0 = intact reference brain RNA; B60 = 60 minute heating at 95°C.

2. DASL-based and IVT-based platforms on postmortem frozen and formalin-fixed brain tissue

Ch2 Table S 1– Frozen and formalin fixed tissue samples assayed.
Case ID, diagnosis, age, gender, cause of death (COD), postmortem interval (PMI), RNA Integrity Number (RIN), and preservation method of the postmortem human brain samples assayed in this study are listed.

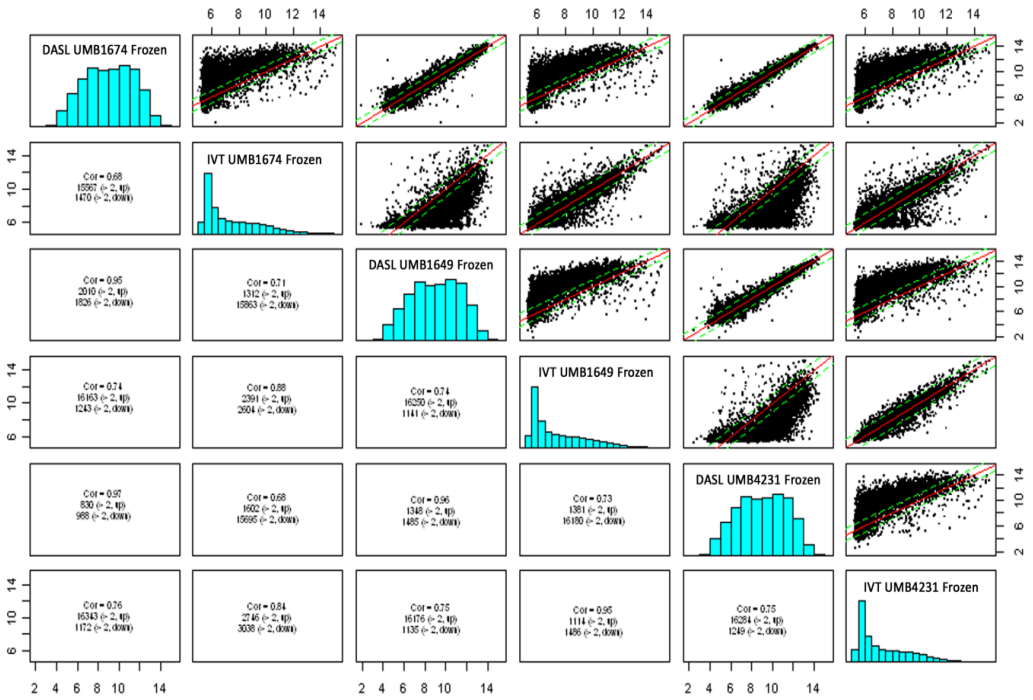
Case ID	Diagnosis	Age	Sex	COD	PMI	RIN	Frozen/Fixed
B6399	Autism	2	M	Drowning	4	7.6	Frozen
UMB4671	Autism	4	F	Accident, multiple injuries	13	8.6	Frozen
B1469	Autism	5	M	Unknown	42.8	2.3	Frozen
B5569	Autism	5	M	Asphyxia due To Drowning	25.5	2.5	Frozen
UMB1349	Autism	5	M	Drowning	39	8.7	Frozen
UMB1174	Autism	7	F	Seizure, Hypotension	14	-	Frozen
UMB4849	Autism	7	M	Drowning	20	6.3	Frozen
B5666	Autism	8	M	Sarcoma	22.2	7.8	Frozen
UMB4231	Autism	8	M	Drowning	12	7.4	Frozen
UMB4721	Autism	8	M	Drowning	16	8.5	Frozen
UMB1182	Autism	9	F	Smoke Inhalation	24	-	Frozen
B4925	Autism	9	M	Seizure Disorder	27	2.2	Frozen
UMB797	Autism	9	M	Drowning	13	6.9	Frozen
UMB4899	Autism	14	M	Drowning	9	8.5	Frozen
B7079	Autism	15	M	Asphyxia	23	5.7	Frozen
B5223 (M1106)	Autism	16	M	Stopped Breathing	47.9	-	Fixed
B6184	Autism	18	F	Seizures	7	3.6	Frozen
B5144	Autism	20	M	Auto Trauma	23.7	-	Frozen
B6337	Autism	22	M	Aspirated on vomit/Seizure	25	-	Frozen
B5000	Autism	27	M	Drowning	8.3	-	Frozen
B6994	Autism	28	M	Seizures	43.25	3	Frozen
B6640	Autism	29	F	Seizures	17.83	-	Frozen
B5173	Autism	30	M	GastroIntestinal Bleeding, seizures	20.3	-	Frozen
B6677	Autism	30	M	Congestive heart failure	16	-	Frozen
B6401	Autism	39	M	Cardiac Tamponade	14	2.3	Frozen
UMB1445	Autism	45	M	Complications of ALS/Autism	23	-	Frozen
B7085	Autism	49	F	Colorectal cancer spread through abdomen	21	3.4	Frozen
B7109	Autism	51	M	Myocardial infarction	22	4	Frozen
B4498	Autism	56	M	Anoxic Encephalopathy	20	-	Frozen
B6736	Control	4	F	Acute bronchipneumonia after tonsillectomy	17	6.3	Frozen
UMB1499	Control	4	F	Lymphocytic Myocarditis	21	6.5	Frozen
UMB1185	Control	4	M	Drowning	17	6.8	Frozen
UMB4670	Control	4	M	CommotioCordis	17	7.9	Frozen
UMB1377	Control	6	F	Drowning	20	-	Frozen
UMB1500	Control	6	M	Multiple Injuries	19	1.8	Frozen
UMB4898	Control	7	M	Drowning	12	7.7	Frozen
UMB1674	Control	8	M	Drowning	36	-	Frozen
UMB1860	Control	8	M	Cardiac arrythmia	5	7.5	Frozen

Table S1, Continued							
UMB1407	Control	9	F	Seizure, Asthma	20	7.6	Frozen
UMB1650	Control	10	M	Sudden Unexpected Death	24	2.1	Frozen
UMB1714	Control	12	M	Cardiac arrythmia	22	7.7	Frozen
UMB4787	Control	12	M	Asthma	15	6.5	Frozen
UMB-1670 (M806)	Control	13	M	Asphyxia By Hanging	5	-	Fixed
UMB4722	Control	14	M	MVA Multiple Injuries	16	7.7	Frozen
UMB4638	Control	15	F	Chest Injuries	5	8.8	Frozen
B6207	Control	16	M	Heart attack/disease	26.2	-	Frozen
B6756	Control	16	M	Myocardial infarction	22	-	Frozen
UMB1796 (EC6)	Control	16	M	Multiple Injuries	16	-	Frozen and Fixed
B5251	Control	19	M	Pneumonia/resp. infection	19	3.5	Frozen
UMB1649	Control	20	M	Multiple Injuries	22	4.9	Frozen
BTB3960	Control	25	F	Gunshot to the Chest	26	-	Frozen
UMB818	Control	27	M	Multiple Injuries	10	1.9	Frozen
B5873	Control	28	M	Unknown	23.3	-	Frozen
B5334	Control	30	M	Asphyxia	14.83	5.7	Frozen
B5352	Control	31	M	Asphyxia	33	3.8	Frozen
B5813	Control	41	M	Unknown	27	5.2	Frozen
BTB-3859 (EC5)	Control	44	M	Unknown	30	-	Fixed
B6208	Control	50	F	Heart attack/disease	20	-	Frozen
B4756	Control	56	M	Myocardial infarction	23	5.9	Frozen
B6860	Control	56	M	Unknown	22	6.3	Frozen



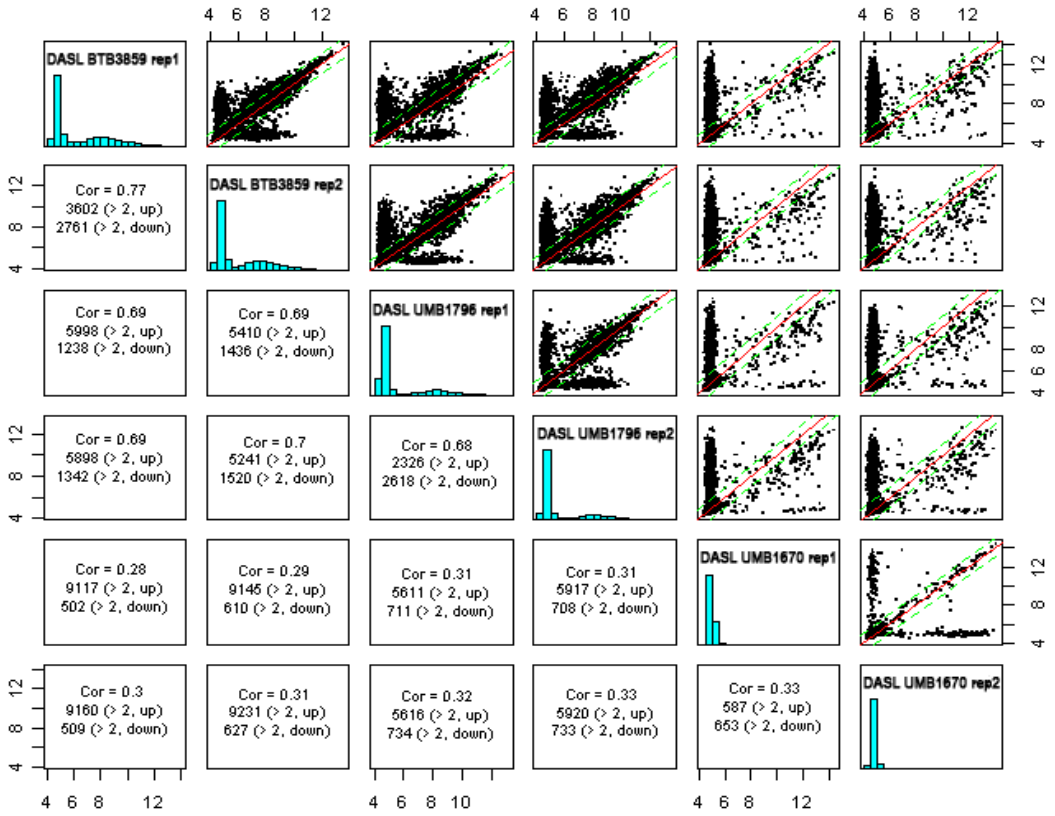
Ch2 Figure S 8-- Comparison of correlations between unnormalized expression assay and preservation methods in one postmortem case, UMB1796.

Scatterplots of UMB1796 replicates, histogram of data distribution, correlation, and number of up- and downregulated genes differing between samples. IVT and DASL-based assays, frozen and fixed tissue from the same postmortem case are represented. Labels indicate assay type (DASL/IVT), preservation method (Frozen/Fixed), sample code, and replicate number (if applicable).

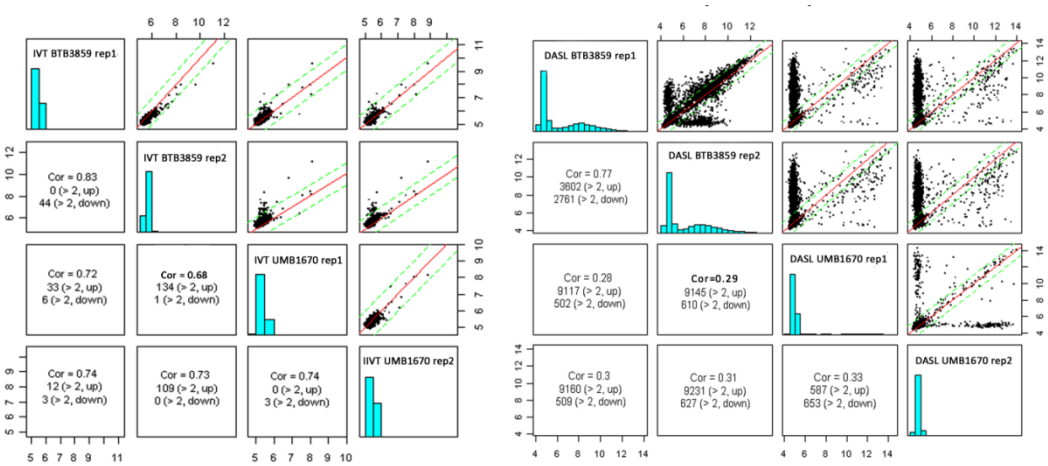


Ch2 Figure S 9-- Correlations between IVT and DASL assays on frozen tissue-extracted RNA samples.

Scatterplots of 3 samples processed by the two assays, histogram of data distribution, correlation, and number of up and downregulated genes after within-assay normalization by log2/quantile method.

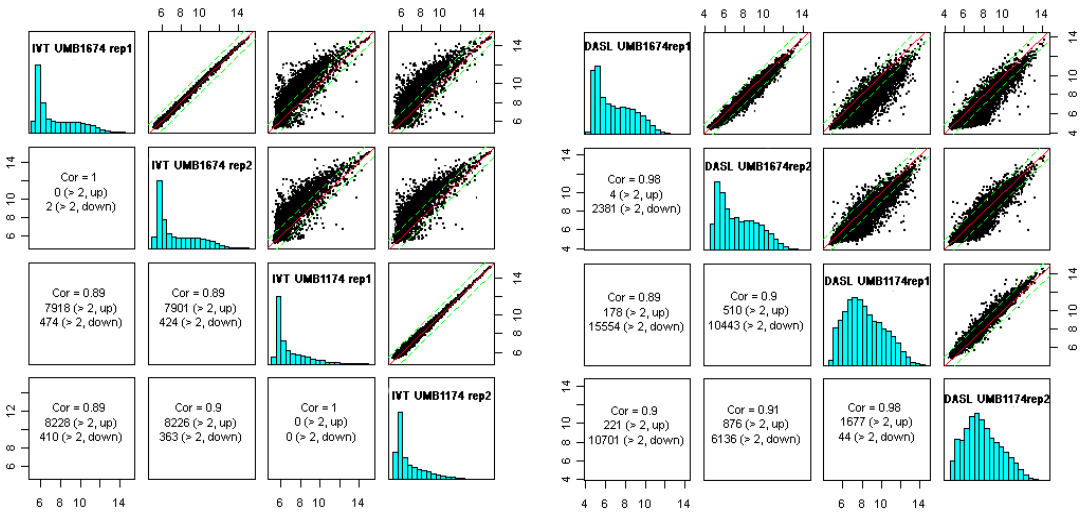


Ch2 Figure S 10-- Correlations between DASL-based fixed tissue-extracted RNA technical replicates. Scatterplots of 2 formalin-fixed samples processed by the DASL-based assay, histogram of data distribution, correlation, and number of up and downregulated genes. Rep = technical replicate.



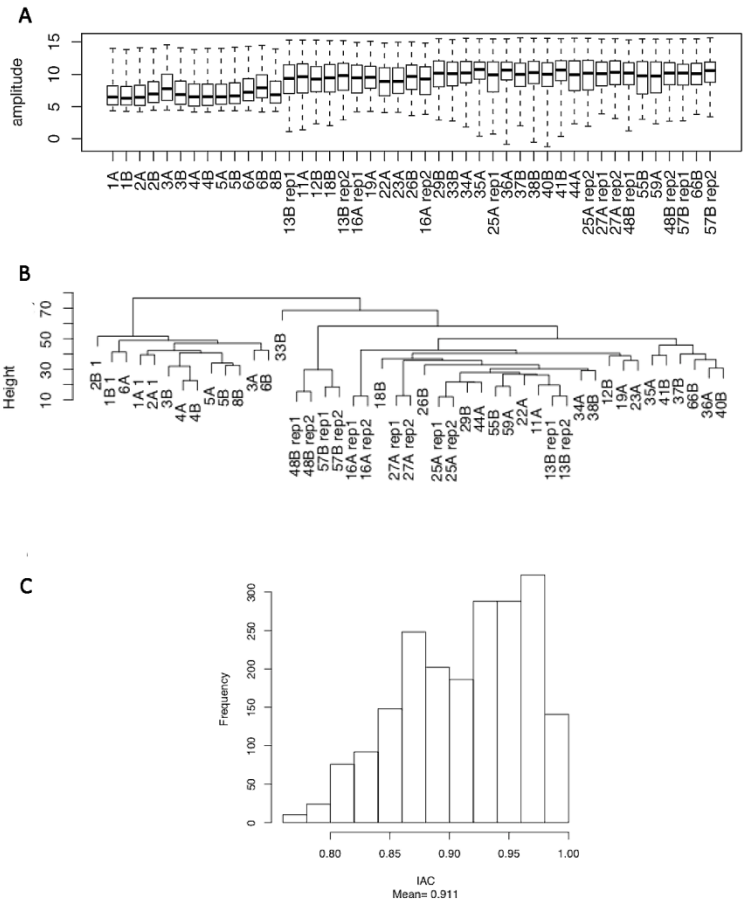
Ch2 Figure S 11-- Correlations between fixed tissue technical replicates and between samples on IVT- (left) and DASL- (right) based platforms.

Scatterplots of 2 formalin-fixed samples processed by the two assays, histogram of data distribution, correlation, and number of up and downregulated genes. Rep = technical replicate.

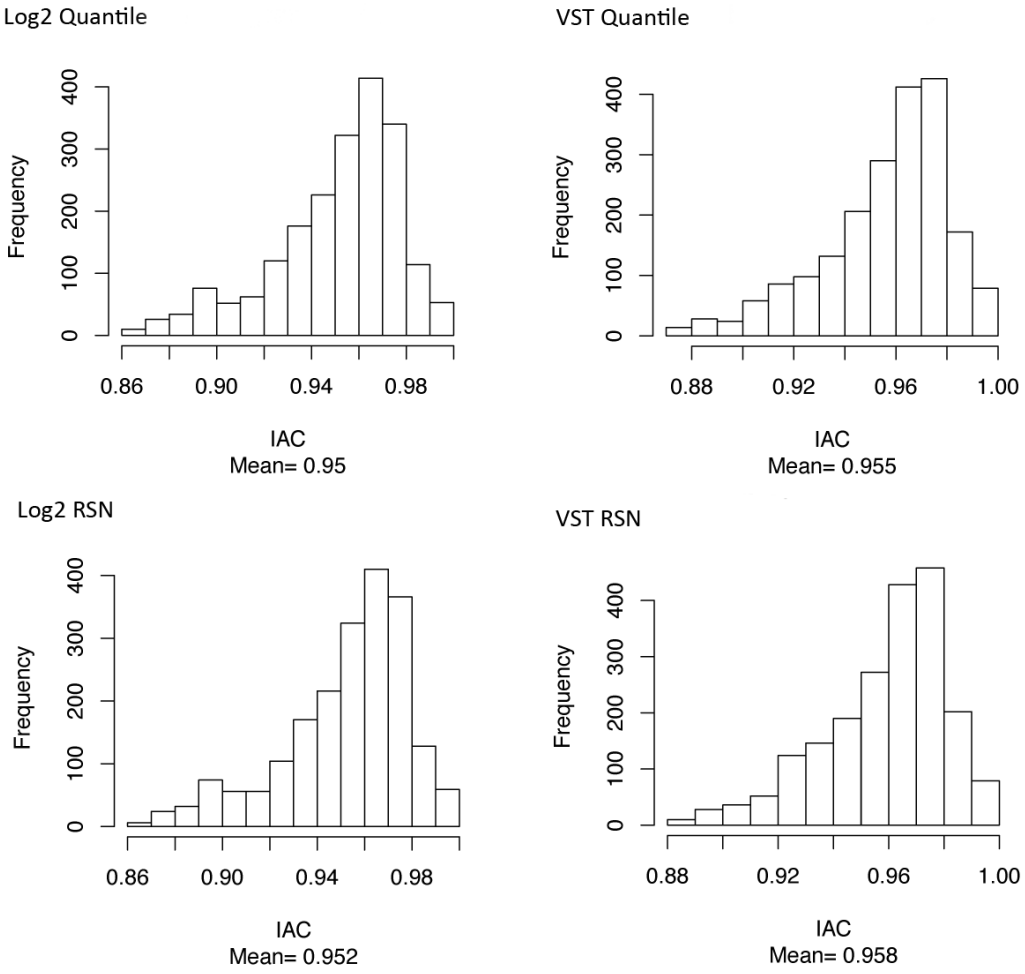


Supplementary Figure S11 -- Correlations between unnormalizedfrozen tissue technical replicates and between samples on IVT- (left) and DASL- (right) based platforms.
Scatterplots of 2 formalin-fixed samples processed by the two assays, histogram of data distribution, correlation, and number of up and downregulated genes. Rep = technical replicate.

3. Exploring transformation, normalization, and other preprocessing techniques on high quality DASL frozen tissue-RNA microarray data



Ch2 Figure S 12-- 33 frozen brain tissue sample dataset assayed on the DASL-based platform.
(A) Boxplots depict amplitude of intensity distributions for each sample passing quality control criteria before normalization. Samples labeled 1-8 were processed as a separate batch from the remaining samples. A and B = biological replicates; rep = technical replicates. (B) Cluster diagram depicts the average clustering of Euclidean distance between 33 samples passing quality control criteria before normalization. (C) Distribution frequency of inter-array correlations in the unnormalized dataset of these 33 samples is shown.



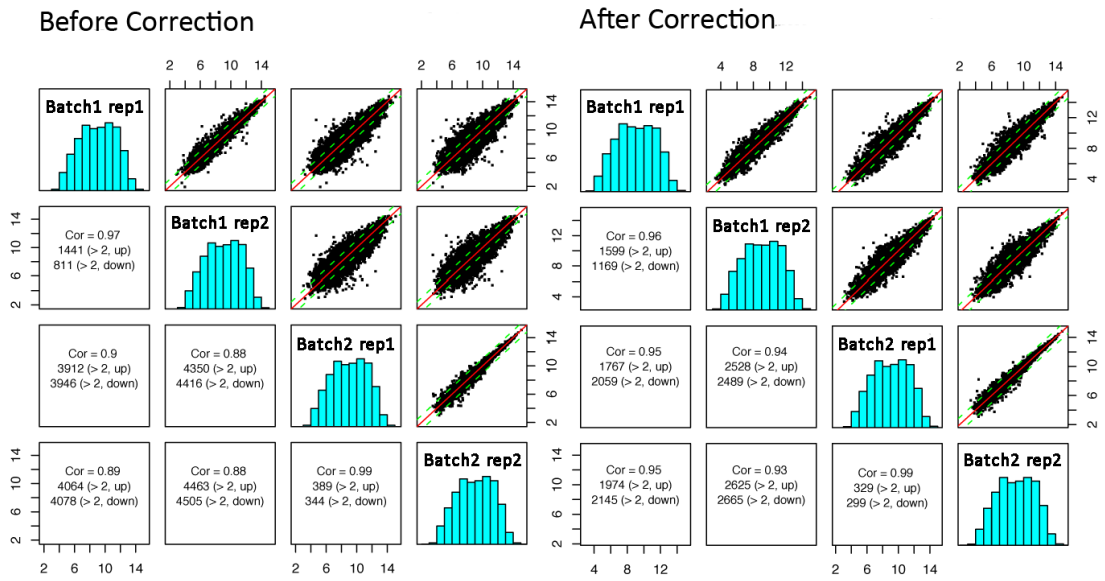
Ch2 Figure S 13-- Inter array correlation frequency distribution for four transformation and normalization methods after batch correction.

The mean inter-array correlations and distributions for the dataset after each of the four procedures, in addition to batch correction, are shown.

Ch2 Figure S 14-- MDMR analysis and mean inter array correlation of 4 transformation and normalization techniques after batch and seizure correction.

Columns 2 and 3 show single and multiple regression results of MDMR analysis before and after batch and seizure correction, with each transformation and normalization analysis. Column 4 lists the mean inter array correlations after batch correction of each transformation and normalization analysis. PVE, percentage of variance explained; IAC, inter array correlation.

VST RSN	Before correction				After Correction			
Single Regression	**F Statistic**	**PVE**	**p-value**	**Single Regression**	**F Statistic**	**PVE**	**p-value**	**Mean**
Diagnosis	1.6729134	0.037448	0.109	Diagnosis	1.8940367	0.042189	0.033	0.958
Age	2.4245125	0.0533745	0.026	Age	1.58669	0.0355866	0.063	
Seizures	2.7582096	0.0602779	0.013	Seizures	2.4793926	0.0545168	0.017	
Batch	13.058957	0.2329504	0	Batch	0.2846881	0.0065771	1	
Multiple Regression				**Multiple Regression**				
Batch	13.058957	0.2329504	0	Seizures	2.4793926	0.0545168	0.015	
Seizures	2.1226654	0.2698518	0.012	Age	1.6660211	0.0905905	0.048	
Age	1.8294695	0.3010403	0.024	Diagnosis	1.6574314	0.1259251	0.018	
Diagnosis	1.6026397	0.327966	0.023	Batch	0.2901352	0.1322195	1	
VST Quantile								
Single Regression	**F Statistic**	**PVE**	**p-value**	**Single Regression**	**F Statistic**	**PVE**	**p-value**	**Mean**
Diagnosis	1.8890465	0.0420826	0.074	Diagnosis	2.1861262	0.0483805	0.022	0.955
Age	2.2405636	0.0495255	0.034	Age	1.5368514	0.0345074	0.115	
Seizures	3.6468514	0.07818	0.005	Seizures	3.383833	0.0729529	0.003	
Batch	12.752688	0.2287367	0	Batch	0.3229249	0.0074539	1	
Multiple Regression				**Multiple Regression**				
Batch	12.752688	0.2287367	0	Seizures	3.383833	0.0729529	0.004	
Seizures	2.8867262	0.2783377	0.002	Age	1.6521697	0.1080402	0.044	
Age	1.8295067	0.3091643	0.019	Diagnosis	1.6128694	0.1418003	0.023	
Diagnosis	1.5452078	0.3348588	0.044	Batch	0.2860049	0.147893	1	
Log2 RSN								
Single Regression	**F Statistic**	**PVE**	**p-value**	**Single Regression**	**F Statistic**	**PVE**	**p-value**	**Mean**
Diagnosis	2.1462506	0.04754	0.049	Diagnosis	2.5611912	0.0562143	0.01	0.952
Age	2.1291878	0.0471798	0.065	Age	1.6129807	0.036155	0.092	
Seizures	4.3589952	0.0920415	0.001	Seizures	4.1035766	0.0871182	0.003	
Batch	15.008355	0.2587275	0	Batch	0.3858177	0.0088927	1	
Multiple Regression				**Multiple Regression**				
Batch	15.008355	0.2587275	0	Seizures	4.1035766	0.0871182	0	
Seizures	3.4994278	0.3157398	0	Age	1.7746043	0.124126	0.025	
Age	1.9815402	0.3472857	0.015	Diagnosis	1.5874065	0.1567734	0.029	
Diagnosis	1.521837	0.3712086	0.064	Batch	0.3383702	0.1638466	1	
Log2 Quantile								
Single Regression	**F Statistic**	**PVE**	**p-value**	**Single Regression**	**F Statistic**	**PVE**	**p-value**	**Mean**
Diagnosis	2.0685712	0.0458983	0.046	Diagnosis	2.4877101	0.0546897	0.01	0.95
Age	2.1157896	0.0468969	0.056	Age	1.6154992	0.0362094	0.088	
Seizures	4.2879443	0.0906773	0.003	Seizures	4.094734	0.0869467	0.002	
Batch	14.483298	0.2519566	0	Batch	0.3862096	0.0089017	1	
Multiple Regression				**Multiple Regression**				
Batch	14.483298	0.2519566	0	Seizures	4.094734	0.0869467	0.001	
Seizures	3.495735	0.3094337	0	Age	1.7776195	0.1240219	0.027	
Age	2.0082104	0.3416788	0.015	Diagnosis	1.5400331	0.155734	0.04	
Diagnosis	1.4739227	0.3650745	0.048	Batch	0.3483945	0.163024	1	

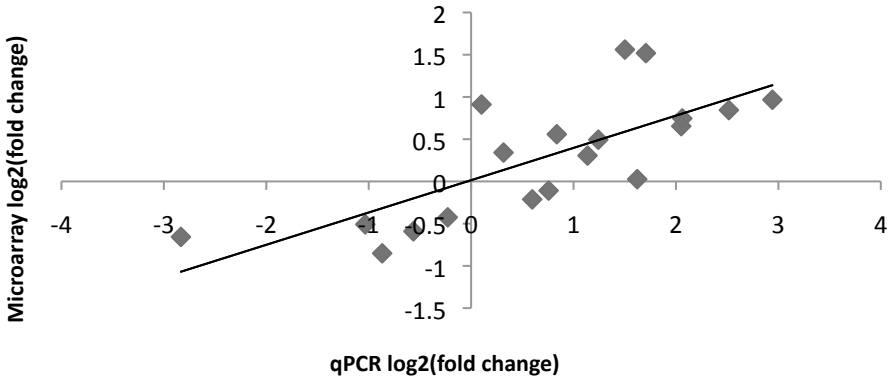


Ch2 Figure S 15-- Correlation between samples before and after data correction by ComBat.
Scatterplots of 2 samples and respective replicates, histogram of data distribution, correlation, and number of up and downregulated genes before and after batch and seizure correction. Samples 1 and 48 were processed in two separate batches. Cor = correlation; Rep = technical replicates; A and B = biological replicates.

4. Validation of microarray results by qPCR.

Ch2 Figure S 16– Primer sequences for RTPCR validation.
Forward and reverse primer sequences for 19 experimental and 3 reference genes used for RT-PCR to validate microarray results are shown.

Gene Name	Forward Primer Sequence	Reverse Primer Sequence
AIF1	CTCCAGCTTGGTCTGTCTCC	TCATCCAGCCTCTCTTCCTG
CACNB1	ACGTCCTCGGATACCACATC	CGGTCCTCCTCCAGAGATAC
CASP10	CTTTGGACCTTGGAGCACAC	GAACTGGAATACCAATGTTGACC
CTTN	GAAACAGGACCAAAAGCTTCC	CATCTGGACACCAAACTTGC
EDEM3	GAATTTGAAGATGCAGTGAGAAAA	AACTGCTTTGCCATTTGGAG
HAP1	GATGGAGGAGAACAGCAAGC	GAATCTGAGTAGAGCTGGAGGAG
IL12RB1	CTGCCTGCAGAACCAGTGAG	CAGCTGTGGGACCCTCATAC
LAMA2	TGTTTCTGTTCAGGGGTTTCA	TGCTGATCTGCTGAGGTGAG
MTNR1B	TCTTGGTGAGTCTGGCATTG	TTGAAGACAGAGCCGATGAC
NEUROG2	CAAAGTCACAGCAACGCTGA	GAGCAGCACTAACACGTCCTC
NFKB2	CCCTCCCATGGAGGACTG	ACCAGACTGTGGGCATGAG
NRXN1	TCAGGAAATTCGCTTTGACC	GTGTTGGTGATGCATTTTGG
OPN4	ACCCAGCTGGTGGGACAG	CTGTGCCCAGGGTATAGTGG
OR2B6	TGAATTGGGTAAATGACAGCA	CATGGGGGTATGAAGTTTGG
PROK1	CACCCCAAGTGACCATGAG	CTCGAAGCCACAGGCTGAT
SRC	AGATCCGCAAGCTGGACA	CTGAGTCTGCGGCTTGGAC
TBX1	GTGTGAGCGTGCAGCTAGAG	TCCATGAGCAGCATATAGTCG
TGFA	CCTTGGTGGTGGTCTCCAT	CGGTTCTTCCCTTCAGGAG
TLR1	AGGCCCTCTTCCTCGTTAGA	AATGGCAAAATGGAAGATGC
RPL13A	GGGAAGGGTTGGTGTTCAT	GGGAAGGGTTGGTGTTCAT
ACTB	GCCGTCTTCCCCTCCATC	CGTCCCAGTTGGTGACGAT
TBP	CGGCTGTTTAACTTCGCTTC	CCAGCACACTCTTCTCAGCA



Ch2 Figure S 17-- Log2 fold change correlations of selected genes detected by microarray and RTPCR.

Log2 Fold change detected by RTPCR is depicted on the x axis, and microarray on the y-axis of 19 up- and down-regulated genes in the dataset spanning a large range. Spearman's rank correlation detected an R=0.78 (p=0.000075, DF=17) correlation between microarray and qPCR detection of fold change.

Chapter 3 –Gene expression abnormalities in the autistic cortex

Introduction

Autism is a highly heritable disorder marked in the early years of life by abnormal brain overgrowth and dysfunction (Courchesne et al., 2001; Sparks et al., 2002; Hazlett et al., 2005; Courchesne et al., 2007; Amaral et al., 2008; Redcay and Courchesne, 2008; Schumann et al., 2010). These abnormalities show an anterior/posterior gradient and are most strongly evident in prefrontal cortex(Carper et al., 2002). However, this difference in brain size is no longer detectedin adulthood and instead, the adult autistic brain often shows signs of cortical thinning and neuron loss (Courchesne et al., 2001; Courchesne and Pierce, 2005; Courchesne et al., 2007; Amaral et al., 2008; van Kooten et al., 2008; Courchesne et al., 2010). Therefore, important age-related molecular and cellular differences may be present in the autistic brain. Despite these differences, forty studies of the postmortem autistic brain in the literature have only studied cases on average of 22 years, with only few case reports of young autistic cases under 14 years of age(Courchesne et al., 2007).

Cell cycle dysregulation, excess neurogenesis and reduced apoptosis have long been hypothesizedto explain brain enlargement in young autistic children(Courchesne et

al., 2001; Courchesne et al., 2007). Some studies have yielded supporting evidence for this hypothesis. Enrichment analysis of genes located within regions of copy number variations (CNVs) in individuals with autism identified cell proliferation and other neurodevelopmental processes to be affected(Pinto et al., 2010). Though DNA analyses can identify potentially static differences in the autistic in the genome, functional genetic studies are required to examine the downstream functional consequences of these differences. Transcript and protein expression studies in adult autistic brain tissue have also implicated pro-apoptotic (Araghi-Niknam and Fatemi, 2003), immune (Garbett et al., 2008), and inflammatory (Vargas et al., 2005)aberrances in addition to dysregulation of neurotransmitter systems (Oblak et al., 2010). Thus, to further understand the molecular mechanisms underlying early brain overgrowth in autism, we performed genome-wide expression profiling, differential analysis, and enrichment analysison the dataset fully described in Chapter 2.

Methods

Using the preprocessed dataset, we analyzed expression differences among of 57 autistic and control DL-PFC frozen tissue blocks. 33 male cases passed quality control measures: 16 were categorized as young (2-14y; n=9 autistic, n=7 controls) and 17 as adult (15-56y, n=6 autistic, n=11 controls) cases (Tables S1 and S2). Two-way analysis of variance (ANOVA) and post-hoc pair-wise ANOVA-based comparisons of genes filtered for variance and minimum intensity were performed to identify (1) genes exhibiting an interaction effect between diagnosis and age group ($p<0.05$) and (2) genes

exhibiting age-independent diagnosis main effect expression differences in autistic DL-PFC ($p<0.05$). Genes identified in a post-hoc analysis of those displaying significant diagnosis x age interaction effects (young autistic vs. young control) and those displaying a main effect of diagnosis were further subjected to enrichment analyses in the MetaCore® software suite. QPCR validation was performed on a subset of genes (Ch3 Figure S1).

Differential Expression analysis

Filtering and differential expression analyses were performed using BRBArrayTools developed by Dr. Richard Simon and BRBArrayTools Development Team (http://linus.nci.nih.gov/BRBArrayTools.html). Filtering was performed based on variance and minimum intensity. Genes with less than 20% of expression values having at least a 1.5-fold up or downregulation from the gene's median value across cases, and genes with a minimum intensity of less than 15 were excluded. Using the analysis of variance (ANOVA) tool, we identified 1086 genes with an interaction effect of age and diagnosis by performing a 2-way ANOVA with 2 levels of diagnosis (autism and control) and 2 levels of categorical age (2-14 years and 15-56 years). Interaction genes of $p<0.05$, corresponding to an FDR of 0.27(Benjamini and Hochberg, 1995), were selected for posthoc analysis to examine which differences were driven by the young autism and young control groups by t-tests ($p<0.05$) to specifically investigate gene expression changes in the young autistic brain. Genes exhibiting a main effect of diagnosis across age groups were also identified ($p<0.05$, FDR=0.13).

Enrichment analysis by Metacore® analysis suite.

Enrichment analyses were performed using the MetaCore software suite (www.genego.com/metacore.php) to examine the biological and functional relevance of the differentially expressed genes in young ASD cases, as well as the preliminary pathway results of the adult ASD cases. These genes were uploaded into Metacore and filtered for known expression specific to the brain or the fetal brain. The default background gene list was used for both pathways and GO process analysis. All pathways witha corrected p-value of less than 0.05 are reported, and all GO processes with a corrected p-value of less than 0.01 are reported.

In Metacore, pathways 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 network processes are network models of main cellular processes that are created manually by GeneGO using information from GO processes and GeneGO pathway maps. GO processes represent a recognized series of interactions or biochemical reactions accomplished by one or more molecular function ordered assemblies with a defined beginning and end, and are network models of main cellular processes. P-values for pathways and processes are calculated using a hypergeometric model to determine the significance of enrichment(Falcon and Gentleman, 2007).

Results

Gene expression abnormalities in young autistic DLPFC

Pathway analyses suggested markedly abnormal expression of genes that regulate cell numbers, genetic integrity, and neural patterning during development in the young autistic cases (Ch3 Figure 1). Pathways mediating DNA damage, cell cycle checkpoint, and apoptotic functions were significantly enriched with differentially expressed genes. Specifically, ATM/ATR regulation of the G2/M checkpoint (p=4.02E-03) and the G1/S checkpoint (p=6.06E-03) were dysregulated in young autistic cases. Genes downregulated in autistic DL-PFC included the checkpoint and DNA-damage response genes BRCA1, CHEK2, and FANCB (Wang, 2007; Sato et al., 2010) and genes mediating cell cycle and cell division, including TRIOBP, ANKRD38, CHP2, NEK3 and CDC20 (Tanaka and Nigg, 1999; Yu et al., 2008; Yu et al., 2008; Cam et al., 2009; Kakinuma et al., 2009). By contrast, the positive cell cycle regulator FOSL2 (Maaser and Borlak, 2008) and the cell cycle chromosome fidelity factor CHTF18 (Hanna et al., 2001) were upregulated. The genes within these cell cycle and DNA-damage pathways expressing aberrantly could affect the regulation of DNA replication efficacy and the numbers of cells that are incorporated into the developing cortex.

These cell cycle and DNA damage checkpoint aberrancies were complemented by evidence of apoptotic dysregulation (Ch3 Figure 1 and Ch3 Table S3), including dysregulation of genes that also participate in immune and inflammatory responses.

Genes controlling cell number and apoptosis were downregulated in young autistic cases. These included PTPRH, TSC22D3, PLP1, CLN8, and FAT2 (Southwood and Gow, 2001; Takada et al., 2002; Cavallaro et al., 2004; Soundararajan et al., 2007; Vantaggiato et al., 2009), and PPP1CA. PPP1CA regulates apoptosis, cell division, and proliferation through WNT/beta-catenin and BRCA1 pathways (Luo et al., 2007; Yu et al., 2008), EIF2 activity (p=8.91E-03), and the endoplasmic reticulum stress response apoptotic pathway (p=1.60E-02). Further, we found upregulation of the NF-κB-regulated genes NOD1 (Jae Gyu et al., 2010) and BCL3 (Brocke-Heidrich et al., 2006). The central apoptosis regulators FAS (Sancho-Martinez and Martin-Villalba, 2009) and MAP4K1, which participate in the JNK pathway (p=1.08E-02), were also upregulated. Taken together, these gene expression aberrances may contribute to the abnormal brain growth trajectories in autism.

In contrast, cell fate, differentiation, and proliferation regulators NTRK3, POU6F2, and WNT3 (Zhou et al., 1996; Liu et al., 1999; Lee et al., 2000; Bartkowska et al., 2007) were upregulated in young autistic cases (Ch3 Figure 1 and Ch3 Table S3). NTRK3 interacts with the WNT pathway (David et al., 2008), mediates positive cell growth and proliferation, and postnatal cortical neuronal localization (Bartkowska et al., 2007). The WNT genes regulate cell fate and proliferation, and patterning during embryogenesis (Freese et al., 2010): WNT3 is required for vertebrate axis formation during embryogenesis (Liu et al., 1999) and influences synapse formation (Freese et al., 2010). Downstream components of the WNT pathway, such as Dvl1, are known to regulate social behavior in mouse models (Lijam et al., 1997). Additional support for

these gene functions was provided by an identified GeneGo Process Network of Neurogenesis in General (Development; p=1.089E-03) in the young autistic brains.

In addition to the genes in the WNT pathway, there was significant downregulation of genes involved in neural patterning and differentiation, such as FGF13, DLX4, HOXD1, NDE1, NODAL, PCSK6 and GREM1 (Constam and Robertson, 2000; Feng and Walsh, 2004; Depew et al., 2005; Bertrand and Dahmane, 2006; Nishimoto and Nishida, 2007; Wordinger et al., 2008; Janssens et al., 2010; Ch3 Figure 1). Dysregulation of these genes that mediate neural patterning and differentiation during neurodevelopment may lead to abnormal global and cellular neural organization and cytoarchitecture. HOX and DLX family genes play important roles in vertebrate craniofacial and brain patterning along dorsal/ventral and rostral/caudal axes and are important for neuronal subtype differentiation (Wigle and Eisenstat, 2008). HOXD1 is induced by the WNT pathway during neurulation (Janssens et al., 2010). Furthermore, ablation of NDE1 in mice results in microcephaly and cortical disorganization due to reduced progenitor cell division and altered neuronal cell fates (Feng and Walsh, 2004). NODAL controls dorsal mesoderm induction, anterior patterning, and formation of left-right asymmetry through primitive streak formation (Schier, 2009). NODAL is also regulated by PCSK6, and knockouts for PCSK6 exhibit craniofacial abnormalities (Constam and Robertson, 2000). Finally, GREM1, a BMP receptor antagonist, plays important roles in several developmental processes (Hsu et al., 1998). These dysregulated proliferation, apoptosis, cell differentiation, and neural patterning functions may be

important contributing factors to the aberrant cortical patterning phenotype in young autistic cases.

Age-independent abnormalities in autistic DLPFC

Next, to identify expression abnormalities common across all young and adult male autistic cases as compared to all young and adult male controls, we analyzed genes that displayed a main effect of diagnosis in the 2-way ANOVA ($p<0.05$) to see if they were enriched in particular pathways (Ch3 Figure 1; Ch3 Table S5 and S6). Significant age-independent dysregulated pathways were identified in autism (Ch3 Figure 1; Table S7 and S8) including cell cycle (14-3-3 (YWHAZ), CDC25A, CDCD25C, ATRX), proliferation [CTNNB1 (beta-catenin), PRKACB, PRKCZ], apoptosis (BAD, CASP8, CASP10, MDM2), cytoskeleton and extracellular matrix remodeling (ErbB4, MMP2, NID1, TIMP1, COL4A3) and growth and development (RELN, ROBO1, ADORA2A, p21 (CDKN1A), 14-3-3, HGF, FGFRL1, TSC1) functions. The p53 signaling pathway ($p=4.90E-05$) and the PTEN pathway ($p=9.95E-04$) were among the top dysregulated pathways in all autistic cases (Ch3 Figure 1).

A number of these genes have known functions in neuronal development, and we highlight a few here. 14-3-3 proteins, which regulate cell cycle, proliferation, apoptosis and cell survival, interact with h-RAS, BAD, CDC25, p21 and MAPK (Darling et al., 2005). One 14-3-3 homologue, 14-3-3 epsilon, is important for neuronal migration and development (Toyo-oka et al., 2003), and is downregulated in autistic cases. CTNNB1, a

key member of the WNT pathway, regulates cerebral cortical size by controlling reentry of neural progenitor cells into the cell cycle. CTNNB1 transgenic mice have enlarged and folded cortices (Chenn and Walsh, 2002). Furthermore, gain or loss of p21-activated kinase (PAK1) function produces migration defects and disrupted cortical organization (Causeret et al., 2009). P53 is a tumor suppressing gene that causes uninhibited cell growth and proliferation when dysregulated (Marchetti et al., 2004). PTEN signaling, which also regulates proliferation (Endersby and Baker, 2008) and has been implicated in macrocephaly in autism (Butler et al., 2005; Page et al., 2009), was also dysregulated. TSC1 acts downstream of PTEN in the mTOR pathway (Hay, 2005) and has been implicated in cortical lamination, neuronal migration, and axon outgrowth and targeting functions in the brain (Orlova and Crino, 2010). This gene is downregulated in autistic cases, and may point to a common pathogenic mechanism shared by autism and tuberous sclerosis that underlies focal cortical lesions (Crino et al., 2006). Finally, RELN is critical for mouse and human neuronal migration (Tissir and Goffinet, 2003). Of note, a majority (18/25) of the top 25 significant pathways commonly dysregulated in all autistic brains involved developmental processes (proliferation, cell cycle, apoptosis, DNA-damage, neural patterning, and migration), consistent with findings in only the young autistic brains. Few pathways (1/25) were strictly immune response-related (Ch3 Figure 1). A full list of genes and reference studies for their functions are provided in Ch3 Figure S9.

Discussion

Using novel gene expression profiling techniques, we discovered that the autistic brain at young ages prominently involves disturbances in pathways regulating cell number, cell differentiation, organization, and DNA damage response. The genes that we specifically examined show an effect of interaction between diagnosis and age, suggesting that these abnormalities change with age in the autistic brain. The autistic brain at young ages may be enlarged due to a lack of acute apoptotic mechanisms that normally remove defective cells (Zhu et al., 2004; Yang and Herrup, 2007; Zhu et al., 2007), and the presence of these cells may in turn affect the organization and connectivity of the brain (Casanova et al., 2002; Courchesne and Pierce, 2005). In fact, gene expression evidence from animal models with severe neuronal migration defects also show severe abnormalities in cell cycle, DNA damage, and cell proliferation processes (Pramparo et al., 2011). We also recently found consistent neuropathological evidence in the cortex of young autism cases that supports this hypothesis, and substantiates the observed molecular cell differentiation and organizational defects (Chow, Pramparo, Boyle et al., *in review*). Normally these functions play important roles during prenatal and early postnatal life as the brain specializes its many regions and establishes its intraconnectivity. Our results point to dysregulation of cell numbers in the autism brain in addition to abnormalities in cellular organization and specification of cell types in young autistic cases.

Dysregulation of DNA damage-induced responses during neurogenesis could lead to the retention of cells lacking functional DNA integrity (Lee et al., 2000) which may contribute to dysfunction at the neural systems level that is well-documented by fMRI

and ERP studies (Minshew and Keller, 2010; Jeste and Nelson, 2009). For example, ATM and ATR signaling mediate apoptosis of proliferating cells (O'Driscoll 2009): postmitotic cells in the subventricular zones of ATM-deficient mice are resistant to p53-dependant apoptosis resulting from DNA damage due to radiation during neurogenesis (Herzog et al. 1998). Further investigation is required to link specific molecular deficits to functional and morphological abnormalities in autism.

Furthermore, abnormalities in the expression of neural patterning genes during the development of vital structures and connectivity in the brain could underlie the abnormal rightward neurofunctional asymmetry documented by numerous imaging studies of autistic subjects (Redcay and Courchesne 2008) as well as the anterior to posterior gradient of pathological overgrowth documented by a number of recent MRI studies (Courchesne et al., 2011). Cerebral lateralization gene PCSK6, which was dysregulated in the young autism group, for example, was recently associated with aberrant language development (Scerri et al., 2010) and could contribute to the language domain of ASD deficits.

Our results complement reports of the role of CNVs in genes regulating neurogenesis and proliferation (Pinto et al., 2010), by showing that complementary expression defects in neurogenesis, cell cycle and apoptosis functions may be responsible for the aberrant numbers of cells in the brain. It is possible that dysregulation of these

CNVs leads to the expression defects observed in our study, but future analyses will be required to show this relationship.

Furthermore, our observation that different growth and development regulators exhibit age by diagnosis gene expression anomalies with pronounced perturbation in young autistic brains suggests that nonspecific genetic abnormalities converging on these pathways may cause similar neurodevelopmental defects, leading to a heterogeneous spectrum of phenotypes. Remarkably, our young cases only showed dysregulation of ~100 genes, while the adult cases showed dysregulation of ~700. Either the young cases in general were more homogenous, or both autistic and control groups showed greater variability in gene expression so that differences were not detected by the ANOVA that was performed. However, the genes that were dysregulated were prominently in pathways that regulate cell numbers in the young cases, while the adult cases were dysregulated in pathways that were more heterogeneous. Our results highlight the importance of considering the progression of neurodevelopment and shifts in biological processes with age in autism research. In the context of these changes, we may further parse the developmental mechanisms involved in the early pathogenesis of autism.

The heterogeneity of genetic and molecular changes observed in our autisticcases also suggests that a variety of insults converging on common pathways regulating neuron numbers, integrity, differentiation, migration and patterning can occur at young ages. It revealed that a single cause of autism is unlikely to be found. Rather, disturbances in

brain growth that result in enlargement and disconnectivity of select regions of the brain affecting language and other higher order functions possibly caused by mismigration or misdifferentiation of cells are the more likely culprits. Thus, a single cause for the disorder across individuals with autism, whether it is a toxin or a gene, is improbable. Our search for the origin of the disorder would be better served by biological subclassification of autistic individuals, whether it is by gene expression in the blood or as a first step by behavioral or cognitive criteria (Hu et al., 2009).

If the pathways that we identified underlie the neurodevelopmental abnormalities in young autistic children, then perhaps they would be even more evident at younger ages and during *in utero* development. There is also a distinct possibility that even the expression changes in the young autistic brain are already reactive modifications due to aberrant formation of the brain, or that we are witnessing the shifted timeframe for gene expression that normally creates a typically functioning brain. For example, the neural patterning genes may either be activated in young autistic children when they should have been activated during an earlier critical period *in utero*. Or, they may have been activated in order to correct the abnormal patterning that has already occurred as part of a corrective process that involves many genes.

We acknowledge that our results are derived from a small sample of young autism and control cases. While our experiments did not directly test relationships between expression dysregulation and brain morphology parameters, the cell cycle and DNA-

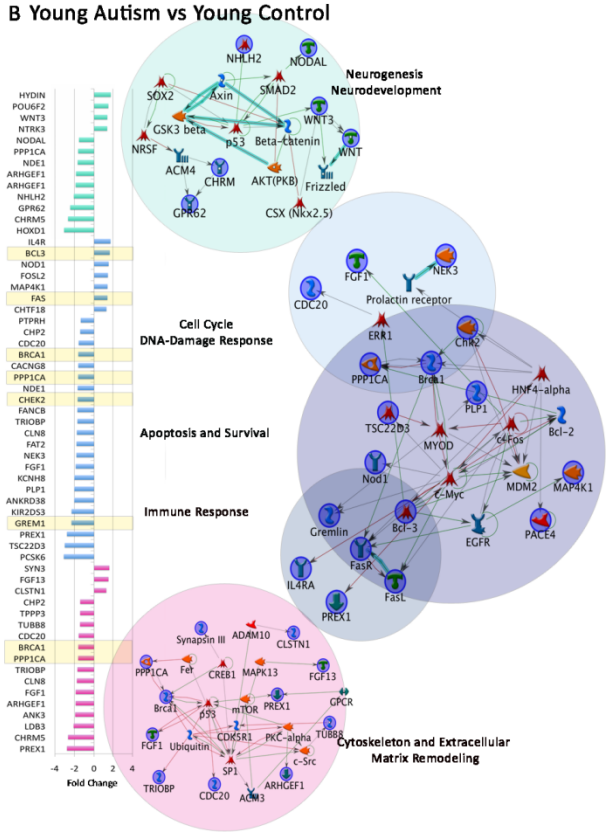
damage checkpoint dysfunctions detected herein may lead to the structural and functional neural defects, including early brain overgrowth, that underlie autism (Courchesne et al., 2001; 2007). Future studies with increased sample size and additional brain regions are required to validate and replicate our results.

In sum, we have identified molecular mechanisms that may underlie early brain overgrowth in autism. These abnormal molecular processes and defects may be among the first steps of early prenatal neural maldevelopment that result in structural, functional, and ultimately the behavioral abnormalities in autism.

A Young Autism vs Young Control

Pathway Category	Pathway Name	pValue	Network
DNA Damage	DNA-damage-induced responses	4.63E-04	2/9
Immune Response	NF-AT signaling and leukocyte interactions	6.14E-04	3/46
Apoptosis and Survival	DNA-damage-induced apoptosis	1.33E-03	2/15
DNA Damage	ATM / ATR regulation of G2 / M checkpoint	4.02E-03	2/26
DNA Damage	ATM/ATR regulation of G1/S checkpoint	6.06E-03	2/32
Cell Cycle	Regulation of G1/S transition (part 1)	8.47E-03	2/38
Translation	Regulation of EIF2 activity	8.91E-03	2/39
Signal Transduction	JNK pathway	1.08E-02	2/43
Transcription	CREB pathway	1.12E-02	2/44
Apoptosis and Survival	Endoplasmic reticulum stress response pathway	1.60E-02	2/53
Regulation of lipid metabolism	Stimulation of Arachidonic acid production by ACM receptors	2.85E-02	2/72

B Young Autism vs Young Control



C ALL Autism vs ALL Control

Pathway Category	Pathway Name	pValue	Network
Development	A2A receptor signaling	1.70E-07	11/43
Development	A2B receptor: action via G-protein alpha s	7.05E-06	10/50
Transcription	P53 signaling pathway	4.90E-05	8/39
Transcription	CREB pathway	1.21E-04	8/44
Development	Thrombopoietin-regulated cell processes	1.43E-04	8/45
Development	PIP3 signaling in cardiac myocytes	1.97E-04	8/47
Cell adhesion	ECM remodeling	4.04E-04	8/52
Cell cycle	Role of 14-3-3 proteins in cell cycle regulation	8.20E-04	5/22
Cytoskeleton remodeling	Cytoskeleton remodeling	8.41E-04	11/102
Reproduction	GnRH signaling	8.58E-04	9/72
Development	Role of IL-8 in angiogenesis	8.58E-04	8/58
Immune response	MIF - the neuroendocrine-macrophage connector	9.95E-04	7/46
Signal transduction	PTEN pathway	9.95E-04	7/46
Development	HGF signaling pathway	1.13E-03	7/47
Development	A3 receptor signaling	1.46E-03	7/49
Development	GM-CSF signaling	1.65E-03	7/50
Cytoskeleton remodeling	TGF, WNT and cytoskeletal remodeling	1.69E-03	11/111
Development	IGF-1 receptor signaling	1.85E-03	7/51
Signal transduction	cAMP signaling	1.88E-03	6/38
Cell adhesion	PLAU signaling	2.16E-03	6/39
DNA damage	DNA damage_Role of SUMO in p53 regulation	2.44E-03	4/17
Cell adhesion	Cell adhesion_Chemokines and adhesion	2.54E-03	10/100
Proteolysis	Proteolysis_Putative SUMO-1 pathway	3.03E-03	5/29
Apoptosis and survival	Apoptosis and survival_BAD phosphorylation	3.18E-03	6/42
Development	Development_Osteopontin signaling in osteoclasts	3.53E-03	5/30

Ch3 Figure 1 – Pathways dysregulated in young autistic cases and all autistic cases.

A) Pathways dysregulated in young autistic cases identified by Metacore® are presented. Pathways in green correspond to the neurogenesis and neurodevelopment domain; pathways in blue correspond to cell cycle/DNA damage, apoptosis and survival, and immune response domains. B) Networks of pathways and additional genes identified by Metacore® and additional literature search, as well as fold changes of genes in each domain are listed. Genes in green correspond to the neurogenesis and neurodevelopment domain; Genes in blue correspond to cell cycle/DNA damage, apoptosis and survival, and immune response domains; genes in pink correspond to the cytoskeleton and extracellular matrix remodeling domain. Genes highlighted in yellow are overlapping between domains. C) Pathways dysregulated in all autistic cases are presented. Pathways in green correspond to the neurogenesis and neurodevelopment domain; pathways in blue correspond to cell cycle/DNA damage, apoptosis and survival, and immune response domains; pathways in pink correspond to the cytoskeleton and extracellular matrix remodeling domain.

Supplementary Figures

Ch3 Table S 1– Demographics of postmortem cases.

ID, diagnosis, age, gender, study, DLPFC RNA integrity number (RIN), basis of diagnosis, ADI measures, and intellectual disability for cases in present study are documented. Red X's indicate cases passing quality control for given study; Black x's indicate cases processed that did not pass quality control for given study. Nine cases (B5000, B5144, B5569, B6184, B6401, B6640, B6736, B7085, UMB1500) did not pass quality control for any study and are not included in this table. ADI-R = Autism Diagnostic Interview-Revised; ADOS = Autism Diagnostic Observation Schedule; CNV = copy number variation; ISH = *in situ* hybridization; Comm = Communication; R & R = Restricted and Repetitive behavior; DL-PFC = Dorsolateral Prefrontal Cortex; RIN = RNA integrity number; M = male; F = female; v = verbal; nv = nonverbal; Y = yes; N = no.Additional clinical data is presented in Table S2. *Records from ATP not yet complete.

Case ID	Alternate ID	Diagnosis	Age (Years)	Gender	DLPFC RIN	Basis of Diagnosis	ADI Social Score	ADI Comm Score	ADI R & R Score	Intellectual Disability
B6399	AN03345	Autism	2	M	7.6	ADI-R	14	9 (nv)	6	N
UMB1349		Autism	5	M	8.7	ADOS				Y
B1469	AN13364	Autism	5	M	2.5	*				
UMB4849		Autism	7	M	6.3	ADI-R	22	18 (v)	8	Y
B5666	AN19511	Autism	8	M	7.8	ADI-R	19	14 (v)	4	N
UMB4231		Autism	8	M	7.4	Medical Records				
UMB797		Autism	9	M	6.9	ADI-R	24	20 (v)	6	N
B4925	AN16641	Autism	9	M	2.2	ADI-R	24	13 (nv)	4	Y
UMB4899		Autism	14	M	8.5	ADI-R	22	14 (nv)	8	Y
B7079	AN04682	Autism	15	M	5.7	*				
B6994	AN08166	Autism	28	M	3	ADI-R	22	16 (v)	5	N
B5173	AN08792	Autism	30	M	-	ADI-R	10	12 (nv)	3	Y
B6677	AN11989	Autism	30	M	-	ADI-R	26	22 (v)	12	Y
UMB1445		Autism	45	M	-	Medical Records				Y
B7109	AN17254	Autism	51	M	4	ADI-R	27	19 (v)	6	N
UMB1185		Control	4	M	6.8					
UMB4898		Control	7	M	7.7					
UMB1674		Control	8	M	-					
UMB1650		Control	10	M	7.3					
UMB1714		Control	12	M	7.7					
UMB4787		Control	12	M	6.5					
UMB4722		Control	14	M	7.7					
UMB1796		Control	16	M						
B6756	AN07591	Control	16	M						
B5251	AN03217	Control	19	M	3.5					
UMB1649		Control	20	M	4.9					
UMB818		Control	27	M	1.9					
B5873	AN19760	Control	28	M	-					
B5334	AN15622	Control	30	M	5.7					
B5352	AN12137	Control	31	M	3.8					
B5813	AN01410	Control	41	M	5.2					
B4756	AN10606	Control	56	M	5.9					
B6860	AN13295	Control	56	M	6.3					

Ch3 Table S2A– Detailed Clinical Information of Autistic Cases.

Available clinical information, including information from the ADI-R assessment, noted seizure activity and intellectual disability, is provided for autisticcases in this study. Assessments were made by a licensed clinical psychologist (C.C.B.). ADI = Autism Diagnostic Interview; RIN = RNA Integrity Score; HX = History; DO = Diagnosis; ADI Comm Score = ADI-R Communication Score; ADI R&R Score = ADI-R Restricted and Repetitive Behavior Score; IVF = In Vitro Fertilization

Case ID	Age	Diagnosis	Basis of Diagnosis	Verbal Compre-hension	Verbal Expression	Age of 1st Concern	Language Regression	IQ*	Self injury	Engagement in Appropriate Activity	Simplex/ Multiplex	Seizures
B-6399	2	Autism	ADI-R		< 5 words total ("I love you"& "no")	15m	N	at 27 months got IQ of 78 on Merrill-Palmer	N	limited constructive play with repetitive activities	Mulitiplex	N
UMB-1349	5	Autism	ADOS	some words and simple familiar commands	< 5 wds total / speech not used daily	<24m	Y	16m age equiv. @ 33m	Y	limited constructive play with repetitive activity and motor stereotypies	Simplex	none reported
B-1469	5	Autism									no info	
UMB-4849	7	Autism	ADI-R		**echollalia: functional use of language, restricted in frequency and contexts used	2 yrs	reported loss of 4 words acquired at age 2 (insufficient language to code true regression on ADI)	<50 overall on Bayley Scales	N	limited constructive play with repetitive activities	Multiplex	N
B-5666	8	Autism	ADI-R		functional use of language; occasional echolalia	36m	N		N	initiates a limited range of appropriate activities	Simplex	teacher thought he was having petite mals -- EEG showed "unusual brain activity but not seizures"
UMB-4231	8	Autism	Medical Records				N				Simplex	
UMB-797	9	Autism	ADI-R		functional use of spontaneous language used on a daily basis	30m	N		N	limited range of appropriate activities, some repetitive	Simplex	N
B-4925	9	Autism	ADI-R	many words and some simple familiar commands	< 5 wds total / speech not used daily	12m	N	Catell = 36; Mullen <39		passive activities (e.g., Barney video's); active play (running, chasing), & repetitive activities	Simplex	Y
UMB-4899	14	Autism	ADI-R		< 5 words total / speech not used daily	12m	Y		Y	repetitive play, no pretend play	Multiplex	Y
B-7079	15	Autism	ADI-R pending									ATP has not yet completed collection of clinical documents
B-6994	28	Autism	ADI-R								Simplex	Y
B-5173	30	Autism	ADI-R		< 5 wds total / speech not used daily	5m	N	no testing reported	Y	repetitive activities	Simplex	Y
B-6677	30	Autism	ADI-R		single words only	24m	N	MA 16 months at CA 34 months			Simplex	Y - "related to brain tumor"
UMB-1445	45	Autism	Medical Records	many words and some simple familiar commands	< 5 wds total / speech not used daily			Severe MR on standardized assessments			Multiplex probably; see notes	none reported
B-7109	51	Autism	ADI-R		functional use of spontaneous language used on a daily basis	9m	N	no IQ measure; described as HFA, drove and had a job as early as age 16	N		Simplex	N

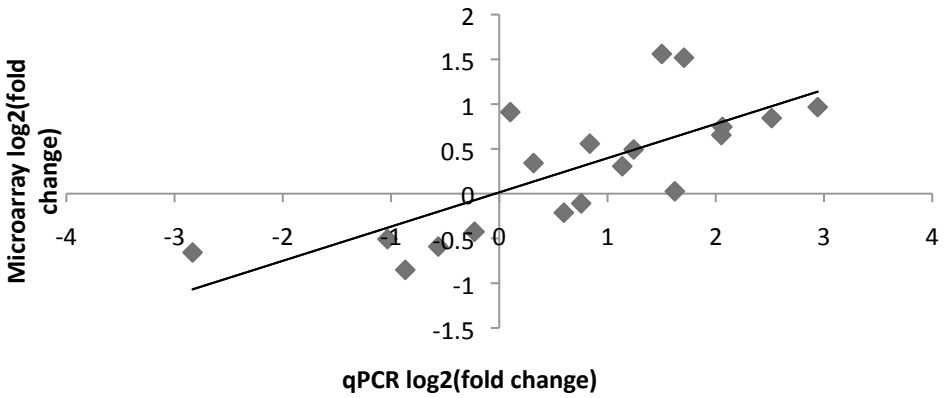
Ch3 Table S 2 B– Detailed Clinical Information of Autistic and Control Cases.

Available clinical information is provided for autistic and control cases in this study. Assessments were made by a licensed clinical psychologist (C.C.B.). HX = History; DO = Diagnosis; IVF = In Vitro Fertilization; PMI = postmortem interv

Case ID	Age	Diagnosis	Race	Hx Meds	Prenatal Hx	IVF/ Fertility Hx	Psych DO in Family	Cause of Death	PMI	Other
B6399	2	Autism	Japanese, Native American, Caucasian, Hispanic	None	Repeated C-Sec, mother miscarried twice; slight jaundice at birth, slightly blue at birth, got oxygen	N	maternal 3rd cousin with autism;			
UMB1349	5	Autism	Caucasian	repeated antibiotics due to ear infections;	fallen cervix during preganancy; occassional cigarette use during pregnancy; no alcohol use; induced labor	none reported	mother reported no psych problems in herself or child's half sibs. Father: "mood swings"; Maternal male cousin= "behavior probs, delayed speech"			marked obesity; hypogonadism; high height/weight percentile before age 1; neurologist wanted to check for chromosomal disorder
B-1469	5	Autism	Caucasian				unknown			
UMB4849	7	Autism	African-American	inpatient chelation due to lead poisoning (plumbism) @ age 3	full term, cesarean; heroine, cocaine, alcohol & cigarette use during pregnancy; donor hospitalized with withdrawal for first 3 weeks of life	none reported	younger female sib = autism			
B5666	8	Autism		Depakote for 1 year; chemotherapy	Nothing reported		paternal uncle = undx aspergers? Is "antisocial" ; paternal cousin = delays; maternal cousin = delays			
UMB4231	8	Autism	African American				None			
UMB797	9	Autism	Caucasian	desipramine (antidepressant) for ADD			None	autopsy said child mistakenly given a double dose of med 1 week before dealth and was treated in the hospital; pm toxicology revealed high level in blood at time of death but about in the normal therapeutic level, but seizures and cardiac arrhythmia are reported complications of the med		only ADI test scores available on portal, no ADI report
B-4925	9	Autism		Fluvozamine, Tegretol, Ritalin, Clonodine, Prozac, Luvox, Fenfluramine, poss. Risperdal	Nothing remarkable reported		sister1 = seizures; sister2 speech delay & learning problems; paternal brother with "long hist of social difficulties & peculiar interests", and in early 20's began having hallucinations & paranoid thoughts and is on meds for that			
UMB4899	14	Autism	Caucasian	Trileptal, 300 mg: Zoloft, 50mg; Clonidine, .06mg tid; Melatonin, hs			fraternal twin bro w/ autism			
B-7079	15	Autism						cause of death was asphyxia by hanging; PMI 23.23, brain weight 1370		
B6994	28	Autism	Caucasian	Seizure, antipsychotic, anxiety, etc. drug history including Geodon, Carbamazepine, Trileptal, methylphenidate, Buspar, Tenex, lithium, Serzone, Effexor, Zyprexa, Risperdal, Stellazine, Clonodine, Abilify, Aricept, amantadine, Ritalin, seroquel + allergy meds…	PREMIE - 32 WKS -- birth complicated by pre-eclampsia in 6th month and toxemia in 8th month, diabetes, c-section,diabetes and HPB, performed as the donor was in breech position. Born at 10 lbs, ICU for 2, days to stabilize his blood sugar and received oxygen for respiratory distress	YES - Clomid	Mother has essential tremor, is anxious and cervical dystonia & spasmodic dystonia; paternal history: nephew with behavior problems, aggression, difficulty with social interaction, impulsive behavior, learning problems and mood swings. PGM = diabetes; PGF = impulsive behavior; cousins = diff w/ social interactions, learning probs, mood swings, speech&language probs, substance abuse.	died of seizure disorder		obesity
B5173	30	Autism		Dilantin; Depakote 1750 mg/day (total dose), Tranxene bid, Cisapride 20 mg. qid, Clorazepate – mg. bid, folic acid 1 mg. qd, Lactulose; Phenobarbital & Trileptal probable	labor induced with oxytocin	N	maternal fam his of Alzheimers, Parkinsons, Depression, OCD, epilepsy; Mother dx with OCD and had a Subependymoma brain tumor (which can reoccur) removed; father abused alcohol; father, brother, and paternal half-brother IQ over 140. Full brother normal with no delays.	death from complications of gastrointestinal bleeding		child had a number of medical issues; ; umbilical cord wrapped around neck 3 times at brith very tightly; odd involuntary movements noticed at 5 months of age, seizures began at around 7 months of age; Myoclonic seizures began later along with falling episodes requiring him to wear a helmet and mouth guard all the time; neruo reported EEG quite abnormal with diffuse slowing and frequent spike discharges more prominent on the left; placed in walker at age 5 and lost ability to sit or stand unsupported by 12; self injury; feeding tube for seizure meds; never acheived bladder/bowel control; lived in nursing home; negative for fragile x

Table S2B, Continued

Case #	Age	Diagnosis	Race	Hx Meds	Prenatal Hx	IVF/ Fertility Hx	Psych DO in Family	Cause of Death	PMI	Other
B6677	30	Autism		unidentified medicaiton for continence at age 6; colipromine, zoloft, number of indentified meds for pain management of congestive heart failure; significant med history incldduing surgery for scoliosis, heart murmur, aortic insufficiency, brain surgery to remove tumor, seizures related to brain tumor; rec'd all vacinations	none reported; ADI only	none reported	mother reported she had "difficulty with social interactions"; unaffected brother	died of congestive heart failure		positve history for seizures related to brain tumor
UMB1445	45	Autism	Caucasian	none reported	no info	none reported	brother in residential facility, likely also autistic but dx not given of brother			reported to also have ALS (amyotrophic laeral sclerosis); lived in residential facility; based on social service notes seems to have one unaffected brother who visited him but could not provide the care he needed and one brother also in the residential facility;
B7109	51	Autism				none reported	Paternal fam history negative for disorders; maternal fami his significant for diabetes, migraines, and SIDS; brother with dyslexia, other brother no difficulties	died of cardiac arrest;		dx as hypoglycemic
UMB-1185	4	Control	Caucasian	atropine and Epi at death scene to revivie heart - no response	no info	no info	no info	Drowning	17	drowned at beach; HIV negative
UMB-4898	7	Control	Caucasian	Concerta & Conodine				Drowning	12	dx with hyperactive disorder and premature at 5-6 months gestation
UMB-1674	8	Control	Caucasian	none reported	no info	no info	no info	Hypothermia & Drowning	36	child fell through ice in a park; remained under ice for 60 minutes; unresponsive to intubation etc. HIV negative. (report says hyperthermia but I changed based on description)
UMB-1650	10	Control	Caucasian	none reported	no info	no info	no info	SUDC	24	dx with polymyosis (uncommon connective tissue disease with inflammation) and undergoing evaluation at time of death; HIV negative
UMB-1714	12	Control	Af-Am	none reported	no info	no info	no info	Cardiac Arrthymia	22	history of high cholestrol and enlarged heart; HIV negative
UMB-4787	12	Control	Af-Am	Singulair, Albuterol, Prednisone, & Claritin	no info	no info	no info	Asthma	15	HIV negative, history of asthma and peanut & apple allergies
UMB-4722	14	Control	Caucasian	none reported	no info	no info	no info	Multiple injuries	16	ATV accident; non smoker; HIV negative
UMB-1796	16	Control	Caucasian	no info	no info	no info	no info	Multiple injuries	16	HIV Negative; passenger in car accident, went into cardiac arrest due to multiple injuries
B-6756	16	Control	Unknown	no info	no info	no info	no info	Heart attack	22	brain weight 1230; pt. had epilepsy
B-5251	19	Control	Unknown	no info	no info	no info	no info	Pneumonia; secondary cause is respiratory infection	18.58	brain weight 1555
UMB-1649	20	Control	(Hispanic)	none reported	no info	no info	no info	Multiple injuries	22	HIV negative
UMB-818	27	Control	Caucasian	none reported	no info	no info	no info	Multiple injuries	10	car accident (driver), open container (beer) in car
B-5873	28	Control	Unknown	no info	no info	no info	no info	unknown	23.25	brain weight 1580; no known disorder, cause of dealth unknown
B-5334	30	Control	no info	no info	no info	no info	no info	no info	14.8	
B-5352	31	Control	no info	no info	no info	no info	no info	no info	33	
B-5813	41	Control	no info	no info	no info	no info	no info	no info	27	
B-4756	56	Control	no info	no info	no info	no info	no info	no info	23	
B-6860	56	Control	no info	no info	no info	no info	no info	no info	22	



Ch3 Figure S 1– Log2 Fold Change Correlations of selected genes detected by microarray and RTPCR.

Log2 Fold change detected by RTPCR is depicted on the x-axis, and change detected by microarray is on the y-axis. Spearman's rank correlation detected a R=0.78 (p=0.000075, DF=17) correlation between microarray and qPCR detection of fold change.

Ch3 Table S 3- Differentially expressed genes in young autistic cases.
All 102 differentially expressed genes in the posthoc comparison passing a threshold of $p<0.05$ between young autism and young control cases of genes showing an effect of interaction between diagnosis and categorical age. Gene symbols, Illumina probe IDs, p-Value of difference, cytogenic bands, and fold changes are listed. Genes are arranged by fold change from the greatest increase to the least increase, followed by the least decrease to the greatest decrease, similar to the organization of Figure 3A. Bolded genes are shown in Figure 3A.

Table S3: Differentially Expressed Genes of Young Autistic Cases					
#	Probe ID	p-Value	Gene Symbol	Cytoband	Fold Change
37	ILMN_1767556	0.0178242	C10orf10	10q11.21c	2.283901423
18	ILMN_1702296	0.0091403	CARTPT	5q13.2b	2.093626699
3	ILMN_2415157	0.0024493	ARID5A	2q11.2a	2.073438838
10	ILMN_1782050	0.0056193	CEBPD	8q11.21a	1.841181227
2	**ILMN_2286379**	**0.0017495**	**HYDIN**	**16q22.1f-q22.3a**	**1.769483623**
39	ILMN_1740426	0.0184694	RASD1	17p11.2g	1.747239056
34	**ILMN_1652185**	**0.0166762**	**IL4R**	**16p12.1a**	**1.74636822**
9	ILMN_1763587	0.0051548	TNMD	Xq22.1b	1.742366729
97	**ILMN_1721774**	**0.0470183**	**MPP7**	**10p11.23c**	**1.711712913**
14	ILMN_2409167	0.0075977	ANXA2	15q22.2a	1.692925858
65	**ILMN_1710514**	**0.0294053**	**BCL3**	**19q13.31b**	**1.669903233**
22	ILMN_1692332	0.0125032	ALOX12B	17p13.1c	1.657099882
87	ILMN_2391912	0.0393724	SEC14L1	17q25.2b	1.64442072
31	**ILMN_2282019**	**0.0164827**	**SYN3**	**22q12.3a**	**1.626937071**
59	ILMN_1761309	0.0266007	ADCK5	8q24.3h	1.595471013
76	ILMN_1694913	0.0340732	LMO3	12p12.3d	1.593570791
33	ILMN_2113728	0.0166613	FLJ31568	22q11.23a	1.580422773
12	**ILMN_2114422**	**0.0071293**	**NOD1**	**7p15.1b**	**1.554913981**
85	ILMN_1655713	0.0388536	OR2M7	1q44f	1.540474863
86	**ILMN_1653826**	**0.0389161**	**FGF13**	**Xq26.3d**	**1.538524147**
56	ILMN_2089616	0.0248697	FBXO10	9p13.2a	1.529865637
60	**ILMN_1773914**	**0.0266055**	**POU6F2**	**7p14.1d**	**1.525413123**
32	ILMN_2086612	0.0165765	CMAH	6p21.32	1.510486073
49	ILMN_1773271	0.0218208	SCN7A	2q24.3d	1.496558223
7	ILMN_1808810	0.0045008	CERKL	2q31.3b	1.492776302
38	**ILMN_1725175**	**0.0180504**	**FOSL2**	**2p23.2b**	**1.476985004**
95	ILMN_1664170	0.0452175	CUGBP2	10p14Bp14a	1.47148914
81	ILMN_1687495	0.0364861	SLC37A1	21q22.3b	1.461054692
54	ILMN_1753111	0.0233274	NAMPT	7q22.2c	1.453358726
67	ILMN_1811608	0.0296929	FLJ14107	8p21.3	1.443912079
82	**ILMN_2365111**	**0.0376773**	**MAP4K1**	**19q13.2a**	**1.443153441**
55	**ILMN_2319077**	**0.0243218**	**FAS**	**10q23.31b**	**1.429930875**
5	**ILMN_1803593**	**0.002886**	**WNT3**	**17q21.32a**	**1.418587714**
66	ILMN_1792094	0.0294248	TMEM63C	14q24.3c	1.418420387
75	**ILMN_1687967**	**0.0340483**	**NTRK3**	**15q25.3d**	**1.405410192**
90	ILMN_2351241	0.0404107	RNF14	5q31.3c	1.363125044
88	ILMN_1692742	0.0397669	DENND3	8q24.3c	1.34144919

Table S3, Continued					
96	**ILMN_1756705**	**0.0453123**	**CHTF18**	**16p13.3f**	**1.330898774**
24	ILMN_1720181	0.0135588	CLSTN1	1p36.22d	1.32232156
102	ILMN_1742968	0.0492937	NMNAT2	1q25.3d	1.31346127
44	**ILMN_1741465**	**0.0199732**	**PTPRH**	**19q13.42b**	**-1.361593416**
41	**ILMN_1695631**	**0.0190538**	**CHP2**	**16p12.1b**	**-1.415551164**
17	ILMN_1795673	0.0089112	LOC441476	9q34.3f	-1.418359605
64	ILMN_1815165	0.0293862	SLC28A1	15q25.3a	-1.420135748
84	ILMN_1695036	0.0383753	TPPP3	16q22.1b	-1.421260234
1	ILMN_2178994	0.0016877	TUBB8	10p15.3d	-1.434781489
77	ILMN_1775136	0.0347722	C3orf45	3p21.31b	-1.47778267
94	ILMN_1726210	0.0444314	GPIHBP1	8q24.3f	-1.502684371
36	ILMN_1785413	0.0174033	ATP2C2	16q24.1a	-1.517927532
35	ILMN_2277334	0.0167047	KCNK2	1q41a	-1.523586114
63	**ILMN_1712537**	**0.0279818**	**NODAL**	**10q22.1b**	**-1.537181567**
74	ILMN_1684742	0.0335874	TCEA2	20q13.33e	-1.547607636
50	ILMN_1754132	0.0220576	FLJ45079	17q25.3a	-1.550516956
57	**ILMN_1663390**	**0.0258167**	**CDC20**	**1p34.2a**	**-1.567522565**
69	ILMN_1673605	0.0303997	PRSSL1	19p13.3j	-1.579873391
52	ILMN_1665311	0.0231118	STH	17q21.31e	-1.580142072
98	ILMN_2102422	0.0470686	ABCB8	7q36.1d	-1.581219165
61	ILMN_1738539	0.0271168	OPLAH	8q24.3g	-1.586292139
91	**ILMN_2311089**	**0.0407431**	**BRCA1**	**17q21.31a**	**-1.603892207**
15	ILMN_2106874	0.0079741	CACNG8	19q13.42a	-1.607977761
8	**ILMN_2377980**	**0.004787**	**PPP1CA**	**11q13.1f**	**-1.621301223**
79	ILMN_1710312	0.0351176	TMEM31	Xq22.2a	-1.632386858
101	ILMN_1688646	0.0484697	LCN10	9q34.3e	-1.659630008
29	ILMN_1810092	0.0156641	TMEM179	14q32.33b	-1.659998524
62	ILMN_1667840	0.0277708	CMTM3	16q21e-q22.1a	-1.661106161
42	**ILMN_1739805**	**0.0194196**	**NDE1**	**16p13.11Bp13.11a**	**-1.669673154**
80	**ILMN_1759585**	**0.0359602**	**CHEK2**	**22q12.1c**	**-1.670873753**
46	**ILMN_1698252**	**0.0203824**	**FANCB**	**Xp22.2**	**-1.697438213**
23	**ILMN_2370588**	**0.0133007**	**TRIOBP**	**22q13.1a**	**-1.697645607**
99	ILMN_1727045	0.047567	RASGRP3	2p22.3d	-1.699373189
27	ILMN_1722070	0.0153361	APOC3	11q23.3b	-1.73837072
71	ILMN_1671362	0.0321787	UBE2J2	1p36.33a	-1.754267722
45	**ILMN_1684576**	**0.0203819**	**CLN8**	**8p23.3a**	**-1.755853888**
48	ILMN_1677108	0.0214474	CAPN13	2p23.1Bp23.1a	-1.78376345
93	**ILMN_2128931**	**0.0442292**	**FAT2**	**5q33.1d**	**-1.79130078**
30	**ILMN_2238795**	**0.0156762**	**NEK3**	**13q14.3d**	**-1.802075509**
40	ILMN_2111932	0.0190218	SERINC2	1p35.2a	-1.819803173
78	ILMN_1698060	0.0349873	FLJ43860	8q24.3d	-1.832144494
28	ILMN_2293131	0.0155328	ARHGEF1	19q13.2c	-1.838419525
83	ILMN_2349982	0.0378258	FGF1	5q31.3d	-1.873113689
26	ILMN_1772370	0.0152546	ARHGEF1	19q13.2c	-1.879996133

Table S3, Continued					
89	**ILMN_1661875**	**0.0400691**	**ANK3**	**10q21.2a**	**-1.92792007**
53	**ILMN_1750941**	**0.0232784**	**DLX4**	**17q21.33a**	**-1.93072079**
100	ILMN_1761965	0.0476763	ZNF200	16p13.3c	-1.940928777
70	ILMN_1775348	0.0308159	KCNH8	3p24.3c	-2.014486404
68	**ILMN_1790106**	**0.0303653**	**PLP1**	**Xq22.2a**	**-2.017485029**
4	**ILMN_1776936**	**0.0026995**	**ANKRD38**	**1p31.3d**	**-2.023986121**
73	ILMN_1803850	0.0333949	TSPAN15	10q21.3e	-2.068264427
25	ILMN_2208777	0.014313	NHLH2	1p13.1d	-2.068656311
47	ILMN_1795300	0.0208269	FLJ41603	5q33.1b	-2.070391027
6	ILMN_2238389	0.004419	LDB3	10q23.2a	-2.09188599
58	ILMN_1672443	0.026461	QDPR	4p15.32b	-2.13894527
51	ILMN_2175715	0.0229608	KIR2DS3	19q13.4	-2.295779916
13	**ILMN_2124585**	**0.007413**	**GREM1**	**15q13.3c-q13.3d**	**-2.305262746**
92	ILMN_1756807	0.0429335	GPR62	3p21.1e	-2.435674441
72	ILMN_1751785	0.0326038	DMRT2	9p24.3b	-2.586787043
21	ILMN_1746646	0.0121327	CHRM5	15q14a	-2.656677881
16	ILMN_1720727	0.0084141	SLCO1A2	12p12.1e	-2.765890212
19	**ILMN_1777342**	**0.0105735**	**PREX1**	**20q13.13b**	**-2.770779816**
11	**ILMN_1781356**	**0.005856**	**TSC22D3**	**Xq22.3b**	**-2.993190062**
20	**ILMN_1717381**	**0.0116866**	**HOXD1**	**2q31.1h**	**-3.063724889**
43	**ILMN_1676384**	**0.0196882**	**PCSK6**	**15q26.3d**	**-3.094558732**

Ch3 Table S 4-- Dysregulated pathways in adult autism cases.
Names of pathways and pathway categories identified by Metacore in the two groups are listed. P-values for each pathway are calculated using a hypergeometric model, and all pathways reached a threshold of corrected $p<0.05$.

Table S4 -- Top pathways dysregulated in adult autistic cases			
#	Name	pValue	Network
1	Development_Role of Activin A in cell differentiation and proliferation	6.44E-05	6/40
2	Signal transduction_PKA signaling	2.57E-04	6/51
3	Development_MAG-dependent inhibition of neurite outgrowth	4.45E-04	5/37
4	Cell cycle_Regulation of G1/S transition (part 1)	5.05E-04	5/38
5	Development_A2A receptor signaling	9.03E-04	5/43
6	Neurophysiological process_PGE2-induced pain processing	9.03E-04	5/43
7	ENaC regulation in airways (normal and CF)	2.15E-03	5/52
8	Apoptosis and survival_Endoplasmic reticulum stress response pathway	2.34E-03	5/53
9	Development_Activation of astroglial cells proliferation by ACM3	2.57E-03	4/33
10	Signal transduction_Activin A signaling regulation	2.57E-03	4/33
11	Oxidative stress_Role of ASK1 under oxidative stress	2.88E-03	4/34
12	Apoptosis and survival_Role of CDK5 in neuronal death and survival	2.88E-03	4/34
13	Fatty Acid Omega Oxidation	2.88E-03	4/34
14	Development_Role of CDK5 in neuronal development	2.88E-03	4/34
15	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	3.43E-03	7/111
16	Regulation of CFTR activity (norm and CF)	3.48E-03	5/58
17	Protein folding_Membrane trafficking and signal transduction of G-alpha (i) heterotrimeric G-protein	4.26E-03	3/19
18	Transcription_PPAR Pathway	4.33E-03	5/61
19	Transcription_P53 signaling pathway	4.76E-03	4/39
20	Development_WNT signaling pathway. Part 1. Degradation of beta-catenin in the absence WNT signaling	4.94E-03	3/20
21	Development_Neurotrophin family signaling	5.22E-03	4/40
22	Immune response_IL-15 signaling	5.32E-03	5/64
23	Transcription_ChREBP regulation pathway	5.70E-03	3/21
24	Apoptosis and survival_Lymphotoxin-beta receptor signaling	5.71E-03	4/41
25	Immune response_PIP3 signaling in B lymphocytes	6.22E-03	4/42
26	Signal transduction_AKT signaling	6.77E-03	4/43
27	Transcription_CREB pathway	7.35E-03	4/44
28	Neurophysiological process_Glutamate regulation of Dopamine D1A receptor signaling	7.96E-03	4/45
29	Reproduction_GnRH signaling	8.72E-03	5/72
30	G-protein signaling_TC21 regulation pathway	9.35E-03	3/25
31	G-protein signaling_K-RAS regulation pathway	9.35E-03	3/25
32	Immune response_IL-23 signaling pathway	9.35E-03	3/25
33	G-protein signaling_R-RAS regulation pathway	9.35E-03	3/25
34	Cytoskeleton remodeling_Neurofilaments	9.35E-03	3/25

Table S4, Continued			
35	Development_Signaling of Beta-adrenergic receptors via Beta-arrestins	1.04E-02	3/26
36	Development_A2B receptor: action via G-protein alpha s	1.15E-02	4/50
37	Chemotaxis_Inhibitory action of lipoxins on IL-8- and Leukotriene B4-induced neutrophil migration	1.23E-02	4/51
38	Development_Beta-adrenergic receptors signaling via cAMP	1.32E-02	4/52
39	Immune response_PGE2 common pathways	1.32E-02	4/52
40	Cell cycle_Role of SCF complex in cell cycle regulation	1.41E-02	3/29
41	Apoptosis and survival_p53-dependent apoptosis	1.41E-02	3/29
42	PGE2 pathways in cancer	1.59E-02	4/55
43	Apoptosis and survival_Role of IAP-proteins in apoptosis	1.69E-02	3/31
44	Inhibitory action of Lipoxins on neutrophil migration	1.79E-02	4/57
45	Apoptosis and survival_Granzyme B signaling	1.84E-02	3/32
46	Cytoskeleton remodeling_Thyroliberin in cytoskeleton remodeling	2.00E-02	3/33
47	Regulation of CFTR gating (normal and CF)	2.00E-02	3/33
48	Delta508-CFTR traffic / ER-to-Golgi in CF	2.13E-02	2/13
49	Normal wtCFTR traffic / ER-to-Golgi	2.13E-02	2/13
50	Signal transduction_Erk Interactions: Inhibition of Erk	2.17E-02	3/34
51	G-protein signaling_RhoA regulation pathway	2.17E-02	3/34
52	Inhibitory action of Lipoxin A4 on PDGF, EGF and LTD4 signaling	2.17E-02	3/34
53	Cell adhesion_Plasmin signaling	2.34E-02	3/35
54	Development_Lipoxin inhibitory action on PDGF, EGF and LTD4 signaling	2.34E-02	3/35
55	Cytoskeleton remodeling_Keratin filaments	2.52E-02	3/36
56	G-protein signaling_G-Protein alpha-12 signaling pathway	2.71E-02	3/37
57	G-protein signaling_H-RAS regulation pathway	2.71E-02	3/37
58	Cytoskeleton remodeling_ACM3 and ACM4 in keratinocyte migration	2.71E-02	3/37
59	Regulation of metabolism_Bile acids regulation of glucose and lipid metabolism via FXR	2.71E-02	3/37
60	Beta-2 adrenergic-dependent CFTR expression	2.80E-02	2/15
61	Transcription_Sin3 and NuRD in transcription regulation	2.91E-02	3/38
62	Signal transduction_cAMP signaling	2.91E-02	3/38
63	HIV-1 signaling via CCR5 in macrophages and T lymphocytes	3.11E-02	3/39
64	Development_ERBB-family signaling	3.11E-02	3/39
65	Development_PACAP signaling in neural cells	3.11E-02	3/39
66	G-protein signaling_Regulation of p38 and JNK signaling mediated by G-proteins	3.11E-02	3/39
67	G-protein signaling_Rac3 regulation pathway	3.17E-02	2/16
68	Cytoskeleton remodeling_Cytoskeleton remodeling	3.42E-02	5/102
69	Development_GH-RH signaling	3.54E-02	3/41
70	Transport_ACM3 in salivary glands	3.54E-02	3/41
71	DNA damage_Role of SUMO in p53 regulation	3.55E-02	2/17
72	Blood coagulation_GPCRs in platelet aggregation	3.66E-02	4/71
73	Neurophysiological process_HTR1A receptor signaling in neuronal cells	3.76E-02	3/42
74	Fructose metabolism	3.99E-02	4/73
75	Apoptosis and survival_FAS signaling cascades	3.99E-02	3/43

Table S4, Continued			
76	Apoptosis and survival_TNFR1 signaling pathway	3.99E-02	3/43
77	Development_EDG3 signaling pathway	3.99E-02	3/43
78	Signal transduction_JNK pathway	3.99E-02	3/43
79	CFTR-dependent regulation of ion channels in Airway Epithelium (norm and CF)	4.23E-02	3/44
80	Development_Ligand-independent activation of ESR1 and ESR2	4.23E-02	3/44
81	Development_EDG1 signaling pathway	4.23E-02	3/44
82	Cytoskeleton remodeling_Alpha-1A adrenergic receptor-dependent inhibition of PI3K	4.36E-02	2/19
83	Development_Alpha-1 adrenergic receptors signaling via cAMP	4.36E-02	2/19
84	Cell adhesion_Ephrin signaling	4.47E-02	3/45
85	Immune response_PGE2 signaling in immune response	4.47E-02	3/45
86	Development_Hedgehog signaling	4.73E-02	3/46
87	Development_GDNF family signaling	4.73E-02	3/46
88	Chemotaxis_Lipoxin inhibitory action on fMLP-induced neutrophil chemotaxis	4.73E-02	3/46
89	Development_Angiotensin activation of Akt	4.73E-02	3/46
90	Glycolysis and gluconeogenesis p. 1	4.73E-02	3/46
91	Signal transduction_PTEN pathway	4.73E-02	3/46
92	Translation_IL-2 regulation of translation	4.79E-02	2/20
93	Vitamin E (alfa-tocopherol) metabolism	4.89E-02	4/78
94	Development_Leptin signaling via PI3K-dependent pathway	4.99E-02	3/47
95	Development_PDGF signaling via MAPK cascades	4.99E-02	3/47

Ch3 Table S 5– Top 100 Downregulated genes of all male autistic cases across all young and older ages (2 to 56 years).

Top 100 downregulated genes showing a main effect of diagnosis passing a threshold of $p<0.05$ between autism and control cases, Illumina probe IDs, pValue of difference, cytogenic bands, and fold changes are listed. Highlighted genes are also presented in Figure 3C.

Table S5: Top 100 Downregulated genes of all autistic cases across all young and older ages (2-56 years)					
#	**Unique id**	**P-value**	**Symbol**	**Cytoband**	**Fold Change**
184	ILMN_2208903	0.0024303	CD52	1p36.11b	-2.410674105
152	ILMN_2052079	0.0020735	ZNF544	19q13.43c	-2.143945694
1773	ILMN_1730256	0.0413185	GNG13	16p13.3f	-2.082915908
641	ILMN_2381020	0.0103796	SLCO1A2	12p12.1e	-2.048057703
1727	ILMN_1767393	0.0395188	GRIA3	Xq25b	-2.023978355
492	ILMN_2393149	0.0078457	ALOX15B	17p13.1d	-2.015891746
343	ILMN_2379823	0.0049367	TNFRSF19	13q12.12a	-2.012101301
422	ILMN_2176625	0.0064175	LOC285735	6q23.2c	-1.992223017
443	ILMN_1757639	0.0067223	C3orf57	3q26.1b	-1.955654811
117	ILMN_1731958	0.0015902	SYT10	12p11.1b	-1.915423598
136	ILMN_1743187	0.0019092	C6orf120	6q27f	-1.912032862
119	ILMN_1663062	0.001667	CCDC67	11q21a	-1.868389293
255	ILMN_2415447	0.0035365	KCNQ4	1p34.2c	-1.815448225
1687	ILMN_1746646	0.0381878	CHRM5	15q14a	-1.766627349
1455	ILMN_1781356	0.0302485	TSC22D3	Xq22.3b	-1.763821158
1135	**ILMN_1753005**	**0.0214601**	**RELN**	**7q22.1g**	**-1.753085136**
1034	ILMN_2118773	0.0193053	ASAH2B	10q11.23b	-1.741433805
114	ILMN_2123557	0.0015271	FAM73A	1p31.1e	-1.721109387
958	ILMN_2043569	0.0168192	MOSPD2	Xp22.2	-1.7130517
191	ILMN_1765257	0.0025085	CINP	14q32.31c	-1.703683781
337	**ILMN_1651617**	**0.0048523**	**CTNNB1**	**3p22.1b**	**-1.691706334**
1649	ILMN_1786239	0.0369945	TACR1	2p13.1a	-1.689192389
710	ILMN_1732189	0.0120642	SYCE1	10q26.3f	-1.685862436
330	ILMN_2162603	0.0047823	SMPX	Xp22.12a	-1.684780938
654	**ILMN_1747129**	**0.010571**	**CASP8AP2**	**6q15d**	**-1.677791686**
618	ILMN_2054652	0.0100063	TYRP1	9p23b	-1.675826976
24	ILMN_1730917	0.0004379	KMO	1q43e	-1.667494409
105	ILMN_1733564	0.0014416	GPLD1	6p22.2b	-1.651537764
761	ILMN_1719309	0.0129776	LRRC39	1p21.2a	-1.648665058
110	ILMN_1787408	0.0014958	NUDT17	1q21.1b	-1.647011505
1485	ILMN_2049184	0.0315735	DNASE1L3	3p14.3a	-1.641646708
120	ILMN_2296011	0.0016837	BRWD1	21q22.2a-q22.2b	-1.641608276
341	ILMN_1738672	0.0049123	TRIM33	1p13.2Bp13.2a	-1.639341873
1618	ILMN_2342835	0.0360879	P2RY14	3q25.1Bq25.1c	-1.636370025
62	ILMN_1678435	0.0009251	SRD5A3	4q12d	-1.632757655

Table S5, Continued					
118	**ILMN_1653728**	**0.0016093**	**ERBB4**	**2q34c-q34e**	**-1.630507485**
590	ILMN_2189815	0.009477	SLC22A10	11q12.3b	-1.628676424
737	ILMN_1713161	0.0124575	USP16	21q21.3c	-1.628597753
1072	ILMN_1752457	0.0200432	DDX4	5q11.2e	-1.626834268
87	ILMN_2262543	0.0012252	C20orf7	20p12.1d	-1.624474811
104	ILMN_2388716	0.0014371	KCNC4	1p13.3a	-1.621522673
1036	ILMN_2405756	0.0193171	VAMP1	12p13.31d	-1.621206674
1574	ILMN_2258409	0.0344905	P2RY14	3q25.1Bq25.1c	-1.617849604
553	ILMN_2082109	0.0089075	ZNF214	11p15.4b	-1.61759371
1140	ILMN_1773485	0.0216142	QKI	6q26c	-1.617285558
434	ILMN_1675053	0.0065985	DMN	15q26.3b	-1.616979002
1862	ILMN_1665714	0.0444812	C4orf22	4q21.21c	-1.609701068
1546	ILMN_1783131	0.0335151	SAMD13	1p31.1a	-1.607617581
1223	ILMN_1679837	0.0236763	SGPP1	14q23.2b	-1.59889703
1577	ILMN_1670193	0.0345835	ACY3	11q13.2a	-1.598672992
66	ILMN_2293012	0.0009626	ADAM32	8p11.23Bp11.23a	-1.590280446
1081	ILMN_2225104	0.020231	ZNF569	19q13.12c	-1.586342253
1651	ILMN_2092232	0.0370329	TSR1	17p13.3c	-1.584913022
584	ILMN_2382974	0.0093547	CCDC7	10p11.22b	-1.583323003
1988	ILMN_1659753	0.0490985	LAMP2	Xq24d	-1.583147012
333	ILMN_2147133	0.0048134	NBPF15	1q21.1d	-1.580338152
79	ILMN_2282366	0.0011305	IQSEC3	12p13.33d	-1.579910353
1809	ILMN_2071737	0.0427711	EIF5A2	3q26.2c	-1.579101323
1644	ILMN_2237474	0.0368357	TBC1D8B	Xq22.3b	-1.578991182
1715	ILMN_2366205	0.0391057	CACNB1	17q12c	-1.574539839
149	ILMN_1753567	0.0020553	ZNF451	6p12.1a	-1.572884474
1972	ILMN_2166582	0.0485356	CAMK4	5q22.1b	-1.572301997
1147	ILMN_2305599	0.0217509	LDB3	10q23.2a	-1.569753875
1623	ILMN_1658805	0.0362422	BRE	2p23.2b	-1.569300373
1000	ILMN_1790230	0.0180802	ZNF181	19q13.11c	-1.568361777
25	ILMN_1692271	0.0004431	CCM2	7p13c	-1.563241268
1453	ILMN_2265082	0.0301822	FUSIP1	1p36.11d	-1.562064858
4	ILMN_2341711	0.0000862	ISCA1L	5q12.1d	-1.559656876
1265	ILMN_1753663	0.0246874	ARL4A	7p21.3a	-1.559389506
1525	ILMN_1785010	0.0326705	TPTE2	13q12.11a	-1.553372491
662	ILMN_1806366	0.0108019	ENOX2	Xq25h-q26.1a	-1.550674744
535	ILMN_1697168	0.0087047	PRPF4B	6p25.2a	-1.55051529
1021	ILMN_1708959	0.0189585	MFN1	3q26.32c	-1.548304332
670	ILMN_2253272	0.010993	C1orf9	1q24.3d	-1.548027528
16	ILMN_1723398	0.000354	CSNK1G1	15q22.31a	-1.547782686
1003	ILMN_2205896	0.0181499	MEIS3P1		-1.545899885
1905	ILMN_1724915	0.0458729	C6orf191	6q22.33e	-1.544661349

Table S5, Continued					
90	**ILMN_1699798**	**0.001259**	**HRAS**	**11p15.5d**	**-1.54465518**
1184	ILMN_1784099	0.0227746	VPS13A	9q21.13c	-1.54437913
37	ILMN_2328952	0.0005605	CLCC1	1p13.3c-p13.3b	-1.542775885
70	ILMN_1717541	0.0010297	CNTNAP3	9p13.1Bp13.1a	-1.540142854
1284	ILMN_2397294	0.0252059	PLCB4	20p12.2b	-1.539821305
985	ILMN_1738572	0.0175794	USP48	1p36.12b	-1.539648149
1837	ILMN_1770857	0.0434953	SYT2	1q32.1d	-1.538811603
815	ILMN_2061641	0.0138677	SGOL2	2q33.1e	-1.538810974
1010	ILMN_1664560	0.0183222	DYRK1A	21q22.13b	-1.538056832
1293	ILMN_1803490	0.0256417	CCDC34	11p14.1d	-1.53722867
1368	ILMN_1813341	0.0274475	PTGFR	1p31.1e	-1.536839634
1241	ILMN_1787988	0.024201	UBE2D1	10q21.1e	-1.536341237
1920	ILMN_2193761	0.0464745	LOC442245		-1.53565212
929	ILMN_2225151	0.0160995	DLX6	7q21.3c	-1.535440803
68	ILMN_1657818	0.0009699	IQCB1	3q13.33c	-1.535400975
975	ILMN_2386040	0.0173869	MYO19	17q12b	-1.533441485
837	ILMN_1741143	0.0142681	TXK	4p12a	-1.530777572
157	ILMN_1731729	0.0021466	OCLN	5q13.2a	-1.530025071
461	ILMN_1803217	0.0073125	KIAA0514	10q11.22a	-1.529675424
781	ILMN_1747577	0.0133895	ALAD	9q32c	-1.529076318
103	ILMN_1798952	0.001412	KDELR3	22q13.1b	-1.528886658
31	ILMN_1769245	0.0004912	GLIPR1	12q21.2a	-1.528265649
1423	ILMN_1783840	0.0294419	FLJ42986	2q11.2e	-1.527526322

Ch3 Table S 6– Top 100 Upregulated genes of autistic cases across all young and older ages (2 to 56 years).

Top 100 upregulated genes showing a main effect of diagnosis passing a threshold of $p<0.05$ between autism and control cases, Illumina probe IDs, pValue of difference, cytogenic bands, and fold changes are listed. Highlighted genes are also presented in Figure 3C.

Table S6: Top 100 Upregulated genes of autistic cases across all young and older ages (2-56 years)					
#	Unique id	P-value	Symbol	Cytoband	Fold Change
760	ILMN_1753166	0.0129621	PREI3	2q33.1b	3.163095105
253	ILMN_1715169	0.0034973	HLA-DRB1	6p21.32b	3.072859818
403	ILMN_1788874	0.0060707	SERPINA3	14q32.13b	2.941806537
487	ILMN_2206126	0.0077479	RAET1L	6q25.1b	2.633618398
972	ILMN_2074477	0.0173429	GPR4	19q13.32a	2.553466997
36	ILMN_1806607	0.0005519	SFN	1p36.11a	2.437586931
260	ILMN_2215418	0.0036355	SERPINA6	14q32.13a	2.435505988
175	ILMN_1689734	0.0023758	IL1RN	2q13d	2.362526116
53	ILMN_1737096	0.000787	CLDN10	13q32.1b	2.361541173
126	ILMN_1789162	0.0017554	MAB21L2	4q31.3a	2.324730682
209	ILMN_1755674	0.0028003	FAM12A	14q11.2b	2.311026679
10	ILMN_2309615	0.0001947	PSG6	19q13.31a	2.219710147
49	**ILMN_1771841**	**0.0007337**	**FOSL1**	**11q13.1d**	**2.210575573**
225	ILMN_2086143	0.0031126	CCR4	3p22.3c	2.208332245
339	ILMN_2313672	0.0048925	IL1RL1	2q12.1a	2.188175227
15	ILMN_1667754	0.0003509	MC3R	20q13.2d	2.186412895
1116	ILMN_1791759	0.0210929	CXCL10	4q21.1a	2.171008349
72	ILMN_1768120	0.0010429	C3orf48	3p24.3c	2.162513314
1024	ILMN_1693976	0.0189992	KRTAP9-3	17q21.2a	2.160325955
1289	ILMN_1781745	0.0253758	C9orf152	9q31.3a	2.153004856
131	ILMN_1722850	0.0018112	VGLL2	6q22.2a	2.146396291
214	ILMN_2071028	0.0028571	C3orf24	3p25.3c-p25.3b	2.139502961
767	ILMN_2275760	0.0131291	TFAP2A	6p24.3a	2.133200071
1175	ILMN_2070375	0.0226226	GPR82	Xp11.4a	2.12887513
1376	ILMN_1697069	0.0278279	CSF3	17q21.1a	2.119800211
459	ILMN_2194619	0.0072459	AMAC1L2	8p23.1b	2.116896693
881	ILMN_1716512	0.0152096	MGC70870		2.106414899
35	ILMN_1758164	0.0005274	STC1	8p21.2d	2.10637289
904	ILMN_1735737	0.0156016	SLC11A1	2q35e	2.101896093
404	ILMN_1653073	0.0060769	OR10Q1	11q12.1b	2.095635241
496	ILMN_1766401	0.0079355	OR1M1	19p13.2d	2.095066016
887	ILMN_2279645	0.0152821	BCL2L14	12p13.2a	2.083421271
99	ILMN_1779890	0.0013657	OTOA	16p12.2a	2.077906662
359	ILMN_1784884	0.0052713	LILRB3	19q13.42a	2.076099817

Table S6, Continued					
182	ILMN_1772179	0.0024228	OR2G2	1q44e	2.073002692
124	ILMN_1813776	0.001746	OR4D6	11q12.1d	2.067387091
6	ILMN_1693487	0.0001593	OR2W1	6p22.1a	2.066688293
107	ILMN_1695658	0.0014547	KIF20A	5q31.2c	2.062731605
366	ILMN_1731529	0.0053882	MS4A12	11q12.2a	2.050919002
593	ILMN_1787509	0.0095384	PRIC285	20q13.33e	2.048410321
424	ILMN_2361810	0.0064279	DRD3	3q13.31a	2.044891521
73	ILMN_1751028	0.0010487	SERPINH1	11q13.5a	2.044843707
116	ILMN_1774219	0.0015884	C2orf51	2p11.2c	2.035096123
248	ILMN_1764402	0.0033944	FAM12B	14q11.2b	2.033908337
647	ILMN_1669119	0.0104821	LOC728946		2.025835019
938	ILMN_1691264	0.0163231	NAT8B	2p13.2a	2.025108439
338	ILMN_1721916	0.0048807	LOC389151	3q22.3c	2.023243277
69	ILMN_2145490	0.0010248	LOC283677	15q24.1a	2.022440581
347	ILMN_1777917	0.00501	DSCR10	21q22.13b	2.016119774
1545	ILMN_1681576	0.0334144	IQCF2	3p21.1e	2.005690025
517	ILMN_1741165	0.008413	SLC11A1	2q35e	1.996124475
291	ILMN_1813206	0.0041172	CP	3q24f-q25.1a	1.99041984
471	ILMN_1815480	0.0074524	NAT8	2p13.2a	1.989906773
159	ILMN_1714841	0.0021933	OR4D9	11q12.1d	1.982635097
58	ILMN_1694166	0.0008844	HIST1H2AA	6p22.2a	1.977219677
267	ILMN_1655565	0.0037057	UGT3A2	5p13.2c	1.975246829
860	ILMN_2092118	0.0147335	FPR1	19q13.33e	1.975030425
17	ILMN_1714446	0.0003614	PLG	6q26a	1.973673776
410	ILMN_1780368	0.0062054	GPR18	13q32.3a	1.968653067
208	ILMN_2123719	0.0027892	LOC651503	6p22.1a	1.967328655
247	ILMN_1774685	0.0033591	IL24	1q32.1h	1.964159629
165	ILMN_1710993	0.0022502	C1orf111	1q23.3b	1.952692909
3	ILMN_1682592	0.0000638	IL19	1q32.1h	1.949276851
215	ILMN_1713927	0.002866	AMTN	4q13.3a	1.948099745
543	**ILMN_2415393**	**0.0088195**	**FSHB**	**11p14.1a**	**1.945617075**
401	ILMN_1787212	0.0060387	CDKN1A	6p21.31a	1.940516202
421	ILMN_1808674	0.0064025	CPN1	10q24.2c	1.935519723
326	ILMN_1696284	0.0046968	CLDN18	3q22.3b	1.931688688
19	ILMN_1741430	0.000385	MAGEA10	Xq28e	1.927681042
12	ILMN_1694400	0.0002441	MSR1	8p22c	1.926152655
1397	ILMN_2322768	0.0285995	CSF3	17q21.1a	1.923600388
274	ILMN_1727459	0.0037901	ORC1L	1p32.3d	1.919166103
254	ILMN_1798000	0.0035331	PSG1	19q13.31a	1.918322512
143	ILMN_1749368	0.0019862	HIST1H3H	6p22.1c	1.916871375
500	ILMN_1783969	0.0080127	FAM24A	10q26.13b	1.916612987
51	ILMN_1775879	0.00076	KRTAP19-2	21q22.11a	1.913584764

Table S6, Continued					
672	ILMN_1672076	0.0110124	WFDC10B	20q13.12b	1.906941589
192	ILMN_1801584	0.0025158	CXCR4	2q21.3b	1.902028509
396	ILMN_1773848	0.0059503	C15orf32	15q26.1d	1.90004053
50	ILMN_2256050	0.0007436	SERPINA1	14q32.13a	1.899160712
173	ILMN_1697466	0.002367	OR5AY1	1q44e	1.898447824
529	ILMN_1686245	0.0085856	OR6Q1	11q12.1b	1.895309148
1275	ILMN_1711111	0.0249492	SLC9A10	3q13.2a	1.893507324
71	ILMN_1701376	0.0010374	MGC34821	11q12.3b	1.893141053
1288	ILMN_1813561	0.025332	SCIN	7p21.3a	1.8816685
342	ILMN_1705551	0.0049136	OR56A4	11p15.4c	1.876572144
170	ILMN_2168421	0.0023309	SPATA8	15q26.2c	1.875293706
453	ILMN_1749341	0.0069923	LOC554226		1.872041954
67	ILMN_1793182	0.0009661	SLC36A2	5q33.1d	1.869309791
1986	ILMN_2138801	0.0490656	TP73L	3q28b	1.866656901
280	ILMN_2357419	0.0039279	LILRA5	19q13.42a	1.865735075
1317	ILMN_2205012	0.0263422	SFTPA2	10q22.3f	1.864205816
93	ILMN_1738691	0.0013038	POU4F1	13q31.1a	1.863802677
648	ILMN_1811289	0.0104964	COL4A6	Xq22.3c	1.863073275
64	ILMN_1775257	0.0009608	PROK2	3p13d	1.862821045
384	ILMN_1731928	0.0057217	LY9	1q23.3a	1.861813297
320	ILMN_1708248	0.0046579	LILRB1	19q13.42a	1.85726951
1615	ILMN_1663793	0.0360043	MIST	4p16.1a	1.856856476
649	ILMN_1652137	0.0105149	PPYR1	10q11.22a	1.854606176
491	ILMN_1656802	0.0078408	LOXL2	8p21.3a	1.853159173

Ch3 Table S 7– Top 10 dysregulated pathways in all autistic cases (ages 2 to 56 years).

The top 10 dysregulated pathways in all autistic cases, p-value, network objects, and genes within each pathway, are listed.

#	Name	pValue	Network objects	Genes in Pathway
1	Development_A2A receptor signaling	1.70E-07	11/43	Adenosine A2a receptor, BAD, Elk1, G-protein alpha s, H-Ras, NF-kB, pard6, PI3K cat class IA, PKA-cat, PKC-zeta, p38 MAPK
2	Development_A2B receptor: action via G-protein alpha s	7.05E-06	10/50	Beta-catenin, Elk1, G-protein alpha-s, H-Ras, NF-kB, Pi3k cat class IA, PKA-cat, PLC-beta, Pyk2, p38 MAPK
3	Transcription_P53 signaling pathway	4.90E-05	8/39	Beta-catenin, MDM2, MMP-2, NF-kb, PIAS2, SUMO-1, XPA, p21
4	Transcription_CREB pathway	1.21E-04	8/44	CA(II) channels, CAMK IV, DREAM, G protein alpha-s, H-Ras, PI3K cat class IA, PKA-cat, p38 MAPK
5	Development_Thrombopoietin-regulated cell processes	1.43E-04	8/45	BAD, C3G, Elk1, H-Ras, PI3K cat class IA, PKC-zeta, Survivin, p21
6	Development_PIP3 signaling in cardiac myocytes	1.97E-04	8/47	14-3-3, BAD, H-Ras, HGF, Hamartin, PARD6, PI3K cat class IA, PKC-zeta
7	Cell adhesion_ECM remodeling	4.04E-04	8/52	CD44, collagen IV, ErbB4, MMP2, Nidogen, PAI1, PLAU, TIMP1
8	Cell cycle_Role of 14-3-3 proteins in cell cycle regulation	8.20E-04	5/22	14-3-3 sigma, 14-3-3 zeta/delta, CDC25A, CDCD25C, p38 MAPK
9	Cytoskeleton remodeling_Cytoskeleton remodeling	8.41E-04	11/102	Beta-catenin, collagen IV, H-Ras, MELC, MyHC, PAI1, PI3K cat class IA, PLAU, eIF4E, p21, p38 MAPK
10	Reproduction_GnRH signaling	8.58E-04	9/72	Elk-1, FSH-beta, Follistatin, G protein alpha-S, H-Ras, MKP-2, PKA-cat, PLC beta, Pyk2

Ch3 Table S 8– Top 100 dysregulated pathways of autistic cases across all young and older ages (2 to 56 years).

Top 100 dysregulated pathways enriched in genes showing a main effect of diagnosis ($p<0.05$) identified using the Metacore® software suite as passing a threshold of $p<0.05$ between autism and control cases, p-value, and network objects are listed. Pathways are sorted by pathway category name. Continued from Figure 3D.

Table S8: Top 100 dysregulated pathways of autistic cases across all young and older ages (2 to 56 years)			
#	Name	pValue	Network
68	Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	1.36E-02	5/41
24	Apoptosis and survival_BAD phosphorylation	3.18E-03	6/42
79	Apoptosis and survival_Granzyme A signaling	1.97E-02	4/30
60	Blood coagulation_Blood coagulation	1.10E-02	5/39
44	Cardiac Hypertrophy_NF-AT signaling in Cardiac Hypertrophy	7.36E-03	7/65
52	Cell adhesion_Cell-matrix glycoconjugates	9.90E-03	5/38
22	Cell adhesion_Chemokines and adhesion	2.54E-03	10/100
7	Cell adhesion_ECM remodeling	4.04E-04	8/52
61	Cell adhesion_Endothelial cell contacts by junctional mechanisms	1.20E-02	4/26
30	Cell adhesion_Histamine H1 receptor signaling in the interruption of cell barrier	4.52E-03	6/45
20	Cell adhesion_PLAU signaling	2.16E-03	6/39
8	Cell cycle_Role of 14-3-3 proteins in cell cycle regulation	8.20E-04	5/22
97	Chemotaxis_CXCR4 signaling pathway	2.99E-02	4/34
74	Chemotaxis_Leukocyte chemotaxis	1.56E-02	7/75
83	Chemotaxis_Lipoxin inhibitory action on fMLP-induced neutrophil chemotaxis	2.16E-02	5/46
9	Cytoskeleton remodeling_Cytoskeleton remodeling	8.41E-04	11/102
50	Cytoskeleton remodeling_RalB regulation pathway	9.36E-03	3/13
17	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	1.69E-03	11/111
1	Development_A2A receptor signaling	1.70E-07	11/43
2	Development_A2B receptor: action via G-protein alpha s	7.05E-06	10/50
15	Development_A3 receptor signaling	1.46E-03	7/49
46	Development_Activation of ERK by Kappa-type opioid receptor	7.87E-03	5/36
35	Development_Angiotensin activation of ERK	5.40E-03	5/33
94	Development_BMP signaling	2.71E-02	4/33
96	Development_EDNRB signaling	2.98E-02	5/50
87	Development_EGFR signaling pathway	2.24E-02	6/63
54	Development_Endothelin-1/EDNRA signaling	1.01E-02	6/53
58	Development_ERBBfamily signaling	1.10E-02	5/39
33	Development_G-Proteins mediated regulation MARK-ERK signaling	5.04E-03	6/46
34	Development_GDNF family signaling	5.04E-03	6/46
16	Development_GM-CSF signaling	1.65E-03	7/50
48	Development_Hedgehog and PTH signaling pathways in bone and cartilage	8.85E-03	5/37
84	Development_Hedgehog signaling	2.16E-02	5/46
14	Development_HGF signaling pathway	1.13E-03	7/47
32	Development_HGF-dependent inhibition of TGF-beta-induced EMT	4.71E-03	5/32
18	Development_IGF-1 receptor signaling	1.85E-03	7/51
55	Development_Leptin signaling via JAK/STAT and MAPK cascades	1.04E-02	4/25
66	Development_Neurotrophin family signaling	1.23E-02	5/40
25	Development_Osteopontin signaling in osteoclasts	3.53E-03	5/30
6	Development_PIP3 signaling in cardiac myocytes	1.97E-04	8/47
72	Development_Prolactin receptor signaling	1.54E-02	6/58
73	Development_Regulation of CDK5 in CNS	1.55E-02	4/28
90	Development_Regulation of epithelial-to-mesenchymal transition (EMT)	2.40E-02	6/64
64	Development_Role of Activin A in cell differentiation and proliferation	1.23E-02	5/40
11	Development_Role of IL-8 in angiogenesis	8.58E-04	8/58

Table S8, Continued			
45	Development_TGF-beta receptor signaling	7.62E-03	6/50
37	Development_TGF-beta-dependent induction of EMT via MAPK	5.62E-03	6/47
41	Development_Thrombopoetin signaling via JAK-STAT pathway	6.54E-03	4/22
5	Development_Thrombopoietin-regulated cell processes	1.43E-04	8/45
91	Development_Transcription regulation of granulocyte development	2.45E-02	4/32
92	DNA damage_ATM/ATR regulation of G1/S checkpoint	2.45E-02	4/32
21	DNA damage_Role of SUMO in p53 regulation	2.44E-03	4/17
38	G-protein signaling_G-Protein alpha-q signaling cascades	6.15E-03	5/34
65	G-protein signaling_Rap1A regulation pathway	1.23E-02	5/40
75	Immune response _Immunological synapse formation	1.66E-02	6/59
57	Immune response_CD28 signaling	1.10E-02	6/54
28	Immune response_ETV3 affect on CSF1-promoted macrophage differentiation	4.09E-03	5/31
62	Immune response_Fc epsilon RI pathway	1.20E-02	6/55
56	Immune response_IFN gamma signaling pathway	1.10E-02	6/54
86	Immune response_IL-3 activation and signaling pathway	2.20E-02	4/31
26	Immune response_IL-4 - antiapoptotic action	3.53E-03	5/30
77	Immune response_IL-4 signaling pathway	1.81E-02	5/44
80	Immune response_Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha	1.98E-02	5/45
12	Immune response_MIF - the neuroendocrine-macrophage connector	9.95E-04	7/46
99	Immune response_Oncostatin M signaling via JAK-Stat in human cells	3.12E-02	3/20
89	Immune response_Oncostatin M signaling via JAK-Stat in mouse cells	2.35E-02	3/18
81	Immune response_PGE2 signaling in immune response	1.98E-02	5/45
98	Immune response_Role of the Membrane attack complex in cell survival	2.99E-02	4/34
67	Immune response_TREM1 signaling pathway	1.31E-02	6/56
47	Membrane-bound ESR1: interaction with G-proteins signaling	8.39E-03	6/51
93	Muscle contraction_ GPCRs in the regulation of smooth muscle tone	2.59E-02	7/83
39	Muscle contraction_Relaxin signaling pathway	6.24E-03	6/48
43	Neurophysiological process_Long-term depression in cerebellum	6.90E-03	6/49
85	Neurophysiological process_NMDA-dependent postsynaptic long-term potentiation	2.16E-02	7/80
76	NGF activation of NF-kB	1.75E-02	4/29
23	Proteolysis_Putative SUMO-1 pathway	3.03E-03	5/29
29	Regulation of lipid metabolism_Regulation of lipid metabolism by niacin and	4.52E-03	6/45
51	Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and	9.90E-03	5/38
10	Reproduction_GnRH signaling	8.58E-04	9/72
82	Role of alpha-6/beta-4 integrins in carcinoma progression	1.98E-02	5/45
49	Signal transduction_Activation of PKC via G-Protein coupled receptor	9.21E-03	6/52
95	Signal transduction_Activin A signaling regulation	2.71E-02	4/33
27	Signal transduction_AKT signaling	3.58E-03	6/43
19	Signal transduction_cAMP signaling	1.88E-03	6/38
42	Signal transduction_IP3 signaling	6.90E-03	6/49
100	Signal transduction_PKA signaling	3.21E-02	5/51
13	Signal transduction_PTEN pathway	9.95E-04	7/46
31	Transcription_Androgen Receptor nuclear signaling	4.52E-03	6/45
36	Transcription_ChREBP regulation pathway	5.50E-03	4/21
4	Transcription_CREB pathway	1.21E-04	8/44
3	Transcription_P53 signaling pathway	4.90E-05	8/39
78	Transcription_PPAR Pathway	1.94E-02	6/61
59	Transcription_Receptor-mediated HIF regulation	1.10E-02	5/39
40	Transcription_Role of heterochromatin protein 1 (HP1) family in transcriptional	6.54E-03	4/22
53	Translation _Regulation of EIF4F activity	1.01E-02	6/53
70	Translation_Insulin regulation of translation	1.50E-02	5/42
63	Translation_Non-genomic (rapid) action of Androgen Receptor	1.23E-02	5/40
88	Transport_Alpha-2 adrenergic receptor regulation of ion channels	2.35E-02	5/47
71	Transport_FXR-regulated cholesterol and bile acids cellular transport	1.50E-02	5/42
69	wtCFTR and deltaF508 traffic / Late endosome and Lysosome (norm and CF)	1.41E-02	3/15

Ch3 Table S 9- References for genes discussed in Chapter 3.
Genes discussed in the gene expression and CNV analysis portions of the main text are listed. Gene category, references, and analysis corresponding to these genes are presented.

Table S9 - References for genes presented in main text			
Gene	**Category**	**Reference**	**Analysis**
FAS	Apoptosis	Sancho-Martinez and Martin-Villalba 2009	gEx young vs young
BCL3	Apoptosis	Brocke-Heidrich et al., 2006	gEx young vs young
NOD1	Apoptosis	Jae Gyu et al., 2010	gEx young vs young
FAT2	Apoptosis	Cavallaro et al., 2004	gEx young vs young
PLP1	Apoptosis	Southwood and Gow, 2001	gEx young vs young
CLN8	Apoptosis	Vantaggiato et al., 2009	gEx young vs young
PTPRH	Apoptosis	Takada et al., 2002	gEx young vs young
BAD	Apoptosis	Danial 2008	gEx diagnosis main effect
CASP8	Apoptosis	Frisch 2008	gEx diagnosis main effect
CASP10	Apoptosis	Muhlethaler-Mottet et al., 2011	gEx diagnosis main effect
MDM2	Apoptosis	Manfredi 2010	gEx diagnosis main effect
TSC22D3	Apoptosis	Soundararajan et al., 2007	gEx young vs young
PPP1CA	Apoptosis, cell division and proliferation	Yu et al., 2008; Luo et al., 2007	gEx young vs young
ROBO1	Axon guidance	Devine and Key 2008	gEx diagnosis main effect
NEK3	Cell cycle and division	Tanaka and Nigg, 1999	gEx young vs young
BRCA1	Cell cycle and division	Wang 2007	gEx young vs young
CHEK2	Cell cycle and division	Sato et al., 2010	gEx young vs young
FANCB	Cell cycle and division	Wang 2007	gEx young vs young
CHP2	Cell cycle and division	Cam et al., 2009	gEx young vs young
CDC20	Cell cycle and division	Yu et al., 2007	gEx young vs young
TRIOBP	Cell cycle and division	Yu et al., 2008	gEx young vs young
ANKRD38	Cell cycle and division	Kakinuma et al., 2009	gEx young vs young
FOSL2	Cell cycle and division	Maaser et al., 2008	gEx young vs young
CHTF18	Cell cycle and division	Hanna et al., 2001	gEx young vs young
CDC25	Cell cycle and division	Ivanova et al., 2011	gEx diagnosis main effect
ATRX	Cell cycle and division	De La Fuente et al., 2004	gEx diagnosis main effect
CTNNB1	Cell cycle and division	Chenn and Walsh 2002	gEx diagnosis main effect
p21	Cell cycle and division	Jung et al., 2010	gEx diagnosis main effect
14-3-3 (YWHAZ)	Cell cycle and division, neuronal migration	Toyo-oka et al., 2003; Darling et al., 2005	gEx diagnosis main effect
BUBR1	Cell cycle_Mitosis and S Phase	Harris et al., 2005	CNV
CCNB1 (cyclin B)	Cell cycle_Mitosis and S Phase	Soni et al., 2008	CNV
RAD51	Cell cycle_Mitosis and S Phase	Scully et al., 1997	CNV
CRM1	Cell cycle_Mitosis and S Phase	Arnaoutov et al., 2005	CNV
RFC2	Cell cycle_Mitosis and S Phase	Noskov et al., 1998	CNV
LIS1	Cell cycle_Mitosis and S Phase	Yingling et al., 2008	CNV
CLIP170	Cell cycle_Spindle and cytoplasmic microtubules	Brunner and Nurse 2000; Wittmann and Desai 2005	CNV

Table S9, Continued			
Alpha tubulin	Cell cycle_Spindle and cytoplasmic microtubules	Rosenbaum 2000	CNV
DNAL1	Cell cycle_Spindle and cytoplasmic microtubules	Horvath et al., 2005	CNV
ROD	Cell cycle_Spindle and cytoplasmic microtubules	Famulski and Chan 2007	CNV
Dynein	Cell cycle_Spindle and cytoplasmic microtubules	Bader and Vaughan 2010; Hirokawa et al., 2010	CNV
PTEN	Cell proliferation	Endersby and Baker, 2008; Bulter et al., 2005; Page et al., 2009	gEx diagnosis main effect
PRKACB	Cell Proliferation	Stork and Schmitt, 2002; Cohen 2003; Tasken and Aandahl, 2004	gEx diagnosis main effect
PRKCZ	Cell Proliferation	Reyland 2009	gEx diagnosis main effect
TSC1	Cortical lamination and neuronal migration	Hay 2005; Orlova and Crino 2010	gEx diagnosis main effect
MMP2	Cytoskeleton and extracellular matrix remodeling	Lukes et al., 1999; Rauch 2004	gEx diagnosis main effect
NID1	Cytoskeleton and extracellular matrix remodeling	Miosge et al., 2001	gEx diagnosis main effect
TIMP1	Cytoskeleton and extracellular matrix remodeling	Lukes et al., 1999; Stamenkovic 2003	gEx diagnosis main effect
COL4A3	Cytoskeleton and extracellular matrix remodeling and Development	Zagris 2001	gEx diagnosis main effect
ErbB4	Cytoskeleton and extracellular matrix remodeling, development	Chong et al., 2008; Stamenkovic 2003	gEx diagnosis main effect
HGF	Development	Achim et al., 1997	gEx diagnosis main effect
FGFRL1	Development	Trueb and Taeschler 2006	gEx diagnosis main effect
ADORA2A	Development and apoptosis	Huang et al., 2001	gEx diagnosis main effect
FGF13	Neural patterning and differentation	Nishimoto et al., 2007	gEx young vs young
DLX4	Neural patterning and differentation	Depew et al., 2005	gEx young vs young
GREM1	Neural patterning and differentation	Hsu et al., 1998	gEx young vs young
NDE1	Neural patterning and differentation	Feng and Walsh 2004	gEx young vs young
NODAL	Neural patterning and differentation	Schier et al., 2003	gEx young vs young
HOXD1	Neural patterning and differentation	Zakany et al., 2001	gEx young vs young
PCSK6	Neural patterning and differentation	Constam and Robertson, 2004	gEx young vs young
NTRK3	Neural patterning and differentation	Bartkowska et al., 2007	gEx young vs young
POU6F2	Neural patterning and differentation	Zhou et al., 1996	gEx young vs young
WNT3	Neural patterning and differentation	Lee et al., 2000	gEx young vs young
Hydin	Neurodevelopment	Dawe et al., 2007	gEx young vs young
RELN	Neuronal migration	Tissir and Goffinet 2003	gEx diagnosis main effect
PAK1	Neuronal migration	Causeret et al., 2009	gEx diagnosis main effect

Chapter 4 - Conclusion

Summary.

In summary, using novel expression profiling technology and systematically-tested statistical methods, we also found gene expression abnormalities of pathways related to cell cycle, cell proliferation, apoptosis, and neuronal migration. These abnormalities may be consistent with the brain overgrowth trajectory in autism and other neuropathological phenotypes as identified in Chow, Pramparo, Boyle et al. (*in review*).

Exploration of statistical methods for preprocessing of DASL-based expression profiling on brain tissue-extracted RNA.

We investigated the relative efficacy ofthe DASL-based expression profiling approach with the IVT-based approach for profiling genome-wide expression in degraded RNA from reference and tissue sources. Subsequently, a preprocessing pipeline specific for brain tissue RNA profiled by DASL was characterized systematically. Following preprocessing, differential expression and pathway analyses were performed. These changes were verified by RT-PCR, showing a significant correlation between microarray profiling and the gold standard approach.

DASL (cDNA-mediated selection, ligation) and RNA quality.

Despite the poor quality of RNA extracted from frozen brain tissue with freeze-thaw artifacts or of artificially degraded reference RNA, the DASL protocol provided a reliable and efficient method of utilizing microarray technology for whole genome expression assaying, superior to conventional methods such as IVT(Fan et al., 2004).

With poor quality RNA, there is a risk of inadvertent spurious results when conventional methods are used to prepare samples for microarray assays. To preclude false reporting due to poor quality tissue, some published studies on autism have excluded cases with poor RNA quality. This led to smaller sample sizes and thus decreased statistical power. Other studies which profiled RNA of questionable quality processed by weaker expression methodology may have confounded degradation and real gene expression effects.

We chose to use DASL, which we show to produce reliable microarray results in comparison to IVT through artificial degradation of reference RNA experiments. Though the results from the tissue-extracted RNA experiment were not conclusive (DASL recovered 1 sample that IVT failed to profile), we may have increased the probability of a successful microarray experiment through using this platform. Thus, we increased the size of our sample, which is important for generalizability and statistical analysis.

We were able to analyze both frozen and fixed hemispheres of a single case, UMB 1796, using both IVT and DASL methodology. The DASL approach using the frozen tissue was the only method that created a reasonable dynamic range and distribution for further analysis. However, even using the DASL approach, the correlation between frozen and fixed samples were very low. The dynamic range of frozen and formalin fixed samples profiled by the IVT-based method was extremely limited. The small sample size prevents us from making conclusive statements about the relative efficacy of DASL- and IVT-based approaches on tissue-extracted RNA. However, the distributions of the data gleaned from the DASL approach do suggest that results may reflect the degraded reference RNA experiments. This result cautions the comparison between tissue preservation techniques and between platforms.

Nonetheless, due to the high reliability of our microarray results using the DASL-based approach, we can confidently compare between autistic and control cases. Furthermore, due to our relatively large sample of postmortem cases, there is a greater chance that our results may be generalized to other autistic individuals.

RASL (RNA-mediated selection, ligation).

Since DASL failed to produce reliable, high quality quality gene expression results on fixed brain tissue, we planned to further investigate the viability of profiling gene expression on this tissue with RNA-mediated annealing, selection and ligation (RASL). The distinction between DASL and RASL is a reverse transcription step that is

unsuccessful in severely degraded, low quantity RNA samples. However, since new RASL oligonucleotides would have to be synthesized in order for a whole genome investigation to be performed whereas DASL had recently become a commercially available product, and cDNA is more stable and easier to work with than RNA, we began with DASL.

Further, due to the instability of RNA and its susceptibility to freeze-thaw artifacts, DASL should be used whenever possible. Preliminary results from small numbers of samples on a subset of genes using RASL appear promising (Li, unpublished data).

Data Preprocessing.

Since the DASL approach had never before been utilized with brain tissue, we explored different methods of preprocessing the data in order to remove systemic experimental error and to prepare the microarray data for differential expression and other downstream analyses. We indeed found that not all normalization and transformation methods produced a dataset that would be suitable for downstream statistical analyses aimed toward deciphering expression differences between two groups (Lim et al., 2007; Schmid et al., 2010). We identified several processing procedures that were apt for our purposes, which included transformation, normalization, and dataset correction steps with multiple outcome measures at each step for quality control.Namely,

these procedures created a dataset that following a normal distribution with high reliability between technical replicates so that analyses of variance would be able to pull out differences of diagnosis.

As discussed in the introduction, premortem, agonal and other postmortemconditions of the donor and the tissue may be responsible for a portion of the differentially expressed genes and greatly confound studies of neuropsychiatric disorders. Disorders like autism and schizophrenia are especially challenging due to the heterogeneous nature of autism that arises from behavioral diagnostic criteria. Furthermore, the deaths of many autistic patients were due to drowning, which was not well-matched with respective controls. However, since postmortem brain tissue is so scarce and precious, especially from younger ages, it is difficult to control for such factors.

Another confounding factor that is not often considered has to do with the microarray experiment itself. Though DASL and other platforms of gene expression profiling often produce highly reliable replicates within the same processing session, but systematic differences in labeling, hybridization, and other variables within different processing sessions are less reliable. We demonstrated this principle using the tissue-extracted RNA. Samples within the same batch tended to cluster together, separately from samples in different batches. Their correlations with the samples in the other batches tended to be lower than those within the same batch. Even the intensity distributions across the probes on the array were distinctly different.

To contend with these two confounds, we applied batch and covariate correction using ComBat, a statistical program by Johnson et al. (2007), on our dataset. We identified samples in the two processing batches that were run and found that ComBat indeed removed variance attributable to batch using MDMR (Zapala and Schork 2006). In addition, because seizures are an important confounding comorbid condition in autism, we also corrected for this variable using clinical information gleaned from medical history reports from the Autism Tissue Program. This variable is so important that MDMR determined it was the primary deciding factor of variance in the dataset. We also found that ComBat reduced the proportion of variance explained by seizures. However, it did not demote seizures from being the number 1 predictor of variance in the gene expression dataset. Although it would be disastrous to introduce new or incorrect variance into the dataset, it appears that perhaps other statistical methods should be applied for this purpose. Future studies will have tocarefully examine these factors in expression profiling for elucidation of mechanisms in neuropsychiatric disorders.

Gene expression results and considerations for interpretation.

We identified expression abnormalities in cellular proliferation, apoptosis, cell cycle, and neural patterning functions in the young autistic brain. These abnormalities were also apparent in older autistic brains. Aberrance in these types of functions may logically lead to brain overgrowth in autism, the heterogeneous anterior/posterior

gradient of neuropathology, and the functional asymmetry that is observed in autistic children. We acknowledge the important considerations in interpreting our results.

It was hypothesized that early brain overgrowth results from dysregulation of multiple processes, including neurogenesis, cell cycle, and apoptosis (Courchesne et al., 2001; Courchesne et al., 2007). The gene expression data indeed points to aberrancies in these functions in the young autistic brain. This dysregulation results in overproliferation of neurons, which may lead to the overgrown autistic brain in the early years of life. Abnormalities in these functions may not be homogeneous throughout the cortex, but only in discrete portions of cortex, which may lead to the patch phenotype observed in young autistic cases (Chow, Pramparo and Boyle, *in review*).

We also hypothesized that some reactive changes also occur in the autistic cortex: the immune dysregulation observed in our whole genome expression analyses independent of age may result from formative abnormalities. It remains to be shown, however, how the balance between the generative and reactive processes causes the behavioral and cognitive phenotypes in the living autistic child.

One obvious interpretational difficulty associated with postmortem gene expression studies is that perhaps the gene expression abnormalities that precipitated aberrant prenatal neurodevelopment has already occurred and the dysregulation of transcript product is no longer in the tissue because the autistic individual has matured past the ages of brain development. Thus, we may be witnessing the molecular aftermath

of the initial insult that caused our subjects to have autism despite the young postnatal ages of our sample. This possibility, perhaps, creates a rationale for *in utero* testing for autism biomarkers.

We were fortunate to be able to study the brains of children from two years of age, during the period of brain overgrowth. Since older cases with autism show a distinctly different pattern of dysregulated processes and networks, it is evident that age plays an important role in the biology of the brain in autism. Though it was possible that even at age 2, the signs of the initial insult in autism have disappeared, we saw evidence of dysregulation of neurogenesis, apoptosis, and other processes that may be important in the formation of the brain postnatally. Nonetheless, even if postmortem brain tissue studies are unable to identify causative gene expression abnormalities, they are invaluable for identifying biomarkers and 'aftermath' genes that remain dysregulated and cause behavioral and cognitive aberrancies. When these genes are identified, therapeutics may be produced to correct the abnormality and perhaps treat certain symptoms in a targeted fashion.

Furthermore, though we were able to take into account the incidence of seizures in our autistic cases, there are many other subject factors affecting brain gene expression that were not controlled for. These factors include antemortem medications and intellectual disability. Due to inadequate information from medical records of the postmortem cases as well as small sample sizes, we were unable to statistically account for these confounds without possibly introducing artificial variance into the dataset.

Thus, we interpret our results with some caution, and will examine the roles of these factors in future investigations.

Due to the heterogeneity of autism, comparisons of autism versus control cases in whole genome expression studies could be more informative if clinical information was available to parse the sample into subgroups. Hu and Steinberg(Hu and Steinberg, 2009)recently used a novel algorithm to cluster the phenotypes within the ADI-R for gene expression analyses of lymphoblastoid cell lines. The four clusters that were identified included one with severe language deficits; the second presented with mild symptoms across ADI-R domains; the third included individuals with high frequency of savant skill; and a fourth presented with intermediate symptoms. We were unable to adequately perform similar analyses due to small sample size and missing ADI-R data and inconsistent medical records for many of our subjects. Though addressing this issue may require coordinated effort between doctors, brain banks, and scientists, it will be imperative to utilize the behavioral and cognitive phenotypes to subtype individuals who fall within the category of autistic disorder.

Moreover, it is possible that the best biomarker for autism is an aberrant brain phenotype, thus the combination of imaging and genetic information should be obtained for diagnostic screening. The goal of this analysis would be to predict clinical factors such as treatment responsiveness, especially as the field of pharmacogenomics moves forward.

Similarities with other disorders.

In the introduction to this document, I reviewed disorders that have already been shown to have an increased rate of ASD symptoms. Our gene expression results yielded molecular evidence in support of common mechanisms between autism and tuberous sclerosis.

In the genome-wide expression analyses, we find that the TSC1 gene is downregulated across autistic cases regardless of age. 10-30% of individuals with tuberous sclerosis have a mutation in this gene (Crino et al., 2006). These mutations affect multiple organ systems, including the central nervous system. Mutations in TSC1 are also known to cause focal cortical dysplasia type II (Becker et al., 2002). Focal lesions are evident in both focal cortical dysplasia and tuberous sclerosis. Given the similarities between the neuropathological phenotypes, dysregulation of TSC1, and clinical features of autism and tuberous sclerosis, it is possible that the disorders share some common pathogenic mechanisms. In fact, Gillberg (2005) reports that tubers in frontal and temporal areas predispose individuals to autistic behaviors. Perhaps the cascades of genetic pathways that are affected by a downregulation of TSC1 are the culprits leading to the focal, patchy nature of the neuropathology in autism and tuberous sclerosis (Chow, Pramparo and Boyle, *in review*).

Other neural patterning genes that have been found to play pathogenic roles in a variety of human genetic disorders also differentially expressed in our autistic cohort

such as reelin and nodal may also provide clues for understanding common mechanisms between these disorders and autism.

Similarities with mouse models.

Animal models that show similarities to the abnormalities we identified in the autistic cortex may also elucidate possible mechanisms giving rise to aspects of the phenotype.

The pattern of cortical organization abnormality, gyrification aberrance (Levitt et al., 2003)and brain overgrowth (Courchesne et al., 2007) in autism is reminiscent of a mouse model of beta-catenin overexpression (Chenn and Walsh, 2002). An increase in the number of neurons, as well as brain size, in the mice is evident due to an expanded population of neural progenitor cells. In addition, an increase in the number of ectopic neurons as well as a decrease in the organization of cytoarchitecture is notable. More importantly, where normal mice lack cortical folds, these beta-catenin mice exhibited folding and buckling of the cortex. These abnormalities were not reported to be homogenous throughout cortex, however, and these mice were anecdotally more easily startled and aggressive, which may be related to symptoms that some autistic individuals have. The Wnt pathway was, in fact, implicated as aberrant in autism in our gene

expression analysis. Beta-catenin was detected as differentially expressed between all autistic and all control cases (Ch3 Figure 3C).

In a related study of the WNT/Notch1 pathway in brain samples of focal cortical dysplasia (FCD) patients, a decrease in nuclear expression, but similar cytoplasmic levels of beta catenin was found in large hypertrophic neurons (Cotter et al., 1999). FCD has been associated with intractable epilepsy (Palmini et al., 1995), so the high incidence of seizures in autistic individuals is consistent with this type of cortical malformation. Thus, perhaps beta catenin or other Wnt-pathway members plays a role in the malformation of the autistic cortex (De Ferrari and Moon, 2006). In the gene expression study, the WNT pathway appears to be dysregulated in the comparison between the young autism group and the young control group; perhaps during postnatal brain development, it is the dysregulation of these pathways that creates aberrant cortical formation in autism (Chow, Pramparo and Boyle et al., *in review*).

The PTEN mouse model is another that may directly inform mechanisms in producing an enlarged brain (Backman et al., 2001). This pathway was implicated in the gene expression analysis. Deletions of the PTEN gene, which negatively regulates PI3K signaling, are prone to seizures and showed brain enlargement. Furthermore, granule cell dysplasia in the cerebellum and dentate gyrus are observed in these mice. Interestingly, undulations in the hippocampus reminiscent of the polymicrogyric regions in our autistic cases have been identified in the PTEN knockout (Figure 7D; Backman et al., 2001). The

autistic brain is well known to show overgrowth during the first years of life (Courchesne et al., 2007). However, the brain tends to decrease in size over time and this observation suggests that a more complex genetic mechanism, which includes timing of pathway activation than the static, state the PTEN model shows. However, it is notable that the cerebellum may be a common pathological location between, and seizures a common side effect of both autism and this model. Furthermore, the PTEN mouse also shows abnormalities in social interaction (Kwon et al., 2006), consistent with the features of autism. Though PTEN deletion itself cannot explain abnormal neurodevelopment in autism, it may contribute to the effects of brain size.

Autism appears to be a disorder with multigenic origins. It is also uniquely human. Thus, the animals described above cannot wholistically model the autistic condition. Nonetheless, our gene expression results point to possible gene candidates for further investigation using animal genetic technology. However, it appears that not only does the functional impact of single aberrant genes have to be investigated, but their interactions and combinatorial effects (including epigenetic factors and genes that are switched on at certain timepoints) have to be taken into consideration to get at the complexity of aberrant neurodevelopment in autism.

Remaining questions.

Our results bring up new questions that will need to be addressed. How do the gene expression abnormalities we detected in our studies lead to the brain overgrowth

and other neuropathological phenotypes in children with autism? What is the timing of these aberrant pathways, and what is their effect on neurodevelopment? What are the triggers that cause the aberrant signaling, which appear to underlie brain development that leads to autism? Are there factors that protect individuals who also have large brains from also having autism? Our understanding of the genome limits us from answering these questions, and thus animal and *in vitro* models must be employed to help us understand the effects of single and interacting genes.

Further, questions of treatment are equally important to investigate. Are there methods of correcting gene expression abnormalities postnatally or prenatally, and will it correct the behavioral and cognitive symptoms of autism? What are the specific genes and timing of expression of these genes that cause the overgrowth brain phenotype? Presumably, in order to be truly effective, prenatal screening methods must be developed to track abnormalities in brain development. This would have to be accompanied by higher resolution brain imaging techniques that could be applied as a fetus develops. If such methods are available, can directed gene therapy be administered before birth to correct the aberrant trajectory of brain growth that precipitates after birth? The genetic technology field has already made great strides in *in utero* gene therapy for congenital neural diseases (Dejneka et al., 2004), and it is my hope that this technology can be applied to devastating genetic neurological disorders like autism in the future.

Future Directions.

All of the analyses that we have performed here are on a restricted sample size using genetic technology that is quickly becoming obsolete. Our studies emphasize the importance of obtaining and preserving donor brain tissue for neuropathological and genetic analyses. Since autism is a disorder of the brain, there is no more direct way to understand the underlying pathogenic mechanisms than through exploration of the tissue. Without minimizing the work that has already been done to collect these precious samples, additional efforts coordinating both scientific and medical fields must be applied in this area to move the field forward.The public needs to be educated on the importance of tissue donation for researching neuropsychiatric disorders. Using these brain samples, the field will need to expand postmortem analyses of the autistic brain using state-of-the-art methods. We also hope that our studies will move the diagnostic and therapeutic fields forward through the identification of biomarkers and encourage changes in thinking of autism pathogenesis.

For an even higher resolution investigation of the autistic cortex, laser capture microdissection should be applied to compare the genetics withindifferent cell populations and within the localized patterns of abnormalities identified by Bauman and Kemper (2005), Hutsler et al., (2009), and Chow, Pramparo and Boyle (*in review*). Expression microarray methods have already been shown to be efficacious in profiling expression abnormalities in small amounts of mRNA samples.

Additionally, as microscope technology continues to refine itself, finer resolution with laser capture of cells within the different lamina of cortex can be applied to study the autistic cortex. With this type of resolution we may be able to infer the stage of neurodevelopment when the abnormalities began, since the prenatal time course of neurodevelopment and markers for each stage is approximately known in humans. Thus, once biological markers of autism can be established and tested for *in utero,* we may develop animal models to examine the specific defects and timing when development goes awry.

It is also our hope that we may use the brain gene expression data to glean insight into changes that may occur in the periphery so that a simple blood test may be able to identify autism. These studies are already underway at the Autism Center of Excellence. Preliminary results have shown interesting comparable cell cycle pathway dysregulation in lymphocytes, suggesting that this line of investigation may be fruitful (Winn et al., *in preparation*). Furthermore, these comparable results may suggest that the dysregulation of these pathways may arise as a result of DNA abnormalities that are expressed both in blood and in the brain.

Finally, though it is beyond the scope of neuroscience and will require collaboration between physicians and scientists, the diagnostic resolution for autism spectrum disorders must be improved. Hu and Steinberg (2009) clustered the symptoms on the ADI-R and showed that these clusters may be able to predict gene expression differences between the groups. This type of subcategorization decreases the

heterogeneity per group. However, it is still based on clinical behavioral measures. This type of analysis should also be performed on biological measures in autism: groups should be subcategorized based on pathways affected. If these pathways lead to common behavioral phenotypes, then these subcategorizations can be leveraged for diagnostic and therapeutic purposes. Tools like MDMR that was applied in the gene expression analysis could also be useful for the proposed analyses to be done. Without greater biological resolution in the diagnostic measures for ASD, it will be difficult to tease out the genetic and environmental etiology of autism in general. The symptoms common to all forms of autism may be caused by different factors. Thus, isolation of biologically-based factors that lead to aberrant brain growth could help us pinpoint phenotypes of a common etiology.

Conclusion.

In sum, there is still much to learn about the autistic condition. Our studies must be followed up with additional cases, with additional brain areas that have been shown to be functionally aberrant, and with different methodology across scientific levels with higher resolution.Though beyond the scope of neuroscience, other medical researchers have found consistent pathological features in organs other than the brain in patients with autism, and it will require the coordinated efforts of many scientists and physicians in order to find the true basis of autism. Our findings thus far are heartening, however, as we have identifiedgenetic patterns in the brain of autistic individuals that may open up many more research possibilities for the field.

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