A TALE OF TWO FAMILIES:
NEURAL PRECURSOR CELLS FROM IDIOPATHIC
AUTISM PATIENTS EXHIBIT PROLIFERATION
PHENOTYPES

BY

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ABSTRACT OF THE DISSERTATION

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BY: MADELINE ELAINE WILLIAMS

DISSERTATION DIRECTION: Emanuel DiCicco-Bloom, MD

Autism spectrum disorder (ASD) is a highly complex, heterogeneous neurodevelopmental disorder characterized by an impairment in social communication and interaction as well as stereotyped/repetitive behavior. While ASD is a highly prevalent disorder (1:68), the underlying molecular mechanisms have yet to be identified and no therapeutics to treat core symptoms exist. As the name suggests, individuals with ASD have a wide range of severity of symptoms, intelligence levels, comorbid disorders, environmental and genetic contributions, different potential etiologies, and pathologies. This human social disorder characterized by vast heterogeneity cannot be properly modelled in a
mouse, a challenge that has hindered the study of ASD. Here, I present methods for examining proliferation in human NPCs as well as a case study on two families examining ASD NPC proliferation in comparison to their sex-matched sibling controls. In comparison to their siblings, ASD patients present patient-specific proliferation phenotypes at baseline, in response to growth factors, and under environmental stressor conditions. A common phenotype emerges after exposure to methyl mercury (MeHg), both ASD patients show a reduction in sensitivity to the negative impact on DNA synthesis. In aggregate, I have created a toolset to measure patient-derived NPC proliferation, applied it to examine differences in ASD-sibling pairs, and identified both patient-specific and common patient phenotypes.
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CHAPTER ONE: INTRODUCTION

1.1. Autism Spectrum Disorder

1.1.1. Significance, clinical diagnosis, and etiological factors

Autism Spectrum Disorder (ASD) is a complex, heterogeneous neurodevelopmental disorder for which there is no current unifying neurobiological or pathological etiology (Geschwind and Levitt 2007). The Centers for Disease Control’s most recent report estimates one in every 68 children has ASD in the US (Autism, Developmental Disabilities Monitoring Network Surveillance Year Principal et al. 2012, Baio 2014). This is a significant increase from previous prevalence reports of one in 150 children (Autism, Developmental Disabilities Monitoring Network Surveillance Year Principal et al. 2007, Autism, Developmental Disabilities Monitoring Network Surveillance Year Principal et al. 2007). Though it is doubtful that increases in prevalence primarily represent growth in numbers of individuals with ASD, it is clear that prevalence is much higher than previously thought thus placing a substantial socioeconomic burden on families and society (Herbert 2010, Autism, Developmental Disabilities Monitoring Network Surveillance Year Principal et al. 2012). Current pharmacotherapy focuses on treating the co-morbidities that occur with autism, such as epilepsy and mood disorders, but fail to treat core symptoms (Posey and McDougle 2000). Thus, it is important for scientists to develop new therapeutic approaches to treat the core features of ASD.
First described in the early 1940s, Leo Kanner’s early observations that children with autism exhibit “aloneness” and “insistence on sameness” still resonate in the clinical presentation of the disorder (Kanner 1968). Under the DSM-V, autism is characterized by deficits in social interaction and communication, and the presence of stereotyped, repetitive behaviors (Battle 2013). These behaviors typically present in early childhood, before 3 years of age, and persist throughout life leading to functional impairment. Notably, across these core features there is a wide range in severity and quality of symptomatology. For example, until the recent DSM-V classification, language problems were fundamental to the diagnosis but delay in spoken language was only present in about half of ASD individuals (Alarcon, Cantor et al. 2002, Spence, Cantor et al. 2006, Geschwind and Levitt 2007). Similarly, social impairments can present in a variety of forms from excessive attention seeking in an odd, socially awkward manner to avoidant and aloof behavior, apathetic to social interaction (Wing and Gould 1979, Volkmar, Cohen et al. 1989). Though not a core symptom, cognitive dysfunction is often associated with ASD and can range from individuals presenting with mental retardation to savant-ism (Howlin, Goode et al. 2009, Matson and Shoemaker 2009).

In equal measure with the complexity of clinical presentations are the multiplex of genetic and environmental factors that contribute to ASD manifestation. Current estimates suggest that roughly 500 to 800 genes play a part in the disorder (Abrahams and Geschwind 2008, De Rubeis and Buxbaum...
These genetic factors include changes in the coding sequence of genes as well as the non-coding changes that alter gene expression, such as epigenetic factors, copy number variants, and other regulators like microRNAs and long non-coding RNAs. However, with the exception of a few Mendelian monogenic disorders such as Fragile-X syndrome, Rett syndrome, Tuberous Sclerosis, and neurofibromatosis, which account for only ~10% of cases, the ASD inheritance pattern is complex (Artigas-Pallares, Gabau-Vila et al. 2005, Abrahams and Geschwind 2008). Concordance rates in twin studies show 38 to 90% among monozygotic twins and only 10 to 17% among dizygotic twins (Rosenberg, Law et al. 2009, Hallmayer, Cleveland et al. 2011). These data suggest that non-genetic factors likely play a role in ASD manifestation. Recent studies suggest that autism risk depends on approximately equal contributions of genes and environmental factors as well as interactions between these components (Herbert 2010, Rossignol, Genuis et al. 2014). Epidemiological studies have observed correlations of ASD prevalence with exposure to environmental factors such as metals, pesticides, and traffic-related air pollution, though further replication is needed (Becerra, Wilhelm et al. 2013, Roberts, Lyall et al. 2013, Volk, Lurmann et al. 2013). Given this information, it is apparent that a multitude of genetic and environmental factors, and their intricate interaction with each other, are instrumental players in the pathological complexity observed in ASD.

Due to the heterogeneity in symptomology and the diversity of contributory factors, it has been proposed that we think of ASD not as one disorder but as
“The Autisms” that are due to specific individual differences that manifest themselves as fewer, more common phenotypes (Figure 1) (Geschwind and Levitt 2007, (IACC) 2012). This may be because the numerous factors and pathways may ultimately impact a smaller network of common pathogenetic pathways or neurobiological processes (Geschwind and Levitt 2007, Voineagu 2012, Pinto, Delaby et al. 2014).

Figure 1: ‘The Autisms’

Figure 1: Representation of the clinical or syndromic, green circles, and the myriad of possible genetic and environmental etiological features, blue circles, associated with autism spectrum disorder genetic. The central arrowed box represents the multitude of neurodevelopmental processes that might be disrupted. Circles with dotted lines around them emphasize areas where the extent of the contribution to ASD remains undefined relative to other contributory factors. Adapted from: Geschwind, D.H. and P. Levitt, Autism spectrum disorders: developmental disconnection syndromes. Curr Opin Neurobiol, 2007. 17(1): p. 103-11.
1.1.2. Post-mortem findings, neuroimaging studies, and altered neurogenesis

Despite the abundance of genetic information, ASD pathology is largely unknown. Numerous studies examining post-mortem brain tissues and utilizing neuroimaging technology have attempted to disentangle ASD and found no consistent neuropathological model (Ritvo, Freeman et al. 1986, Courchesne, Yeung-Courchesne et al. 1988, Piven, Saliba et al. 1997, Bailey, Luthert et al. 1998, Courchesne, Karns et al. 2001, Casanova, Buxhoeveden et al. 2002, Hazlett, Poe et al. 2006, van Kooten, Palmen et al. 2008, Wegiel, Kuchna et al. 2010, Stoner, Chow et al. 2014). Multiple studies examining postmortem brain tissue of autism patients have found a variety of abnormalities including dysplasia in the cortex, the brain stem, cornu Ammonis, the cerebellum, and the dentate gyrus; the presence of heterotopias in cortical and cerebellar brain regions, as well as, vast reductions in Purkinje cell number (Ritvo, Freeman et al. 1986, Bailey, Luthert et al. 1998, Amaral, Schumann et al. 2008, Wegiel, Kuchna et al. 2010). Though no two brains presented the same phenotypic abnormalities, these results suggest that there is a multiregional dysregulation of basic processes involved in neurogenesis, neuronal migration, and neuronal maturation underlying autism pathophysiology. More specifically, increased brain size and increased cortical thickness in many ASD individuals may point to an alteration in neurogenesis (Bailey, Luthert et al. 1998, Wegiel, Kuchna et al. 2010). The presence of heterotopias, a region of misplaced or mis-oriented neurons that can cause epilepsy, may suggest a disruption in neuronal migration.
Some studies have found signs of neuronal immaturity in a number of the patients which could point to defects in neuronal precursors capacity to differentiate (Wegiel, Kuchna et al. 2010). This wide range of brain abnormalities suggests a fundamental disruption in ASD brain development. In spite of this heterogeneity, some differences have been observed with greater frequency, including reductions in Purkinje cell number, abnormal minicolumn morphology, and an early accelerated brain growth, all of which may contribute to ASD etiology.

One of the most consistent findings regarding autism neuropathology is the reduction in Purkinje cell numbers in the cerebellum (Ritvo, Freeman et al. 1986, Fatemi, Halt et al. 2002, Amaral, Schumann et al. 2008, Fatemi 2013). Classically the cerebellum functions to coordinate and regulate motor abilities; however, it also plays diverse roles in language, emotional regulation, social interaction, and cognition (Leiner, Leiner et al. 1991). Studies of nonhuman primates have shown that the cerebellum also interacts indirectly with the cerebral cortex. This interaction occurs through a network involving Purkinje neurons, deep cerebellar nuclei, the thalamus, the cortex and the pons. A decrease in Purkinje cell number could affect the interaction of this network and account for some of the cognitive and motor deficits present in autism (Fatemi, Aldinger et al. 2012). Further, it may be a sign of a more global dysregulation of brain development.
The cellular minicolumn is the basic functional unit of the brain cerebral cortex and changes to this unit can alter neuronal circuitry. In one study (Casanova, Buxhoeveden et al. 2002), researchers examined whether differences existed in the cytoarchitecture of minicolumns using a computerized imaging program to measure minicolumn morphologic features. They found cellular minicolumns in autism patients were more numerous, smaller, and less compact in their configuration in the areas examined—area 9 in the prefrontal cortex and area 22 and 21 in the temporal lobe (Casanova, Buxhoeveden et al. 2002, Casanova, Buxhoeveden et al. 2002). This novel minicolumnar arrangement most likely arose during brain development rather than in an insult or injury to the brain. An increase in these processing units/connections may explain an increase in excitatory activity and a subsequent decrease in inhibitory interneuronal activity. Current theory on autism pathology suggests that there may be an increased ratio of excitatory to inhibitory neurons, this could potentially be explained by the increase in minicolumn density (Nelson and Valakh 2015). However, we must observe some caution, because minicolumn analyses have been performed by using complex computer imaging techniques whereas direct cellular analysis has yet to be done.

As neuroimaging technology has advanced, studies have found abnormalities in the volume of forebrain and hindbrain regions, head circumference, and developmental brain growth in autistic individuals. In Kanner’s early descriptions of autism, he observed that a larger head
circumference was common in children with ASD (Kanner 1968). In the years since then a number of neuroimaging studies assessing cranial circumference and brain volume have found that individuals with autism have an accelerated increase in brain and therefore head growth (Courchesne, Carper et al. 2003). A theory has emerged that in autism there exists a consistent accelerated brain growth phenotype that 1) occurs in early development, 2) is not initially present at birth, and 3) fails to persist later in development (Figure 2) (Lainhart, Piven et al. 1997, Courchesne, Karns et al. 2001, Courchesne, Carper et al. 2003, Hazlett, Poe et al. 2005, Courchesne, Campbell et al. 2011). Additionally, it has been suggested that a subset of individuals with autism are macrocephalic—defined as greater than 2 standard deviations above the population mean (Woodhouse, Bailey et al. 1996, Fombonne, Roge et al. 1999, McBride, Varga et al. 2010). These studies have suggested that there may be a common brain growth phenotype linking some of ‘the Autisms’.
**Figure 2**: One phenotype that a proportion of ASD patients exhibit is an accelerated brain growth phenotype which 1) appears early in development, 2) is not present at birth, and 3) does not persist into adulthood.


Despite these assertions, this field has been challenged by the limited reports of neuropathology as well as small sample sizes and low-resolution observations in the numerous, non-invasive neuroimaging studies. Moreover, the majority of research linking autism to a brain growth phenotype or macrocephaly has relied on comparison with standardized growth charts that have now been called into question (Raznahan, Wallace et al. 2013). Two typically developing growth charts commonly used as comparison in head circumference studies, one from World Health Organization (WHO) and another from the US Center for Disease Control (CDC), were found to over identify the number of children with abnormally large heads (Daymont, Hwang et al. 2010).
When studies using these growth charts as their typical developing comparison were excluded, the percentage of ASD children with macrocephaly dropped from 14% to 4% (Raznahan, Wallace et al. 2013). This percentage is only slightly higher compared to the population average of 3% macrocephaly (Dementieva, Vance et al. 2005). Additionally, some recent findings indicate skull size has grown roughly 7% over the past 100 years which really throws a wrench into everything.

With this knowledge base in mind, including limitations of postmortem and neuroimaging studies, there still exists an extraordinary observation that some perturbation occurs during brain development in autism. Mechanistically, a number of the abnormalities in ASD brains could be due to a dysregulation of neurogenesis or normal programmed cell death. A loss of Purkinje neurons may be due to a reduction in the number that are born or due to postnatal cell death. If these processes are disrupted in one region of the brain they may also be affected in another region. The observed increase in minicolumn number could indicate increased, aberrant neurogenesis or increased cell survival. A temporary increase in brain size in individuals with autism could occur through excess proliferation of neural precursor cells (NPCs) or due to a failure of normal programmed cell death. Indeed, studies employing ASD mouse models of autism have found abnormal regulation of neurogenesis, including cell proliferation and apoptosis, in neuroproliferative regions of the brain (Agulhon, Blanchet et al. 1999, Groszer, Erickson et al. 2001, DiCicco-Bloom, Lord et al.)
findings in studies of syndromic autisms, or autism-like disorders with known genetic causes such as fragile-X syndrome (FXS) or tuberous sclerosis (TS), have reported dysregulation of pathways known to regulate cell growth (Tee, Fingar et al. 2002, Bagni and Greenough 2005, Crino, Nathanson et al. 2006, Marcotte and Crino 2006, Sharma, Hoeffer et al. 2010, Pinto, Delaby et al. 2014, Pucilowska, Vithayathil et al. 2015). If ASD arises, in part, from a dysregulation of typical neurogenesis then signaling pathways involved in regulating proliferation may be perturbed.

Though much of the field has focused on an increase in early brain growth, it is important to reflect that in examining changes in brain size, there are a vast number of mechanisms by which this may occur other than increased neurogenesis. To name a few, these may include changes in neuron size, number of neuronal processes, numbers of dendritic spines, how densely cells may pack together, and size and number of glia. Given the degree of genotypic and phenotypic heterogeneity of the disorder it is unsurprising that no consistent anatomical or pathological findings have emerged. Different cognitive and functional features of autism may be due to differences in affected brain regions, differences in direction or type of underlying affected processes, or some amalgamation of such, all dependent on specific genetic and environmental factors that shape an individual’s development.
1.1.3. **Environmental factors and oxidative stress**

The idea that autism is a set of multiple etiologically-distinct disorders arises in part from the phenotypic heterogeneity as well as the array of genetic and environmental factors that contribute to this disorder (Jones and Klin 2009, Herbert 2010, Rossignol, Genuis et al. 2014). An increasing amount of evidence suggests there is a role for environmental factors in ASD disease pathogenesis (Rossignol, Genuis et al. 2014). A recent study of 192 pairs of genetically identical twins found that environmental factors were estimated to account for 55% of the risk of developing ASD compared with 37% for genetic factors (Hallmayer, Cleveland et al. 2011). A comprehensive analysis of genetic risk now suggests that 49% of disease risk can be attributed to common genetic variants, 10% to rare variants and that 41% is undefined and certainly includes environmental factors (Gaugler, Klei et al. 2014). Despite the apparent connection between ASD and environmental factors, very few studies have directly examined this possible relationship with the exception of gestational exposure to anticonvulsant valproic acid (VPA) (Williams, King et al. 2001, Harden 2013, Meador and Loring 2013, Singh 2013, Baxter 2014, Wood 2014). Some studies examining risk factors for neurodevelopmental disorders have found an association with metals, such as lead and mercury, though we should be careful in interpreting this due to lack of consensus in the field (2000).

Conversely, a number of population studies have found potential associations between ASD and environmental toxicant exposures (Becerra,
Wilhelm et al. 2013, Roberts, Lyall et al. 2013, Volk, Lurmann et al. 2013). The Nurses’ Health Study II on air pollutants and ASD found that exposure to metals increased the odds ratio to 1.5 and exposure to diesel and mercury increased the odds ratio to 2.0 that a boy will have ASD. Heavy metals and traffic-related toxicants are neurotoxicants that can induce inflammatory responses and lead to oxidative stress, an output of mitochondrial dysfunction, which can disrupt biological processes or pathways implicated in autism. Furthermore, greater genetic vulnerability to ASD in combination with exposure to environmental toxicants in utero and during development may cause children to cross biological or behavioral thresholds into ASD.

Notably, population studies examining associations between ASD and environmental toxicants are retrospective in nature, based on environmental sampling of what was in the neighborhood air, and unable to examine direct relationships of the toxicants in the mothers or the affected children. A number of studies have investigated the theory that ASD pathology may be in part due to a physiological and genetic vulnerability to environmental factors by examining biomarkers for these toxicants and genetic-toxicant interactions. They suggest that interactions between genes and environmental toxicants may act in parallel or synergistically to cause alterations in critical periods of neurodevelopment (Herbert 2010, Rossignol, Genuis et al. 2014). However, these studies are limited by their relatively small sample sizes and lack of control for dietary intake or medication use. Additionally, samples for biomarker analysis were taken from
blood, urine, hair, or teeth—areas quite distant from the human brain. Others have suggested that alterations in oxidative stress markers in ASD may indicate mitochondrial dysfunction, and in some cases direct mitochondrial assays of the brain support this theory (Oliveira, Diogo et al. 2005, Correia, Coutinho et al. 2006, Giulivi, Zhang et al. 2010, Rossignol and Frye 2012, Rossignol and Frye 2012, Tang, Gudsnuk et al. 2014). Clinical and experimental studies of ASD pathology clearly implicate a role for altered synaptogenesis and function (Stamou, Streifel et al. 2013). Similarly, studies have shown that toxicants can interfere with synapse formation directly through interaction with synaptic molecules or indirectly through modulation of developmental Ca^{2+} signaling (Stamou, Streifel et al. 2013).
1.2. Brain Development

Over the last few million years of hominid evolution (humans and great apes) the brain and notably, the neocortex, have dramatically increased in relative size and with this has come a striking acquisition of higher order cognitive functions (Hrvoj-Mihic, Bienvenu et al. 2013). In comparing the cortex of human and non-human primates, studies have uncovered an increased number and diversity of human cortical neurons (Nimchinsky, Gilissen et al. 1999, Hill and Walsh 2005, Roth and Dicke 2005, Bystron, Rakic et al. 2006) and specific patterns of neuronal morphology, such as increased size and density of dendritic spines in particular regions (Benavides-Piccione, Ballesteros-Yanez et al. 2002). Many uniquely human features of the cortex are most likely linked to differences in underlying mechanisms that generate, specify, and differentiate cortical neurons, i.e. neurogenesis, (Bystron, Blakemore et al. 2008, Fish, Dehay et al. 2008, Lui, Hansen et al. 2011, Taverna, Gotz et al. 2014), with a direct impact on the number and diversity of cortical neurons (Rakic 1988, Rakic 1995, Bystron, Rakic et al. 2006, Lui, Hansen et al. 2011, Suzuki and Vanderhaeghen 2015).

Over the past 25 or so odd years, the neuroscience field has been radically revolutionized with the discovery and conception of “neural stem cells”. In the 1980’s stem cells were still not thought of as builders’ of the brain but rather as building blocks for “other” tissues; tools, including in vitro culture media, growth factors, antibodies, were limited or not yet discovered (Gage and
Landmark neurobiology discoveries coupled with new technologies such as, retroviral lineage-tracing techniques and advancements in culturing cells *in vitro*, unearthed subpopulations of cells that were multipotential and could later give rise to mature neuronal and glial cells (Gage and Temple 2013). Furthermore, some of these cells had a capacity for self-renewal—a trademark of stem cells. Neural stem cells refer to a subtype of cells that can self-renew and also have the ability to differentiate into one of the three neural lineages—neurons, astrocytes, and oligodendrocytes (Temple 2001). Their discovery and definition have become critical to how we think about how the brain grows and develops.

The development of the human brain is a dynamic and adaptive affair that involves the interaction of a number of different levels and kinds of processes—from gene expression to environmental input. Human brain development first begins during the third gestational week of pregnancy and continues until an individual reaches their mid-twenties, however, brain size reaches 90% potential by 4 to 6 years old (Reiss, Abrams et al. 1996, Lenroot and Giedd 2006). Given that autism is a neurodevelopmental disorder with implications of alterations in early developmental processes, we must take into consideration specific elements of brain ontogenesis.
1.2.1. **Neural induction and patterning of the CNS**

Brain development first begins when the multipotent ectoderm cells of a blastocyst enter the neural lineage, a process known as neural induction (**Figure 3A**). Novel and elegant studies in amphibian models found that neural induction occurs through a ‘default pathway’ where extrinsic cues that would normally suppress neural induction are inhibited by other signaling molecules, chiefly, through inhibition of TGFβ and BMP signaling (**Figure 3A**) (Smith and Harland 1992, Hemmati-Brivanlou, Kelly et al. 1994, Sasai, Lu et al. 1994). In other words, ectodermal tissues have a tendency to autonomously differentiate into neural tissue (Hemmati-Brivanlou, Kelly et al. 1994).  

**Figure 3: Neural induction and regional patterning of the CNS**

**Figure 3:** A) Brain development or neural induction occurs when the multipotent ectoderm cells autonomously differentiate into neural tissue, or the ‘default’ pathway. This occurs via inhibition of BMP/Nodal signaling. B) Regional patterning of the CNS are determined along the dorsoventral and rostrocaudal axes via gradients of morphogens supplied from different organizing centers (yellow: FP, floor plate; MHB, midbrain-hindbrain boundary).
With technological advances in culturing mouse embryonic stem cells and then later, in human induced pluripotent stem cells (iPSCs) (Chambers, Fasano et al. 2009), this model has been shown to be largely evolutionarily conserved across species (Valenzuela, Economides et al. 1995, Kawasaki, Mizuseki et al. 2000, Elkabetz, Panagiotakos et al. 2008, Smith, Vallier et al. 2008, Chambers, Fasano et al. 2009). Indeed, these early studies have informed current in vitro approaches to differentiate embryonic cells or iPSCs into neural stem cells.

Following formation and closure of the neural tube, the central nervous system is patterned through complex molecular signaling cascades. Different organizing centers (Figure 3B, yellow regions) supply gradients of morphogenic molecules that act as positional cues to determine and refine the rostro-caudal and dorso-ventral axes within the neural tube allowing for the emergence of all brain regions and domains (Figure 3B) (Suzuki and Vanderhaeghen 2015). Primitive cell identity can be differentiated into more caudal fates by cues such as retinoic acid, Wnts, or FGFs (Wilson and Houart 2004). Further, different concentrations of various morphogenic molecules, such as Sonic Hedge Hog (Shh), can induce specific expression of transcription factors in discrete domains to form different regions of the brain (Suzuki and Vanderhaeghen 2015). Gradients of gene expression also play a major role in arealization formation of the neocortex. In particular, transcription factors EMX2 and PAX6, are produced in opposing gradients from the rostro-lateral to caudomedial regions of the neocortex.
(Bishop, Rubenstein et al. 2002). Pax6 is expressed from high to low gradient from rostrolateral to caudomedial and Emx2 is expressed from high to low gradient from caudomedial to rostrolateral (Bishop, Rubenstein et al. 2002). These graded patterns of molecular signaling create the basic structural and functional identity of the brain. However, keep in mind that the brain development occurs over a long period of time and that it is a malleable, plastic structure—subject to not only pre-determined genetic identity but also to the effects of environmental input and experience.

1.2.2. Cell cycle regulation

Neural stem cell division, like other mitotic cell types, follows the different stages of the cell cycle. Quite simply, the cell cycle is the process through which a cell replicates its DNA and divides to produce two daughter cells (Schafer 1998). This process is composed of four biochemically distinct stages or phases, G1, S, G2, and M (Figure 4) (Tury, Mairet-Coello et al. 2012). The first and also the longest phase is G1 or Gap 1, this is the initial growth phase where cells increase in size as they synthesize the necessary mRNA and protein needed to prepare for subsequent phases and ultimately for cell division. In the second phase, S or Synthesis, cells actively replicate their DNA. The next phase, G2 or Gap 2, is the second growth phase where cells continue to grow and prepare for cell division or mitosis. In the last phase, M or Mitosis, cell growth ceases and all cellular energy is directed towards separation of the duplicated chromosomes.
and division into two daughter cells (Schafer 1998). At the end of mitosis, cells either re-enter the cell cycle at G1 or they enter a quiescent non-dividing state, termed G0 or the resting phase. Typically, as cells differentiate into their mature forms they enter G0 where they are still metabolically active and performing specialized functions but not in the active cell cycle (Duronio and Marzluff 2017).

**Figure 4: The mammalian cell cycle**

Figure 4: The mammalian cell cycle and its regulation. There are four phases in the cell cycle: Gap1 (G1), DNA synthesis (S), and Gap2 (G2) phases and mitosis (M phase). Regulation of the cell cycle occurs through cyclin-cyclin dependent kinase (CDK) complexes which lead to cell cycle progression, and inhibition of these complexes via CDK inhibitory proteins (CKIs). CIP/KIP proteins are regulated by mitogenic and anti-mitogenic extracellular signals. Extracellular mitogenic and anti-mitogenic signals regulate levels of cyclins and CKIs.


The transition through these four phases is regulated through ‘cell cycle checkpoints’ that ensure cell division occurs at the appropriate time and is done correctly. Checkpoints prevent the cell cycle from progressing to verify that
important stage-specific processes have occurred, check and repair potential DNA mutations, and to arrest the cell cycle/prevent division in a variety of cases, including presence of DNA damage, lack of adequate growth factor requirements, differentiation, senescence, and/or stress (Schafer 1998). There are three main checkpoints, each at a transitions between phases, including: from G1/S, G2/M, and the metaphase (mitotic) checkpoint. The first checkpoint, G1/S, is the rate limiting step of the cell cycle and the most tightly regulated; inappropriate entrance into the cell cycle can have potentially devastating effects from improper development to tumorigenesis. At checkpoints exist a team of regulatory proteins, cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CKIs), and they consort together to regulate progression through these cell cycle checkpoints.

Cyclins, the regulatory subunit, and CDKs, a non-active catalytic subunit, bind together in an active heterodimer to phosphorylate target proteins that cause cell cycle progression (Schafer 1998). With the exception of D Cyclins, which have a gradual rise in levels at the beginning of G1 and gradual decrease after G2, Cyclins E, A, and B levels oscillate at different points during the cell cycle. In contrast, CDK levels remain relatively stable throughout. Thus, different expression levels of different cyclins can act as a point of regulation for cell cycle progression.

CKIs, play an integral role in inhibiting or arresting the cell cycle. Two core families of CKIs exist, the cip/kip and the INK4a/ARF family, and they
operate by different mechanisms to inhibit cell cycle progression. Members of the cip/kip family of inhibitors bind to the cyclin-CDK complex to inhibit kinase activity; whereas, members of the INK4a/ARF family selectively bind to catalytic subunits, CDK4 or CDK6 (Duronio and Xiong 2013). The more stable proteins of the two families, members of the INK4a/ARF family of inhibitors play important roles in human development by promoting quiescence in stem and progenitor cells (Duronio and Xiong 2013). An example of such is p18, which deletion of results in spontaneous tumors in mice (Duronio and Xiong 2013). A transcription factor, GATA3, can repress transcription of p18 and allow cells to re-enter the cell cycle through G1 and continue to proliferate, an example of a lineage specifying factor that regulates cell differentiation in part by inhibiting the inhibitor, p18 (Duronio and Xiong 2013).

Oftentimes, there are a number of intracellular and extracellular cues that interact with this machinery to regulate proliferation. In this context, the environment in which proliferating cells live in has a deep impact on the control and coordination of their progression through the cell cycle. As mentioned previously, levels of CDKs remain at a relatively steady state throughout the cell cycle so signaling pathways controlling proliferation often do so through altering levels of Cyclins and CKIs (Duronio and Xiong 2013).
1.2.3. **Signaling pathways regulate proliferation**

In reaction to extracellular stimuli, molecular cascades or signal transduction pathways within the cell are launched and ultimately initiate gene transcription that is translated into a cellular response. There are a number of different signaling pathways that control cell proliferation and these mostly involve increasing levels or degradation of Cyclins and/or CKIs. The most extensively studied in relation to proliferation is the canonical Ras–Raf–MEK–ERK mitogen-activated protein kinase (MAPK) pathway which activates transcription of Cyclin D (Duronio and Xiong 2013). However, D-type cyclin transcription can also be induced by other pathways including through mitogen-activated Rac and NF-κβ signaling, Wnt and Notch pathways, cytokine signaling, and signaling by receptors for extracellular matrix proteins such as integrins (Kopan 2012, Nusse 2012). In regulation of CKIs, the Ras/MAPK pathway can also acts as a key player. For example, Ras/MAPK can activate p21 in some cells to promote cell cycle exit (Clayton, van den Heuvel et al. 2008). While we often point to specific pathways as regulating specific processes such as cell death or proliferation, these pathways and the molecules that activate them are promiscuous and have diverse roles. In considering potential mechanisms for alterations in a cell process it is important to note that responses to specific signals are often cell-type and cell-maturation-stage dependent.

Mouse models of autism, including knockouts of Engrailed-2 studied by our lab, and PTEN and FMR1 by others, have found dysregulation of
neurogenesis, including cell proliferation and apoptosis, in neuroproliferative regions of the brain (Agulhon, Blanchet et al. 1999, Groszer, Erickson et al. 2001, DiCicco-Bloom, Lord et al. 2006, Sheridan, Theriault et al. 2011, Rossman, Lin et al. 2014). Further, multiple studies of syndromic autism such as Tuberous Sclerosis (TS) and Fragile-X Syndrome (FXS), and CNV 16p11.2 deletion report disruption of signaling pathways, namely P-AKT/mTOR/S6K, PI3K, cAMP, and ERK/MAPK, that are known regulators of cell growth (Tee, Fingar et al. 2002, Bagni and Greenough 2005, Crino, Nathanson et al. 2006, Marcotte and Crino 2006, Sharma, Hoeffer et al. 2010, Pinto, Delaby et al. 2014, Pucilowska, Vithayathil et al. 2015). If brain development is perturbed in ASD then signaling pathways involved in regulating neurogenesis may be perturbed.
1.3. Cellular Reprogramming: Human Induced Pluripotent Stem Cells

1.3.1. History

In 2012 Yamanaka was awarded the Nobel Prize in Physiology or Medicine for his monumental discovery that mature cells can be reprogrammed into embryonic-like stem cells after forced expression of four transcription factors: c-MYC, SOX2, OCT3/4, and KLF4 (Takahashi and Yamanaka 2006). The Yamanaka factors regulate expression of a number of genes and in doing so, activate a gene pluripotency network that turns on “stem-ness” of a cell (Wasik, Grabarek et al. 2014). More specifically, c-Myc is a proto-oncogene transcription factor that regulates genes involved in DNA replication and proliferation (Wasik, Grabarek et al. 2014). c-MYC is often dysregulated in cancer and tumor formation. SOX2 heterodimerizes with OCT3/4 and together they activate genes crucial for stem cell maintenance (Wasik, Grabarek et al. 2014). Klf-4 has a number of roles, sometimes opposing, which regulate proliferation, apoptosis, and differentiation (Wasik, Grabarek et al. 2014). After forced expression of these factors, somatic cells (blood, skin, hair, dental pulp, etc.) are reprogrammed and can become virtually any cell in the body i.e. heart cells, brain cells, gut cells, etc. Yamanaka named these newly created cells: induced pluripotent stem cells (iPSCs). To model neuropsychiatric development and disease in vitro, researchers can differentiate these iPSCs into neural stem cells and ‘mature’ neurons. Neural stem cells and neurons can be differentiated from iPSCs either by using cocktails of developmentally relevant growth factors and...
morphogens or by viral transfection of transcription factors that activate gene expression of specific cell types. These derived cells can be used to examine development and disease mechanisms or to screen for potential therapeutics. Since Yamanaka’s 2006 groundbreaking discovery, the technique has revolutionized the way medical research has been conducted. Human iPSC research has already begun to deepen our understanding of human specific features of brain development as well as helped to elucidate the biological signatures of various disease pathologies, including ASD, and may provide insight into patient-specific differences (Aigner, Heckel et al. 2014).

Since its emergence, multiple studies have demonstrated that human iPSCs can be used to model neuropsychiatric diseases, including familial dysautonomia, Rett Syndrome, Timothy syndrome, Fragile X syndrome, Phelan-McDermid syndrome, Parkinson’s Disease, Alzheimer’s Disease, schizophrenia and bipolar disorder (Lee, Papapetrou et al. 2009, Brennand, Simone et al. 2011, Pasca, Portmann et al. 2011, Sheridan, Theriault et al. 2011, Cooper, Seo et al. 2012, Israel, Yuan et al. 2012, Lee, Ramirez et al. 2012, Kondo, Asai et al. 2013, Shcheglovitov, Shcheglovitova et al. 2013, Tran, Ladran et al. 2013, Brennand, Savas et al. 2014, Chen, DeLong et al. 2014, Sanders, Laganiere et al. 2014, Mertens, Wang et al. 2016, Stern, Santos et al. 2017). These studies have identified a number of phenotypes including altered neurite outgrowth, changes in synaptic architecture, and altered neurophysiology that may contribute to pathophysiology showing the value of human iPSC models to study
neuropsychiatric disorders (Marchetto, Carromeu et al. 2010, Urbach, Bar-Nur et al. 2010, Doers, Musser et al. 2014). In neuropsychiatric disorders, iPSC models can recapitulate phenotypes observed in human post-mortem samples and mouse models and also, identify human-specific abnormalities and therapeutic targets.

1.3.2. Studying syndromic ASD using patient-derived cells

The majority of studies using iPSCs to study ASD have focused on monogenic forms of the disorder. For example, in Rett Syndrome, a monogenic form of autism caused by mutations in X-linked methyl CpG binding protein (MECP2), iPSC derived neurons exhibited reduced soma size and spine density, reduced production of synapses and postsynaptic receptors, as well as, alterations in calcium imaging and electrophysiology properties (Marchetto, Carromeu et al. 2010). Re-expression of MECP2 in RTT neurons was able to rescue the number of glutamatergic synapses (Marchetto, Carromeu et al. 2010). Further, using RTT iPSCs as a system has helped to discover therapeutic agents for the disorder, like insulin-like growth factor-1 (IGF-1), which has shown promise in Phase I clinical trials and has been approved for Phase II clinical trials (Khwaja, Ho et al. 2014).
1.3.3. **Using patient-derived cells to study idiopathic disorders**

Despite advances in understanding of pathophysiology in monogenic and syndromic ASDs, these constitute only a small fraction of total ASD cases. Initially, iPSC models were considered of little use in idiopathic disorders because of the challenge in showing that observed *in vitro* phenotypes were related to disease-state without a specific gene to point the finger at and rescue. Equally difficult in studying idiopathic disorders, is tackling the low sample size of patients and attempting to understand whether phenotypes seen in culture are patient-specific or disease-specific. In studying sporadic diseases with wide ranges in heterogeneity of symptomology and pathology, it seems unlikely that all patients would exhibit common underlying deficits. However, four studies examining idiopathic schizophrenia in human iPSC-derived NSCs and neurons found common and reproducible reductions in neurites, neuronal connectivity, and expression of the synaptic proteins in affected neurons (Brennand, Simone et al. 2011, Brennand and Gage 2012, Paulsen Bda, de Moraes Maciel et al. 2012, Robicsek, Karry et al. 2013). Further studies in idiopathic schizophrenia have found increases in extra-mitochondrial oxygen consumption, elevated levels of reactive oxygen species, and gene expression changes in Wnt and cAMP pathways (Brennand, Simone et al. 2011, Brennand and Gage 2012, Paulsen Bda, de Moraes Maciel et al. 2012, Robicsek, Karry et al. 2013). This is encouraging because, like autism, schizophrenia is a complex disease plagued with heterogeneity in symptom onset, severity, genetic profile and potential roles.
of environmental factors. Thus, these studies provide evidence that iPSCs can be used to uncover common phenotypes and potentially dysregulated common pathways in disorders that are genetically and phenotypically complex (Thaker and Carpenter 2001, Kannan, Sawa et al. 2013).

In recent years, an increasing number of human iPSC studies have begun to address idiopathic ASD and have uncovered interesting phenotypes and potential disease mechanisms (Liu, Campanac et al. 2016, Marchetto, Belinson et al. 2016). With regard to this dissertation, two studies examining cohorts of ASD patients selected due to brain enlargement or macrocephaly have found changes in proliferation of ASD cells (Mariani, Coppola et al. 2015, Marchetto, Belinson et al. 2016). For example, Mariani et. al. examined four ASD patients with macrocephaly, using siblings or parents as controls, and found that ASD organoids exhibited an accelerated cell cycle, and an overproduction of inhibitory neurons caused by increased FOXG1 gene expression (Mariani, Coppola et al. 2015). Of note, the accelerated cell cycle in ASD NPCs was specific to 11 days post differentiation of organoids and not present at 31 days post differentiation. In Marchetto et. al. 2017, they selected eight ASD patients who exhibited a larger than normal brain volume and found increases in ASD NPC proliferation related to dysregulation of a β-catenin/BRN2 transcriptional cascade (Marchetto, Belinson et al. 2016). Additionally, they found a reduction of synaptogenesis and the presence of functional defects in ASD neurons which could be rescued via IGF-1. Similar to studies on schizophrenia, this research suggests that hiPSCs
can be used to study idiopathic diseases and identify common cellular phenotypes or biochemical pathways that can act as targets to test potential therapeutics.

While these studies offer seemingly simple and elegant solutions, it is unlikely that all idiopathic autism cases will have a common underlying neurobiology or if they do, that they will all respond to the same therapeutics. Interestingly, in iPSC studies comparing individuals with bipolar disorder who are responsive and nonresponsive to lithium, studies have shown that though neurons derived from both groups display a hyperexcitability phenotype when compared to controls; however, this defect is only reversed by lithium treatment in the neurons derived from bipolar patients who were lithium responsive in a clinical setting (Mertens, Wang et al. 2016, Stern, Santos et al. 2017).

These findings in monogenic and idiopathic disorders offer great promise in deepening our understanding of autism neuropathology. iPSC models can be used to study the developmental consequence of an individual’s genetic profile on cellular and molecular phenotypes. Further, these models allow for the identification of pathway specific drugs and testing of patient-specific drug responsiveness, a popular idea coined ‘personalized medicine’ (Figure 5). Given the idea that there may be several autisms united by clinical phenotypes but caused by different underlying etiologies, this will be particularly useful as it is unlikely that there will be a ‘one-size-fits-all’ drug that can alleviate core symptoms in all ASD patients.
Figure 5: Currently, physicians use a ‘one size fits all’ approach to prescribe medicines to individuals; however, not all individuals have the same response to the same dose of a medicine nor do they necessarily respond to one medication that elicits a response in another individual. For this reason, targets are moving more towards creating ‘personalized medicine’ whereby a person’s genome is used to test different therapeutics.

Adapted from: Alpha Genomix; http://www.alphagenomix.com/empowering-personalized-medicine/

1.3.4. Using environmental stressors to identify diagnosis-dependent vulnerabilities in patient-derived cells

The role for complex gene-by-environment interactions is well-accepted as contributing to disease pathogenesis or progression in a number of neurodevelopmental disorders and neurodegenerative diseases. Exposure to environmental toxicants can lead to inflammatory cellular responses and induce formation of ROS. Reactive oxygen species (ROS) under physiological conditions are a natural by-product of cellular metabolism playing a fundamental role in signal transduction and cell homeostasis (Schieber and Chandel 2014).
Under pathological conditions ROS act as an oxidative stressor that can damage cells and induce cell death (Schieber and Chandel 2014). Signs of oxidative stress often indicate mitochondrial dysfunction, which has been implicated in the pathogenesis of a number of neuropsychiatric disorders. Studying direct relationships of consequences of specific genetic backgrounds and vulnerability to environmental factors in humans has been challenging for obvious reasons. Human iPSCs offer an attractive solution to this problem, allowing for the study of gene, environment, and gene-by-environment interactions. Using iPSC patient-derived cells, it is possible to directly examine the effects of toxicants on cells from patients with neuropsychiatric disorders and see how these cells may respond differently in comparison to normal controls. Indeed, a number of studies have done precisely this and begun to examine phenotypes and effects of stressors on human cells from a wide range of diseases, including patients with Huntington’s, Parkinson’s, Alzheimer’s, and schizophrenia.

Early studies examined iPSCs and NSCs derived from patients with Huntington’s Disease (HD), a neurodegenerative disorder caused by increases of CAG triplet repeats in the coding region of the Huntingtin (HTT) gene which results in an abnormal protein. These studies showed that HD-derived cells exhibited greater cell death than controls both after growth factor withdrawal and after exposure to environmental stressors, such as hydrogen peroxide ($\text{H}_2\text{O}_2$) (Zhang, An et al. 2010, Consortium 2012). HD NSCs also exhibited a significantly lower maximal respiration capacity, indicative of dysfunctional
mitochondria (An, Zhang et al. 2012). Many of these phenotypes were shown to be dose-dependent with the number of CAG repeats present; the longer the repeat lines were, the more vulnerable the cells were to stressors (Consortium 2012). Further, targeted correction of the expanded HTT gene reversed the susceptibility to cell death and altered mitochondrial bioenergenetics (An, Zhang et al. 2012).

Other studies have examined cells from patients with Parkinson’s disease (PD), a neurodegenerative disorder caused by genetic and environmental factors that lead to progressive degeneration of the nigrostriatal dopaminergic pathway in the brain. A fraction of PD cases are inherited through mutations in specific genes, LRRK2 and PINK1, which code for kinases, leucine-rich repeat kinase 2 and PTEN-induced putative kinase, and these mutations can result in altered functional activity of these enzymes. However, similar to ASD, the majority of PD cases are sporadic which strongly implicates a role for environmental factors in contributing to PD pathogenesis. In a study examining dopamine neurons derived from PD patients with mutations in LRRK2, researchers found increased susceptibility to cell death after exposure to several different oxidative stressors, such as 6-hydroxydopamine (6-OHDA), H$_2$O$_2$, and MG-132, a proteasome inhibitor (Nguyen, Byers et al. 2011). Another study examining different PD patients with different mutations in PINK1 or LRRK2 found that exposure to substances that produce mitochondrial dysfunction, such as valinomycin, concamycin, MPP+, and H$_2$O$_2$, induced greater increases in ROS in PD derived neural cells (Cooper,
Seo et al. 2012). PD neural cells also exhibited altered mitochondrial bioenergetics in comparison to controls (Cooper, Seo et al. 2012). Further, some of the phenotypes that arose after exposure to chemicals inducing mitochondrial dysfunction could be rescued in PD cells after pharmacological application of antioxidant Coenzyme Q or LRRK2 kinase inhibitor, GW5074 (Cooper, Seo et al. 2012).

These studies demonstrate the advantages in using hiPSC derived cells as a model system to examine gene-by-environment relationships and the potential for individuals to have a genetic susceptibility to pathological phenotypes after exposure to environmental stressors. In some cases, they provide evidence that neuroprotective candidate molecules can be used to mitigate the negative consequences of these stressors. Using this same approach to examine autism and sibling neural precursor cells (NPCs) after application of known environmental toxicants and oxidative stressors might reveal a physiological vulnerability in either functional oxidative abnormalities and/or mitochondrial dysfunction. Given the heterogeneity of autism, susceptibility to oxidative stress may be a general feature of the disorder, but it is more likely a factor in individual cases. This would provide support for patient-specific interventions.
CHAPTER 2: RATIONALE

Autism Spectrum Disorder (ASD) is a highly prevalent (1:68), heritable, and complex neurodevelopmental disorder characterized by deficits in social communication and interaction and the presence of stereotyped/repetitive behavior. Clinical manifestations of ASD have a wide range in severity and quality. In line with this, a number of different genetic and environmental factors have been implicated in contributing to the pathogenesis of ASD. Due to this heterogeneity, it has been proposed that we think of ASD as not one disorder but as ‘The Autisms’, a set of different but similar disorders with specific individual differences that manifest themselves as fewer, more common phenotypes. This may be due to commonalities in dysregulation of cellular processes or signal transduction pathways that lead to similar behavioral outcomes.

Technological advances in iPSC technology have radically transformed how we study neurodevelopment and disease processes. Now researchers can not only study live human cells, but they can also retain a specific individual’s unique genetic signature on their, albeit simplified, neural development in dish. A multiplicity of studies examining neuropsychiatric disorders, including autism, have increased current understanding of disease pathogenesis. The majority of these studies have primarily focused on terminally differentiated, post-mitotic neurons and the use of 3D culture systems called organoids. While mature
neurons and organoids allow study of disease-related process including dendrite outgrowth and synaptogenesis, they preclude study of earlier processes like proliferation and migration. In neurodevelopmental disorders, such as autism, abundant genetic and post-mortem evidence indicates defects in early developmental processes. Neural precursor cells (NPCs), a highly proliferative cell population, may be a suitable model in which to ask questions about ontogenetic processes and disease initiation.

Thus, I hypothesized that patient-derived ASD NPCs may exhibit a dysregulation of proliferation in comparison to their sibling. As a corollary, we may expect to see differences in ASD NPC responses to regulators of proliferation, such as growth factors, and/or in their responses to environmental stressors which can disrupt proliferation. To support this hypothesis, I began by creating a series of methodologies that would allow me to investigate human NPC proliferation and applied these to two autism-sibling pairs. In both families, I found evidence of patient-specific NPC proliferation differences in comparison to their sibling controls. In examining responses to growth factors, one autism-sibling pair displayed differential sensitivity—a further suggestion of patient-specific phenotypes. Lastly, I examined autism and sibling NPC responses to environmental stressors. Interestingly, in examining NPC DNA synthesis I found a common reduced sensitivity after exposure to methyl mercury (MeHg) in three patients with ASD (both idiopathic patients and one additional patient with a 16p11.2 CNV deletion) compared to controls. These observations indicate that
there may be ASD patient-specific differences as well as common phenotypes in comparison to controls. These studies are described in detail in the following pages.
CHAPTER 3: MATERIALS AND METHODS

3.1. Patient Cohort

Patients were chosen from a cohort recruited by Dr. Brzustowicz of 51 New Jersey families, each having at least 5 members (265 subjects total) (Bartlett, Flax et al., Bartlett, Hou et al. 2014). In each family, one individual was ADI-R/ADOS-confirmed proband with autism and contained a first-degree relative with Children’s Environmental Literacy Foundation (CELF)-confirmed specific language impairment (SLI) (Bartlett, Flax et al. 2012, Bartlett, Hou et al. 2014). Extensive and comprehensive medical and behavioral assessment was performed for the individual with autism (Bartlett, Flax et al. 2012, Bartlett, Hou et al. 2014). Additionally, 3 to 5 hours of direct behavioral testing was performed for each family member (Bartlett, Flax et al. 2012, Bartlett, Hou et al. 2014). These families were chosen for this study because SLI, a language endophenotype for ASD, is likely to reduce the underlying heterogeneity. The studies described here are based on two families from this larger cohort, each containing a sex-matched, sibling control (male). A minimum of 2 – 5 induced pluripotent stem cell (iPSC) clones were used per individual to induce into neural precursor cells (NPCs) (Figure 6A). Where specified, in some cases multiple NPC lines were derived from the same iPSC clone (Figure 6B).
**Figure 6:** Two families were chosen from a larger cohort of New Jersey families that have 1st degree relatives with SLI, A) blood was drawn and iPSCs were created from a boy child with autism and his unaffected brother, B) in following studies between 2 and 5 iPSC clones were used to derive NPCs, in some cases multiple NPCs were derived from 1 iPSC clone.
3.2. Cell Derivation and Culture

3.2.1. Derivation of iPSC lines and culture maintenance

iPSC lines were derived from individuals in the laboratory of Dr. Lu. Blood samples from subjects were collected and defined as pathologically normal before isolating peripheral blood mononucleocytes (PMBCs). PMBCs cells were then either cryopreserved or plated for iPSC derivation. iPSCs were derived by following a protocol that reprograms activated T-cells via a temperature-sensitive mutant of Sendai virus containing Oct3/4, Sox2, Klf4, and c-Myc, Figure 7 (Seki, Yuasa et al. 2012).

Following reprogramming, iPSCs were grown under conditions to promote their outgrowth and colonies formed which were then propagated as cell lines. These iPSC lines were fully characterized by 1) assessing pluripotency mRNA and protein markers, 2) confirming genetic homology to original tissue by DNA fingerprinting, 3) checking for chromosomal abnormalities by karyotype analysis, and 4) examining multi-lineage differentiation by assessing embryoid body and teratoma formation (Wu, Xu et al. 2007).

To maintain iPSC lines, cells were plated on hESC-qualified extracellular matrix-mimic gel or Matrigel (Corning, 354277) and maintained in mTeSR™1 (Stem Cell Technologies, 85850) media. Media was changed daily. After cells reached 70–90% confluence, cells were incubated with 0.5 mM filter-sterilized EDTA diluted in 1XPBS for a minimum of 10 min. When iPSCs lifted, they were centrifuged at 150xg for 5 min, re-suspended in media, and plated at
approximately 15% confluence. For the first 24h in culture, cells were incubated with 5 µM ROCK Inhibitor, Y27632 solution (Stem Cell Technologies, 72302). For more detail, see manufacturers protocol: (https://cdn.stemcell.com/media/files/manual/MA29106-Maintenance_Human_Pluripotent_Stem_Cells_mTeSR1.pdf)

**Figure 7**: Converting T-Cells to iPSCs

3.2.2. NSC induction and culture maintenance

To induce NSCs, 2.5 x 10⁴ – 3x10⁴ cells/cm² iPSCs were plated in one well of a 6 well plate in mTeSR™1 media with 5 µM Y27632 solution for 24h. After 24h, media was replaced with Neural Induction Media (ThermoFisher Scientific, A1647801). Media was changed every other day for 7 days. After 7 days in Neural Induction Media, cells were passaged and considered passage 0 (P0) (Figure 8A). For more information see manufacturers protocol: https://goo.gl/euub7a.
ThermoFisher: Gibco Induction of Neural Stem Cells from Human Pluripotent Stem Cells Using PSC Neural Induction Medium:

https://tools.thermofisher.com/content/sfs/manuals/MAN0008031.pdf

In order to maintain lines, cells were cultured on Matrigel and maintained in Neural Expansion Media (ThermoFisher Scientific, A1647801). Media was changed every 48h until cells reached confluence. Once cells were 100% confluent, they were dissociated using Accutase (Gibco, A11105-01) at 37°C for 10 min, re-suspended with 1XPBS (3 – 5 mL), centrifuged at 300xg for 5 min, re-suspended in media, and counted before plating at a density of 1x10^5 – 1.5x10^5 cells/cm^2 per well of a 6 well plate. For the first 24h of passages 0 – 4, cells were incubated with 5 µM Y27632 solution.

**Figure 8:** iPSCs differentiated into NSCs via monolayer method express NSC markers

*Figure 8:* A) iPSCs plated for 7 days in neural induction media express markers specific to neural stem cells including: transcription factors B) SOX2 (red), C) PAX6 (red) and cytoskeletal marker, nestin (green).
3.2.3. NSC marker expression and differentiation

To define NSC identity, immunocytochemistry (ICC) was conducted to verify that cells expressed NSC transcription factors (Sox2, Pax6) and cytoskeletal proteins (Nestin) inherent in NSCs (Figure 8B, C). Additionally, ICC was performed to ensure that NSCs did not express iPSC transcription factors (Oct4).

To functionally verify NSC identity, NSCs were cultured in cell-type specific media (Table 1) and differentiated into the 3 neural lineages: neurons, oligodendrocytes, and astrocytes (Figure 9). Their identity was confirmed using ICC to stain for cell type specific markers. See manufacturer protocol for detailed information:


**Table 1**: Media and substrate for differentiating NSCs into three neural lineages

<table>
<thead>
<tr>
<th>Desired Cell Type</th>
<th>Media Component</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuron</td>
<td>Neurobasal (1X)</td>
<td>Poly-d-lysine + Laminin</td>
</tr>
<tr>
<td></td>
<td>B27 (2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GlutaMax (2 mM)</td>
<td></td>
</tr>
<tr>
<td>Astrocyte</td>
<td>DMEM (1X)</td>
<td>Matrigel</td>
</tr>
<tr>
<td></td>
<td>GlutaMax (2 mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2 (1%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal Bovine Serum (1%)</td>
<td></td>
</tr>
<tr>
<td>Oligodendrocyte</td>
<td>Neurobasal (1X)</td>
<td>Poly-d-lysine + Laminin</td>
</tr>
<tr>
<td></td>
<td>B27 (2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GlutaMax (2 mM)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thyroid Hormone, T3 (30 μM)</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 9**: NSC directed differentiation into 3 neural lineages for 7 days

<table>
<thead>
<tr>
<th>Neuron</th>
<th>Astrocyte</th>
<th>Oligodendrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>TuJ1</td>
<td>S100β</td>
<td>Olig2</td>
</tr>
<tr>
<td>Tau</td>
<td>GFAP</td>
<td>GalC</td>
</tr>
</tbody>
</table>

**Figure 9**: NSCs cultured for 7 days in cell type specific conditions can be differentiated into neurons expressing immature neuronal cytoskeletal marker β-III tubulin (TuJ1, red) and mature neuronal cytoskeletal marker Tau (green); astrocytes expressing immature marker S100β (green) and glial fibrillary acidic protein (GFAP, red); and oligodendrocytes expressing immature and mature markers Olig2 (red) and GalC (green), respectively.

### 3.2.4. Cell cryopreservation and recovery

Cells were dissociated and re-suspended in appropriate cell type dependent media (mTeSR™ 1 for iPSCs and Neural Expansion Media for NPCs). A 20% DMSO solution was diluted in media and added drop wise to an equal volume of cell suspension to create a final 10% DMSO—cell solution. This solution was distributed in cryovials and placed in freezing containers (Thermofischer Scientific, 5100-0001) with isopropanol in -80°C freezer. After 24h, cryovials were transferred to liquid nitrogen.
To recover cryopreserved cell, cryovials were retrieved from liquid nitrogen, they were swirled in a 37°C water bath until only an ice crystal remained. Cells were re-suspended in pre-warmed Advanced DMEM/F12 Medium (ThermoFischer, 12634-010), which was added drop wise to prevent osmotic shock. Cells were then centrifuged at 300xg for 5 min, re-suspended in cell-type-dependent media with 5 µM Y27632 solution, and counted before plating at cell-type-dependent density on Matrigel-coated plates.
3.3. Experimental Conditions and Preparation

3.3.1. Media preparation (30% Expansion Media)

30% Expansion Media was prepared by diluting 100% Expansion Media by 70% using a 1:1 advanced DMEM/F12 + Neurobasal solution (ThermoFischer Scientific, 21103049) to make media for experimental conditions. Growth factors were added directly to media at time of plating.

3.3.2. Coating plates

24 well plates or 35 mm dishes were coated with 0.1 mg/mL filter-sterilized poly-D-lysine (PDL, Sigma, P0899) for 20 min at room temperature (RT) before washing twice with dH2O for 5 min each. Then dishes were incubated overnight at RT or for 1h in 37°C incubator with 5 µg/mL laminin (LN, Invitrogen, 23017-015) diluted in 1XPBS. After incubation, dishes were washed twice with 1XPBS for 5 min each before adding appropriate media without or with growth factors. All plates and dishes for experiments were coated under these PDL/LN conditions with the exception of hydrogen peroxide (H₂O₂) treatments which directly added media containing 5 µg/mL fibronectin (Sigma, F1141) after PDL coating and 2 dH2O washes to plates. Proliferation assays involving iPSCs used Matrigel coated plates according to manufacturer’s protocol:

3.3.3 NPC toxicant treatment

2h post-plating cells were incubated with either hydrogen peroxide (H$_2$O$_2$) or methylmercury (MeHg) for assay duration. H$_2$O$_2$ (Sigma, H1009) was aliquoted, parafilmed, and stored at 4°C in the dark. Methylmercury chloride (CH$_3$HgCl, Sigma, 115-09-3) was pre-measured and stored in glass vials. A 5 mM stock solution of MeHg was diluted in 0.1 M phosphate-buffered saline (PBS) and prepared by agitation for 1.5h immediately before use (Sokolowski, Falluel-Morel et al. 2011).
3.4. Assays for Assessing Proliferation

3.4.1. DNA synthesis via tritiated \( ^{3}H \)-thymidine radioactive incorporation

NPCs from control and ASD samples were plated in triplicate or quadruplicate into 24 well plates at 5x10^4 cells/cm^2 (for iPSCs, 2.5x10^4 cells/cm^2). 48h post-plating, cells were incubated with 0.5 μCi/mL of tritiated \( ^{3}H \)-thymidine (PerkinElmer, NET027E001) for the final 2h. An automatic harvester collected cells and tritiated \( ^{3}H \)-thymidine incorporation was assessed using scintillation spectroscopy.

3.4.2. NPC S-phase entry via EdU incorporation

In parallel cultures with radioactive thymidine incorporation, cells were plated at 5x10^4 cells/cm^2 in 35 mm dishes. At 46h cells were incubated with 5 mM EdU (ThermoFischer, C10337) for 2h, dissociated using Accutase, and re-plated in 35 mm dishes at 1x10^4 cells/cm^2 in 35 mm dishes to allow for single cell analysis (Figure 10). 2h post-plating, cells were fixed with 4% paraformaldehyde (PFA), assayed using an EdU Click-It reaction, and imaged using fluorescent confocal microscopy. The mitotic index was assessed blind in 10 systematically random fields (10X).
**Figure 10:** S-Phase entry schematic

**Figure 10**: Timeline of S-phase entry. NPCs are culture at 5x10^4 cells/cm² in 35 mm dishes for 48h. At 46h, they are incubated with thymidine analog, EdU, for 2h. At 48h, cells are dissociated into single cells and plated for 2h before fixing with 4% PFA.


3.4.3. **Enumeration of cell numbers**

2.5x10^4 cells/cm² NPCs or iPSCs were plated in duplicate or triplicate per well of a 24 well plate. To perform counting, cells were enzymatically dissociated with Accutase and quantified every 2 days for a 6-day period (or for iPSCs, once a day for 3 days), via hemocytometer in the presence of Trypan Blue (1:10, Sigma, 15250061) to ensure only live cells were included.
For greater detail on Methods 3.4.1 to 3.4.3:


3.4.4. **Cell death**

$5 \times 10^4$ cells/cm$^2$ were plated in triplicate in a 24 well plate for 24h and fixed with 4% PFA at 24h. ICC for apoptotic marker, cleaved caspase-3 (CC-3, 1:5000, Cell Signaling Technology, 9661) was performed and visualized using secondary biotinylated secondary antibody and Vectastain ABC Kit (1:100; Vector Laboratories, PK-4000). The horseradish peroxidase reaction was detected with 0.05% diaminobenzidine (DAB) and 0.02% H$_2$O$_2$. The reaction was stopped by washing 2X with 1XPBS. Cell death was assessed by systematically counting the total number of CC-3 positive cells in 3 X 0.3 cm rows per well via bright field microscopy at 32X.
3.5. Immunocytochemistry (ICC)

After 4% PFA fixation for 20 min at RT, NPCs were permeabilized with 0.3% Triton X-100 in PBS for 10 min. Then NPCs were blocked with 5% normal goat serum (NGS) for 1h before overnight incubation with primary antibodies specific to:

- **pluripotent stem cells:** Sox2 (1:1000, Abcam, ab92494); Oct4 (1:250, Santa Cruz, Sc-5279)
- **neural precursor markers:** Nestin (1:2000–1:5000, R&D Systems, MAB1259), Pax6 (1:300, Covance, PRB-278P)
- **neuronal markers:** β-III tubulin (TuJ1, 1:2000–1:5000, Covance, MMS-435P), Tau (1:500, Santa Cruz, Sc-5587)
- **oligodendrocyte markers:** Olig2 (unknown); GalC (unknown)
- **astrocyte markers:** Glial fibrillary acidic protein (GFAP, 1:1000, Dako, G9269)
- **proliferation markers:** Ki67 (1:100, BD Bioscience, 550609)

Staining was visualized by using FITC- or Texas Red-conjugated fluorescent secondary antibodies (DiCicco-Bloom, Deutsch et al. 2000, Nicot and DiCicco-Bloom 2001) (Yan, Zhou et al. 2013).
3.6. Quantification of Reactive Oxygen Species (ROS)

NPCs were seeded in 96 well plates for 24h. At 24h NPCs were loaded with 125 µM 2′,7′-dichlorofluorescin diacetate (H$_2$DCFDA, Ex./Em. 495/520 nm, Sigma D6883) and 1 µM Hoechst 33342 (Sigma) for 30 min at 37°C. NPCs were washed 3 times with 1XPBS. Fluorescence signals for H$_2$DCFDA at Ex./Em. 485/520 nm and Hoechst 33342 were acquired immediately using a microplate reader (Synergy HT, Biotek). Relative H$_2$DCFDA fluorescence intensities were normalized to Hoechst 33342.
The majority of studies using hiPSCs to investigate neuropsychiatric disease differentiate cells into neuronal subtypes or use a 3D culture system called ‘an organoid’ to recapitulate aspects of human brain development (Dolmetsch and Geschwind 2011, Pang, Yang et al. 2011, Lancaster and Knoblich 2014). These systems have been critical in studying and uncovering unique aspects of human development and disease (Marchetto, Carromeu et al. 2010, Vaccarino, Stevens et al. 2011, Bershteyn, Nowakowski et al. 2017, Giandomenico and Lancaster 2017). However, neuronal cultures and organoids frequently necessitate maintaining cells anywhere from weeks to months in culture before study. The lengthy culture time of these protocols and the required amount of resources to maintain these culture systems may limit the number of possible experiments that can be performed and the number of potential variables that can be tested. Further, many studies utilizing post-mitotic neurons and organoids have focused on processes such as synaptogenesis or neuronal function, which occur at later stages in development. Though there is a great deal of evidence that these processes may be involved in the pathology of developmental disorders such as autism and schizophrenia, earlier
developmental events that occur before neuronal differentiation are also important for disease pathogenesis (Walsh, McClellan et al. 2008, Guilmatre, Dubourg et al. 2009, Voineagu 2012, Parikshak, Luo et al. 2013, Birnbaum, Jaffe et al. 2014, Pinto, Delaby et al. 2014). To be sure, recent genomic studies show that the mid-fetal period, which is comprised of proliferation, neurite outgrowth, and migration are certainly important in the pathogenesis of autism (Walsh, McClellan et al. 2008, Guilmatre, Dubourg et al. 2009, Voineagu 2012, Parikshak, Luo et al. 2013, Birnbaum, Jaffe et al. 2014, Pinto, Delaby et al. 2014, Waltereit, Banaschewski et al. 2014). Thus, studying neural stem and progenitor cell populations may help elucidate these earlier developmental processes. Organoid systems, considered to better recapitulate human brain development because of their 3D nature and self-organizing structure, contain a progenitor population that can be used to study these earlier events. However, the organoid progenitor pool is often limited and thought to be more similar to radial glial cells than to neural stem or progenitor cells (Qian, Nguyen et al. 2016, Bershteyn, Nowakowski et al. 2017). Thus, neural precursor cells (NPCs), a highly proliferative cell population, may be a suitable model in which to ask questions about ontogenetic processes and disease initiation.

It is only in the past 25 or so years that ‘neural stem cells’ have been discovered and defined as multipotential cells that have the capacity for self-renewal. In the literature, there is sometimes confusion in terminology and differences between neural stem cells and neural progenitor cells. Neural stem
cells refer to a subtype of cells that can self-renew and have the ability to become one of the 3 neural lineages—neurons, astrocytes, and oligodendrocytes (Temple 2001). The term ‘progenitor cells’ refer to immature cells which can proliferate; thus, ‘neural progenitor cells’ are a class of immature neuronal cells that are still proliferating (Temple 2001). For this thesis, it is important to be clear on this terminology as I use the term ‘neural precursor cells’ or NPCs. This vague language is intentional due to the fact that the differentiated patient-derived cells we are studying in culture are not a homogenous population but rather a mixture of both neural stem and progenitor cells, similar but distinct cell early neural cell types.

The DiCicco-Bloom lab has an extensive history of examining the effects of growth factors on rat and mouse cells in culture (DiCicco-Bloom and Black 1988, Lu and DiCicco-Bloom 1997, Mairet-Coello, Tury et al. 2009, Yan, Zhou et al. 2013), but before the inception of this project, had no previous experience working with human cells. Due to this history, early experiments were designed to characterize the human NPC model system by examining specific cell type markers and cell proliferation, to identify variables in culture that would impact these characteristics, and to create a defined system that could be used to study differences in proliferation between autism and sibling NPCs. I spent my first year in the laboratory carefully characterizing the model in a detailed and thorough manner, examining a number of combinations of different media, substrates, effects across passages, cell densities, and time points. In many of
these early experiments, it is important to give credit to Xiaofeng Zhou, who was working alongside me to define conditions to assess the human NPCs. Here, I present a small selection of experiments to demonstrate the thought processes behind what created the basic platform the lab now uses for its experiments on human NPCs.
4.1. Identify media composition to define NPC markers and proliferation

To create and maintain human NPCs the DiCicco-Bloom Lab uses a commercially available media from Gibco called ‘Neural Induction Media’ and ‘Neural Expansion Media’, respectively (Yan, Shin et al. 2013, Fujie, Fusaki et al. 2014, Pertek, Meier et al. 2014). Induced pluripotent stem cells (iPSCs) are cultured in the Neural Induction Media for 7 to 10 days (Figure 8A). After this period cells begin to express markers for NPCs, such as cytoskeletal marker Nestin, transcription factor for pluripotent stem cells, Sox2, and transcription factor for cortical forebrain progenitors, Pax6 (Figure 8B, C). The exact composition of the Neural Induction Media is propriety and due to this, we do not know definitively what factors are in it. However, we can guess that it is composed of a combination of various growth factors and dual SMAD inhibitors to create and maintain NPC identity (Chambers, Fasano et al. 2009). Additionally, I have spoken to the company and they have informed us that it does not contain basic fibroblast growth factor 2 (bFGF)—which will be important later in this manuscript. In order to create a more defined culture system, I examined the effects of altering media composition on human NPC characteristics.

Early experiments examined the effects of a wide variety of different media on cell identity and proliferation, the breadth and scope of which can be
seen in **Table 2**. Some media were not adequate for cell survival, such as ‘NB’ and ‘Defined’, and these media were eliminated in subsequent experiments.

**Table 2**: Various media examined to define conditions

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neural Induction Media (NIM)</td>
<td>100% Neurobasal Medium, 2% Neural Induction Supplement, 2 mM GlutaMax</td>
</tr>
<tr>
<td>100% Expansion (Expansion Media)</td>
<td>25% DMEM, 25% F12, and 50% Neurobasal Medium, 2% NIS, 2 mM GlutaMax</td>
</tr>
<tr>
<td>30% Expansion</td>
<td>25% DMEM, 25% F12, and 50% Neurobasal Medium, 0.6% NIS, 2 mM GlutaMax</td>
</tr>
<tr>
<td>Neurobasal + FGF (NBF+)</td>
<td>100% Neurobasal; 2% B27, 0.1% BSA, 2mM GlutaMax, FGF (10ng/mL)</td>
</tr>
<tr>
<td>Neurobasal (NB)</td>
<td>100% Neurobasal; 2% B27, 0.1% BSA, 2mM GlutaMax</td>
</tr>
<tr>
<td>Define</td>
<td>50% DMEM, 50% F12; 1% N2</td>
</tr>
<tr>
<td>Define + FGF (Define +)</td>
<td>50% DMEM, 50% F12; 1% N2, FGF (10 ng/mL), Insulin</td>
</tr>
</tbody>
</table>

In examining the effects of a panel of different media on a control subject’s NPCs, **Figure 11A** shows that reducing the percentage of Neural Induction Supplement (NIS) in the Expansion Media to as low as 20% of the original composition had little to no impact on DNA synthesis or S-phase entry at 48h. Furthermore, using a defined media composition, ‘NBF+’, increased DNA synthesis 3-fold. Cells in ‘Defined’ and ‘Defined+’ conditions exhibited a 50% reduction in DNA synthesis and significant amounts of cell death were observed; thus, these media were not used in future experiments. This experiment demonstrated that we could use different media conditions to alter the magnitude of DNA synthesis (**Figure 11A**) and this was confirmed by a second measure, examining the proportion of cells entering S-phase (**Figure 11B**). For future experimental conditions, I chose to use a reduced version of 100% Expansion
media—30% Expansion. This reduction of the neural induction supplement still supported cell health and survival, but also created a more defined and limiting environment. Culturing cells in a limiting environment is a tried and true culture method which provided the opportunity for us to manipulate experimental conditions by adding back in various growth factors that could potentially untangle a differential response or sensitivity in ASD versus sibling NPCs. There was also positive economic value due to the high cost of the commercial media. Thus, 30% Expansion became our standard media for control experimental conditions.

**Figure 11:** Effect of media on DNA synthesis and S-phase entry

*A.*

<table>
<thead>
<tr>
<th>Media</th>
<th>DNA Synthesis (CPMs, 1x10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Exp</td>
<td>2</td>
</tr>
<tr>
<td>50% Exp</td>
<td>4</td>
</tr>
<tr>
<td>20% Exp</td>
<td>8</td>
</tr>
<tr>
<td>20% Exp + 80% Define+</td>
<td>** ****</td>
</tr>
</tbody>
</table>

*B.*

<table>
<thead>
<tr>
<th>Media</th>
<th>S-Phase Entry (Edu LI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Exp</td>
<td>25</td>
</tr>
<tr>
<td>50% Exp</td>
<td>25</td>
</tr>
<tr>
<td>20% Exp</td>
<td>25</td>
</tr>
<tr>
<td>20% Exp + 80% Define+</td>
<td>**</td>
</tr>
</tbody>
</table>

**Figure 11:** Varying media can alter A) DNA synthesis (***, p=0.0001) and B) S-phase entry (**, p=0.004).
4.2. Establish a defined substrate to assay NPC identity and behavior

The stem cell field traditionally cultures cells on a growth factor rich, basement membrane matrix substrate commercially called Matrigel. Matrigel is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells (Benton, George et al. 2009, Hughes, Postovit et al. 2010). It is heterogeneous in nature and contains a number of extracellular matrix proteins including collagen, laminin, entactin, heparan sulfate proteoglycans as well as growth factors such as TGF-β and EGF (Hughes, Postovit et al. 2010). Composition of Matrigel can vary drastically from lot-to-lot or from batch-to-batch. In order to create a defined culture system, I examined the effects of altering various plate substrate conditions on human NPC characteristics. For different substrates examined see Table 3.

**Table 3**: Substrates examined to define experimental conditions

<table>
<thead>
<tr>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrigel</td>
</tr>
<tr>
<td>0.1 mg/mL Poly-D-lysine (PDL)</td>
</tr>
<tr>
<td>0.1 mg/mL PDL + 5 to 10 μg/mL Laminin</td>
</tr>
<tr>
<td>0.1 mg/mL PDL + 2.5 – 5 μg/mL Fibronectin</td>
</tr>
</tbody>
</table>

In one of my earliest experiments, I examined the effect, across multiple passages, that plating NPCs for 48h on Matrigel or poly-d-lysine (PDL), a polymer of a basic amino acid, had on cell numbers, proportions of cells in S-Phase, and cell type specific markers (Table 4). In primary neuronal cell culture, PDL is commonly used as a substrate because it promotes adhesion of neurons...
adhesion to the dish. This experiment had several notable conclusions. First, at P0, numbers of cells plated on PDL were too low to count due to massive cell death; therefore, no analysis was done for this specific condition. In contrast, cells plated on PDL at P3 had significantly greater survival than cells had at P0. This suggests that P3 cells were ‘sturdier’ in comparison to P0 cells. What I mean by this, is that PDL is a very limiting substrate and has no growth or survival factors yet cells at P3 were still able to survive in this environment. This is interesting because it suggests that NPC passage number can have dramatic effects on different NPC characteristics or behaviors and is essential to track when studying patient-derived NPCs. Secondly, this experiment showed that altering substrate from either Matrigel or PDL can drastically alter the identity and proliferative capacity of NPCs. In contrast to PDL conditions, at both P0 and P3, cells plated on Matrigel had significantly greater cell numbers. At P3, cells plated on Matrigel exhibited 2-fold greater proportions of cells entering S-phase and 1.7 fold more Sox2 and Pax6 positive cells. Cells plated on PDL had ~4 times greater proportions of cells expressing immature neuronal marker TuJ1. These results indicate Matrigel may maintain a proliferative NPC state; whereas, NPCs plated on PDL may be exiting the cell cycle and differentiating. Thus, altering the substrate NPCs were plated on allowed us to alter NPC markers and their capacity to proliferate.
Table 4: Effect of passage and substrate on markers of self-renewal and cell fate

<table>
<thead>
<tr>
<th>Passage</th>
<th>Substrate</th>
<th>Cell #s</th>
<th>EdU+ (%)</th>
<th>Nestin+ (%)</th>
<th>Sox2+ (%)</th>
<th>Pax6+ (%)</th>
<th>Tuj1+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Matrigel</td>
<td>166</td>
<td>30.8</td>
<td>91</td>
<td>---</td>
<td>---</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>PDL</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>Matrigel</td>
<td>189</td>
<td>34.04</td>
<td>82.72</td>
<td>85.1</td>
<td>72.8</td>
<td>12.08</td>
</tr>
<tr>
<td></td>
<td>PDL</td>
<td>126</td>
<td>16.2</td>
<td>78.1</td>
<td>48.8</td>
<td>41.9</td>
<td>48</td>
</tr>
</tbody>
</table>

Table 4: Effect of substrate and passage on cell numbers, proportions of cells in S-phase, neural precursor parkers nestin, sox2, and pax6, and immature neuronal marker b-III tubulin (TuJ1). (11/4/13, 100% Expansion media, 48h in culture, 100k cells/35 mm dish)

Following this experiment, I spent considerable time exhaustively examining the effects of different combinations of media, cell densities, and coating substrates to better understand the system as well as to see if there were any combinations which were more advantageous in studying human NPCs. From the literature, I found that human NPCs and neurons were commonly plated on either laminin, fibronectin, poly-L-ornithine or some combination of these. Both laminin and fibronectin were readily available in the lab, so I first coated plates using our standard 0.1 mg/mL PDL condition and then coated plates with either laminin or fibronectin to ascertain their effect on NPC proliferation. In Figure 12, I found no significant difference between laminin and fibronectin when assessing NPCs at 48hrs in DNA synthesis, S-phase entry, or cell numbers. Further, I examined different cell densities for this comparison of substrates. Both the low density, 25K, where cells have little to no cell-to-cell contact and the high density, 100K, where cells are roughly 50 to 75% confluent, showed no difference in NPC DNA synthesis, S-phase entry, or cell numbers in
comparing laminin and fibronectin coatings. Importantly, in these assessments of different substrates and cell densities, altering the coating to either PDL + laminin or PDL + fibronectin overcame the low mitotic index observed in cells plated on PDL alone, 16%, bringing it up to 30%. This higher mitotic index, 30%, compares favorably to observations of cells when plated on Matrigel, but avoids the complexity and potential variability found in this cell line derived product.

**Figure 12:** Comparison of effect of different substrates and different cell densities on markers of proliferation.

**Figure 12:** No difference in comparison of substrates on two cell densities 25K (1.25x10^4 cells/cm²) and 100K (5x10^4 cells/cm²) at 48h in A) DNA synthesis (n= 1/3/10), B) S-phase entry (n= 1/2/5), and C) cell numbers (n= 1/1/3; cell line/expt/wells)
Through my literature review on human NPCs and findings in my own research of similarities in many NPC characteristics despite laminin or fibronectin substrates, I arbitrarily chose to coat plates with a combination of PDL and laminin to use as a base for my studies of proliferation.

In order to ascertain the importance of a number of different laminin coating variables such as, laminin concentration (5 µg/mL vs. 10 µg/mL), coating time (1.5h at 37°C vs. 12h at RT), plate ‘freshness’ (made fresh that day or frozen for 1 week at -20°C), and what the laminin was diluted in (1x calcium magnesium free PBS, CMF vs. 1XPBS), I conducted a large experiment examining the effects these variables had on DNA synthesis. For good measure, we examined multiple media conditions without and with growth factors to ensure there was no combinatorial effect. Figure 13 shows no significant effect on DNA synthesis when plates were made fresh versus frozen for a week. This meant that we could coat plates ahead of time and store them for ease of use and planning. There was also no significant effect based on whether laminin was diluted in 1XPBS or CMF-PBS (Tyrode’s solution). We chose to use 1XPBS over CMF-PBS because it does not contain glucose and would decrease the possibility of bacterial contamination. Changing the concentration of laminin from 10 µg/mL to 5 µg/mL decreased DNA synthesis by 50% in both 30% Expansion without and with bFGF (10 ng/mL). As the decrease was consistent across different media, we chose to use 5 µg/mL laminin to create a more limiting environment as well as to reduce costs of supplies.
**Figure 13:** Effect of different substrate coating conditions on DNA synthesis

**Figure 13:** Differences in altering method of preparation of substrate, substrate compositions, concentration, and kind of substrate on 48h DNA synthesis
4.3. Determine optimal cell density to measure NPC markers and proliferation

In the stem cell field, given wisdom is that stem cells must be grown and assayed in very dense cultures in order for them to maintain their “stem-ness”. Having not come from this background, our lab experimented with different cell densities, from single cells to cells in a confluent monolayer, to see how this would affect various cell markers and measures of proliferation.

I plated cells at densities 50K, 100K, and 200K ($2.5 \times 10^4$ — $1 \times 10^5$ cells/cm$^2$) in a 24 well plate, and examined how this effected S-phase entry and differentiation. It is important to note that in this experiment, even though 50K is a lower density, there is still a decent amount of cell-to-cell contact as NPCs tend to cluster together. **Figure 14** demonstrates that the various densities plated had no effect on the percentage of cells entering S-phase, but did have a significant effect on the percentage of cells expressing immature neuronal marker β-III tubulin. This experiment was performed multiple times on multiple passages of the same cell line. This suggests that at 48h, altering cell density could impact cell type specific markers but did not significantly affect S-phase entry.
Figure 14: Effect of NPC density on S-phase entry and percentage of cells expressing β-III tubulin expression

Figure 14: At 48h, proportion of cells entering S-phase is unchanged across densities whereas the percentage of cells expressing TuJ1 is inversely related to cell density 50K (2.5x10^4 cells/cm^2), 100K (5x10^4 cells/cm^2), and 200K (10x10^4 cells/cm^2); (** p<0.0001, ** p=0.005, * p=0.03; n= 1/3/6; cell line/expt/wells)

As the results of this experiment suggested that cells may be beginning to differentiate at 48h at lower densities and I was interested in studying NPC self-renewal and proliferation at this time point, I wanted to keep the cells in higher cell-to-cell contact conditions. After many months of counting cells in a monolayer, I observed high variability in single experiments because of small micro-environment effects and different cell-to-cell contact effects. I, along with another graduate student in the lab, Smrithi Prem, began to experiment with plating cells as single cells for 48h and comparing these single cell cultures (Low Density, LD) to cells grown at a higher “monolayer-like” density (High Density, HD) before dissociating them and replating them for 2h to ease enumeration and reduce variability. Figure 15 shows the results of these experiments. This is an example from a single experiment showing only the control media condition, but keep in mind, this was replicated across multiple different media conditions as well as multiple cell lines. Cells grown in either system had similar proportions of
cells entering S-phase and expressing proliferation marker Ki67. Cells grown at a low density for 48 hours contained greater proportions of cells expressing TuJ1, which further confirmed my previous findings albeit using slightly altered paradigms. Despite similarities in proliferation markers in the two paradigms, I conservatively concluded that I would maintain higher densities for proliferation studies and would use the dissociation method for enumeration of proportions of cells.

**Figure 15:** Comparison of low density (LD) and acutely dissociated high density (AD) culture conditions at 48h

**Figure 15:** At 48h single cell densities or LD (5x10^3 cells/cm^2) and cells plated at higher densities (5x10^4 cells/cm^2) and dissociated then replated for 2h to allow for single cell analysis or AD, exhibit no difference in S-phase entry or proportion of cells expressing proliferative marker Ki67; however, cells plated at LD exhibit 2-fold greater proportion of cells expressing TuJ1 (**p<0.0001**).
4.4. **Examine effect of passage number on NPC proliferation**

To attempt to understand differences across passages in our NPC model, we examined a number of different markers as well as effects on proliferation. In examining DNA synthesis at 48hs, some cell lines had strong passage-dependent consequences, while others did not (Figure 16). A control NPC line (CTRL-1, blue) showed a marked decrease in DNA synthesis over several passages whereas another control NPC line derived from a different individual (CTRL-2, orange) had no change in DNA synthesis over the same number of passages. In addition, some NPC lines exhibited increased DNA synthesis as passage number increased (data not shown). To limit variability in our studies we restricted our experiments from passage 3 to passage 8. Though not regularly reported, this dramatic change in proliferation between lower and higher passages has also been the experience of others. The exact reason for this is currently unresolved but may be reflective of cell lines gaining mutations over time or the limited self-renewal properties of NPCs. Understanding why and how proliferation rates change across different passages may be informative in understanding development and disease pathology or perhaps in defining human-derived cell cultures but more research needs to be done to understand this phenomenon.
Figure 16: Cell line and passage-dependent effects on DNA synthesis

Figure 16: Some cell lines exhibit passage dependent effects on DNA synthesis
4.5. Experimental conditions to measure proliferation

The methods that resulted from the experiments presented here have been published in a manuscript and can be read about (and seen!) in greater detail:

CHAPTER 5: Idiopathic Autism
Patient-Derived NPCs Exhibit
Patient-Specific Proliferation
Differences

Autism Spectrum Disorder (ASD) is a highly complex, heterogeneous
neurodevelopmental disorder with high heritability and genetic involvements
(Folstein and Rutter 1977, Ritvo, Freeman et al. 1985). Though ASD
pathogenesis is unknown, the multiplicity of genetic and environmental factors
likely interact and result in complex gene-by-environment interactions
(Hallmayer, Cleveland et al. 2011). Genetic and post-mortem evidence support
the idea that autism arises in part due to a dysregulation of brain development
2010, Pinto, Delaby et al. 2014).

Cell proliferation, an integral part of brain development, is the process that
results in more cells from one day to the next. Albeit simple on it’s face, this
encompasses a number of different complex processes including cell division,
cell survival, and cell death.
5.1. Family 1 ASD NPCs exhibit reduced proliferation in comparison to sibling NPCs

In comparison to sibling NPCs, ASD NPCs from a single-family comparison exhibit a robust and reproducible reduction in proliferation. In 67 experiments (SIB-31, ASD-36), utilizing multiple derivations of NPCs (SIB-5, ASD-9) derived from multiple iPSC clones per individual (SIB-4, ASD-5), ASD NPCs display a 65% reduction in DNA synthesis as evidenced by radioactive thymidine incorporation (**Figure 17**). In **Figure 17B**, the data is represented by clone to demonstrate variability across NPC lines derived from different iPSC clones. **Figure 17C** delineates the number of NPC derivations, experiments, and wells for each NPC line per individual. It is important to communicate this kind of information because one of the current challenges in the patient-derived iPSC field is identifying the appropriate ‘n-value’.
**Figure 17:** Family 1 ASD NPCs exhibit reduced DNA synthesis in comparison to sibling NPCs.

![Graph A](image1)

**Graph A:** A) At 48h, Family 1 ASD NPCs exhibit a 65% reduction in DNA synthesis in comparison to SIB NPCs (****, p <0.0001, SIB n=4/5/31/116, ASD n=5/9/36/125; clones/NPC deriv/expt/wells). B) Data shown by clone to indicate individual variability. C) N-values for NPCs derived from different iPSC clones, number of NPCs derived from a single clone, numbers of experiments, and total numbers of wells.

The proportion of cells entering S-phase was assessed and ASD NPCs exhibited a 40% reduction in the proportion of cells in S-Phase (**Figure 18**).

**Figure 18 B, C** show the data broken down by diagnosis and clone.
To see if changes in DNA synthesis and proportions of cells in S-Phase translated into cellular biology, I examined cell numbers at 2, 4 and 6 days. At both 4 and 6 days in culture, ASD NPCs displayed a 60% reduction in numbers of cells (Figure 19). I think it is of some note that cell numbers at 2 days were not statistically different when examined using a one-way ANOVA, but if evaluating data via a student’s t-test to examine the two groups, there was a significant difference. This early difference in cell numbers caused me to inquire whether or not cell death may be playing a role in this proliferation defect.
**Figure 19**: Family 1 ASD NPCs exhibit a reduction in proliferation in comparison to sibling NPCs.

A. **Representative image of SIB and ASD NPCs at days 2, 4, 6.**

B. **Graph showing cell numbers.**

In order to inquire about whether apoptosis may be playing an additional role in this reduction in cell numbers and the proportion of cells entering S-phase, an undergraduate honors thesis student, Anna Markov, examined expression of a well-known activator of apoptosis, cleaved caspase 3 (CC-3). At 24h, ASD NPCs display no difference in cell death in comparison to sibling NPCs (**Figure**...
This indicates that the reduction in cell numbers after 6 days in culture was due to a smaller proportion of cells entering S-phase and a reduced proliferative population.

**Figure 20:** Family 1 ASD NPCs display no difference in cell death

**Figure 20:** A) Representative image of CC-3 immunostaining, red arrows indicate cells negative for CC-3 and black arrows indicate cells expressing CC-3 B) At 24h Family 1 sibling and ASD NPCs display no difference in numbers of cells expressing apoptotic marker, cleaved-caspase 3 (SIB n=2/2/4/12, ASD n=2/3/5/14; clones/NPC deriv/expt/wells). "Anna Markov"

Lastly, I was curious as to whether this reduction in ASD NPC proliferation in comparison to sibling NPCs was specific to cell-type and stage, merely the NPCs growing and dividing in the brain or if it was a systemic defect that affected the proliferation of other cell types. In order to investigate this, I examined the proliferation of iPSCs using similar methods that were adapted to their culture specific conditions and faster growth rates. Interestingly, there was no difference in ASD iPSC DNA synthesis at 48h (**Figure 21B**) or in cell numbers at 3 days (**Figure 21C**) in comparison to sibling iPSCs. This suggests that the reduction in
proliferation in this ASD patient was specific to NPCs rather than a systemic defect.

**Figure 21:** No difference in Family 1 ASD and sibling iPSC proliferation

**A.**

![Image of SIB and ASD iPSCs](image)

**B.**

![Graph showing no difference in DNA synthesis](image)

**C.**

![Graph showing no difference in cell numbers](image)

**Figure 21:** Family 1 Sibling and ASD iPSCs display no difference in proliferation. A) Shows a representative image of SIB and ASD iPSCs. B) SIB and ASD iPSCs exhibit no differences in DNA synthesis at 48h (SIB n=4/10/33, ASD n=4/11/42; clones/expt/wells) and C) no difference in cell numbers at days 1, 2, and 3 (SIB n=4/11/23, ASD n=4/10/21; clones/expt/wells).
5.2. Family 2 ASD NPCs display hyperproliferation in comparison to sibling NPCs

In examining NPC proliferation in a 2nd family, ASD NPCs exhibit an increase in proliferation in comparison to SIB NPCs. In 31 experiments (SIB-22, ASD-9), utilizing multiple derivations of NPCs (SIB-4, ASD-4) derived from multiple iPSC clones per individual (SIB-2, ASD-3), ASD NPCS display a 60% increase in DNA synthesis as evidenced by radioactive thymidine incorporation (Figure 22).

Figure 22: Family 2: ASD NPCs have increased DNA synthesis in comparison to sibling NPCs

<table>
<thead>
<tr>
<th>Dx</th>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone #</td>
<td>07c16</td>
<td>07c06</td>
</tr>
<tr>
<td>NPC deriv.</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>N= Expt.</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>N= Well</td>
<td>42</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 22: A) At 48h, Family 2 ASD NPCs exhibit a 60% increase in DNA synthesis in comparison to SIB NPCs (****, p <0.0001, SIB n=2/4/22/67, ASD n=3/4/9/27; clones/NPC deriv/expt/wells). B) Data shown by clone to indicate individual variability. C) N-values for NPCs derived from different iPSC clones, number of NPCs derived from a single clone, numbers of experiments, and total numbers of wells.
Interestingly, when examining the proportion of cells entering S-phase, there was no difference between ASD and sibling NPCs (Figure 23). This may suggest an increase in cell survival in ASD NPCs that is responsible for the increase in DNA synthesis. It is important to note that n-values for this assay were low and to have more confidence in the data, additional experiments should be performed.

**Figure 23:** Family 2 ASD NPCs display no difference in S-phase entry

![Figure 23: A) Representative images of EdU labelling in Family 2 ASD and SIB NPCs at 48h post-acute dissociation B) Family 2: ASD NPCs display a no change in S-phase entry at 48h (SIB n=2/2/5/15, ASD n=2/2/2/6; clones/NPC deriv/expt/wells). C) Data indicated by clone/diagnosis.](image)

In examining cell numbers at 2, 4, and 6 days, ASD NPCs display a 30% increase in cell numbers at 6 days (Figure 24).
**Figure 24:** Family 2 ASD NPCs exhibit hyperproliferation in comparison to sibling NPCs

In the same fashion as the previous family, I wanted to identify whether this change in ASD NPC proliferation was present at an earlier stage in development, in the iPSCs, or if it was specific to the neural cells in the brain that
grow and divide. Family 2 ASD and sibling iPSCs displayed no difference in 48h DNA synthesis (Figure 25B) or in cell numbers at 3 days (Figure 25C). Again, these data suggest that the hyperproliferation phenotype observed in ASD NPCs is cell-type specific.

**Figure 25:** Family 2 displays no effect of diagnosis on iPSC proliferation

![Figure 25: Family 2 SIB and ASD iPSCs display no difference in proliferation. A) Shows a representative image of Family 2 SIB and ASD iPSCs. B) SIB and ASD iPSCs exhibit no differences in DNA synthesis at 48h (SIB n=1/3/12, ASD n=2/6/23; clones/expt/wells) and C) no difference in cell numbers at 3 days (SIB n=1/3/9, ASD n=2/6/18; clones/expt/wells).]

**Conclusions**

The findings from this series of experiments suggest we were able to identify a bidirectional dysregulation of proliferation in idiopathic ASD NPCs in comparison to their sibling control. Namely in 48h DNA synthesis, which is predictive of cell numbers, Family 1 ASD NPCs show a 60% reduction in comparison to Family 1 sibling NPCs, or are hypopreliferative (Figure 26A). In
Family 2, ASD NPCs display a 60% increase in 48h DNA synthesis in comparison to Family 2 sibling NPCs, or are hyperproliferative (Figure 26A). Further, these studies have found that this dysregulation of proliferation is cell-type specific as it is not present in the iPSCs which suggests the defect in proliferation may be brain-specific.

When thinking about what differences in NPC proliferation between sibling and autism may mean it is natural to put the data into a broader context and question how these measures may compare across families. In Figure 26B, the raw values of 48h DNA synthesis are plotted of both ASD and sibling in both Family 1 and Family 2. Family 1 sibling NPCs have comparable raw values of CPMS in 48h DNA synthesis as Family 2 ASD NPCs and the same is true of Family 1 ASD and Family 2 sibling NPCs. This raises questions about how to think about the data and will be addressed further in the discussion.

Figure 26: Comparing 48h DNA synthesis in and across families
By definition growth factors are substances that can stimulate cellular survival, growth, proliferation, and/or differentiation. They play important roles in the development and functioning of the human brain. Abnormalities in growth factor levels or accompanying signals may cause disease states. In ASD, there is genetic evidence and clinical data that suggests a potential involvement of dysregulation of growth factor signaling or levels in the pathophysiology of the disorder.

Strong, specific genetic evidence points to an association of hepatocyte growth factor receptor proto-oncogene \textit{MET} with autism (Campbell, Sutcliffe et al. 2006, Geschwind and Levitt 2007). MET tyrosine kinase signaling has been found to regulate a variety of aspects of neocortical and cerebellar neuronal growth and maturation (Geschwind and Levitt 2007). In a single nucleotide polymorphism (SNP) analysis examining 252 families with a male autism child for growth factor genes, researchers found a haplotypic association of epidermal growth factor (EGF) with autism (Toyoda, Nakamura et al. 2007). Activation of the EGF receptor is essential for proliferation of neural precursor cells (NPCs) and later is supportive of cell survival, migration, and differentiation of immature
neurons (Janigro 2006). Clinical studies of ASD children and adults examining a multitude of growth factors have indicated decreases in insulin-like growth factor-1 and -2 (IGF-1,-2), transforming growth factor-β1, epithelial growth factor, epidermal growth factor (EGF), and vascular endothelial growth factor; while other studies have found increases in IGF-1 and -2, insulin-like growth factor-binding protein 3, growth hormone binding protein, vasoactive intestinal peptide, brain-derived neurotrophic factor, neurotrophin 4/5, calcitonin gene–related peptide, and EGF (Nelson, Grether et al. 2001, Vanhala, Turpeinen et al. 2001, Riikonen, Makkonen et al. 2006, Mills, Hediger et al. 2007, Okada, Hashimoto et al. 2007, Suzuki, Hashimoto et al. 2007, Ashwood, Enstrom et al. 2008, Emanuele, Orsi et al. 2010, Iseri, Guney et al. 2011, Onore, Van de Water et al. 2012, Russo 2013). Despite the sometimes-contradictory evidence in these clinical studies and the limitations in scope due to low sample sizes and measurement of growth factor levels from sources far removed from the brain—such as blood, urine, or cerebrospinal fluid—they serve as potential human indications of abnormalities in regulation of growth factor levels in ASD patients and suggest potential targets for manipulation in iPSC studies.

Additionally, application of growth factors can serve as a tool to investigate potential differences in proliferative responses (blunted, increased sensitivity, no response, differential) that may not appear under control growth conditions in ASD versus sibling NPCs. The DiCicco-Bloom lab has successfully employed this strategy with mouse cultures. For example, in the Engrailed2 knock out
mouse (KO), a mouse model of autism, Rossman et. al. found that En2 KO cerebellar granule neuron precursors *in vitro* had a greater response to insulin-like growth factor 1 (IGF-1) in DNA synthesis and this finding was also reproduced *in vivo* (Rossman, Lin et al. 2014). This finding suggests that genetic alterations can change mitogenic responses to growth factors and lead to dysregulation of proliferation.

A difference in response to a growth factor could also indicate alterations in pathogenetic pathways that could ultimately serve as a molecular gateway to therapeutic interventions. For example, studies examining patients with Rett Syndrome, a monogenic disorder with autism-like presentations, have found that deletion of methyl CpG-binding protein 2 leads to alterations in the PI3-Kinase pathway that can be at least partially rescued using IGF-1 (Marchetto, Carromeu et al. 2010).

Due to these reasons, I investigated the effects of a number of different developmentally-relevant growth factors on ASD NPC proliferation (*Table 5*).
Table 5: Growth factors with concentrations and effects on DNA synthesis of patient-derived NPCs

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Abbrev.</th>
<th>Dose</th>
<th>Units</th>
<th>Effect on DNA Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>fibroblast growth factor</td>
<td>FGF</td>
<td>0.03, 0.1, 0.3, 1, 3, 10, 30</td>
<td>ng/mL</td>
<td>+</td>
</tr>
<tr>
<td>epidermal growth factor</td>
<td>EGF</td>
<td>0.003, 0.01, 0.03, 0.1 0.3, 1, 3, 10, 20, 30</td>
<td>ng/mL</td>
<td>+</td>
</tr>
<tr>
<td>serotonin</td>
<td>5-HT</td>
<td>10, 30, 100, 200, 300, 600</td>
<td>µg/mL</td>
<td>− / +</td>
</tr>
<tr>
<td>pituitary adenylate cyclase activating peptide</td>
<td>PACAP</td>
<td>3, 6, 10, 30</td>
<td>nM</td>
<td>− / +</td>
</tr>
<tr>
<td>insulin</td>
<td></td>
<td>10</td>
<td>ng/mL</td>
<td>none</td>
</tr>
<tr>
<td>brain-derived neurotrophic factor</td>
<td>BDNF</td>
<td>1, 3, 10, 30</td>
<td>ng/mL</td>
<td>+</td>
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<td>nerve growth factor</td>
<td>NGF</td>
<td>10</td>
<td>ng/mL</td>
<td>none</td>
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<tr>
<td>neurotrophin-3</td>
<td>NT3</td>
<td>3, 10, 30</td>
<td>ng/mL</td>
<td>−</td>
</tr>
<tr>
<td>oxytocin</td>
<td>OT</td>
<td>3, 10, 30</td>
<td>nM</td>
<td>none</td>
</tr>
<tr>
<td>ciliary neurotrophic factor</td>
<td>CNTF</td>
<td>20</td>
<td>ng/mL</td>
<td>+</td>
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<td>leukemia Inhibitory factor</td>
<td>LIF</td>
<td>10</td>
<td>ng/mL</td>
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<tr>
<td>valproic acid</td>
<td>VPA</td>
<td>0.3, 1, 2</td>
<td>mM</td>
<td>−</td>
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<tr>
<td>AKT agonist, SC-79</td>
<td>SC-79</td>
<td>0, 0.001, 0.01, 0.03, 0.1, 0.3, 1, 2, 3, 10, 30</td>
<td>µg/mL</td>
<td>− / +</td>
</tr>
<tr>
<td>AKT antagonist, MK2206</td>
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<td>nM</td>
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</tbody>
</table>

The majority of growth factors applied to NPCs had small effect sizes and were not investigated further. For the purposes of my dissertation I am focusing on findings related to basic fibroblast growth factor 2 (bFGF) effects, which is not included in the commercially available Neural Expansion media. For more detailed information on responses to various growth factors see Appendix.
6.1. bFGF stimulation reverses proliferation defect in Family 1 ASD NPCs

Both sibling and ASD NPCs in Family 1 respond to bFGF stimulation with increased DNA synthesis, S-phase entry, and cell numbers at 4 and 6 days in culture (Figure 27). 48h post introducing bFGF to cultures, NPCs exhibit a 45% increase in DNA synthesis (Figure 27A), a ~20% increase in S-phase entry (Figure 27B), and this is accompanied by ~50% increases in cell numbers at 4 and 6 days (Figure 27C). Interestingly, bFGF stimulation reverses the proliferation defect identified in ASD NPCs bringing cell numbers up to that of sibling NPCs (Figure 27). In examining a dose-response to bFGF to identify a potential difference in magnitude of stimulation at lower doses, both ASD and sibling NPCs exhibit the same percent increase in DNA synthesis at 48h after application of bFGF at all doses examined (Figure 28).
**Figure 27:** bFGF reverses proliferation defect in Family 1 ASD NPCs

Figure 27: In Family 1, both SIB and ASD NPCs respond to bFGF stimulation (10 ng/mL) through increases in A) DNA synthesis at 48h (****, p <0.0001, SIB n=4/5/23/86; ** p=0.002, ASD n=5/9/28/89; clones/NPC deriv/expt/wells), B) proportions of cells entering S-phase (****, p <0.0001, SIB n=3/4/8/22; ** ASD n=4/5/8/22; clones/NPC deriv/expt/wells), and C) increases in cell numbers at 4 (** p=0.001 SIB; * p=0.02 ASD) and 6 days (**** p<0.0001, SIB n=4/5/14/29, ASD n=5/7/20/41; clones/NPC deriv/expt/wells).

**Figure 28:** No differential response to bFGF in Family 1 NPCs

Figure 28: In Family 1, both SIB and ASD NPCs exhibit no differential response to bFGF stimulation (10 ng/mL) in DNA synthesis at 48h (SIB n=3/3/5/14; ASD n=4/5/8/20; clones/NPC deriv/expt/wells)
6.2. Family 2 ASD NPCs exhibit a blunted proliferative response to bFGF

Similar to Family 1, Family 2 sibling and ASD NPCs exhibit increased proliferation in response to bFGF (Figure 29). 48h post bFGF application, sibling NPCs display a ~45% increase in DNA synthesis (Figure 29A), 30% increase in S-phase entry (Figure 29B), and a 65% increase in cell numbers at 6 days (Figure 29C). Under the same conditions, ASD NPCs exhibit a 30% increase in DNA synthesis (Figure 29A), 30% increase in S-phase entry (Figure 29B), and a 30% and 50% increase in cell numbers at 4 and 6 days (Figure 29C). Noting the significantly reduced response to bFGF stimulation after 48h DNA synthesis, I performed a dose-response to see if this was present at lower doses as well. In 48h DNA synthesis assays examining a range of doses of bFGF from 0.03 ng/mL to 10 ng/mL, ASD NPCs show a significantly blunted response at 0.3, 1, and 3 ng/mL bFGF (Figure 30). Interestingly, despite this blunted response to bFGF 48h, ASD NPCs have significantly greater numbers of cells in bFGF conditions than sibling NPCs at day 6 (Figure 29C). A blunted response to bFGF could indicate that cells are already proliferating at their maximum capacity or perhaps there is an alteration in FGF2 signaling.
**Figure 29**: Family 2 sibling and ASD NPCs exhibit increased proliferation in response to bFGF.

A. 
![DNA Synthesis Graph](image)

B. 
![S-phase Entry Graph](image)

C. 
![Cell Numbers Graph](image)

**Figure 29**: In Family 2, both SIB and ASD NPCs respond to bFGF stimulation (10 ng/mL) through increases in A) DNA synthesis at 48h (*** p =0.0007, SIB n=2/4/17/52; **** p =0.0003, ASD n=3/4/9/26; clones/NPC deriv/expt/wells), B) proportions of cells entering S-phase (*** p =0.0001, SIB n=2/2/4/12; * p=0.01, ASD n=2/2/6; clones/NPC deriv/expt/dishes), and C) increases in cell numbers at 6 days (**** p <0.0001, SIB n=2/3/12/24; ASD n=3/4/7/14; clones/NPC deriv/expt/wells).
**Figure 30:** Family 2 ASD NPCs have blunted response to bFGF in DNA synthesis.

In response to 48h bFGF stimulation, Family 2 ASD NPCs have a blunted response in DNA synthesis at 0.3 ng/mL (** p=0.01), 1 ng/mL (**** p<0.0001), and 3 ng/mL (**** p<0.0001) concentrations in comparison to SIB NPCs (SIB n=2/3/6/16, ASD n=2/2/4/13; clones/NPC deriv/expt/wells).
CHAPTER 7: Examine ASD NPC Responses to Environmental Stressors

Though the involvement of genetic abnormalities in ASD pathogenesis is well accepted, contributions of environmental factors have been less well-defined (Folstein and Rutter 1977, Ritvo, Freeman et al. 1985, Hallmayer, Cleveland et al. 2011). An increasing amount of evidence suggests that autism risk depends on approximately equal contributions of genes and environmental factors as well as interactions between these components (Herbert 2010, Hallmayer, Cleveland et al. 2011, Rossignol, Genuis et al. 2014). Epidemiological studies have observed correlations of ASD prevalence with exposure to environmental factors such as metals, pesticides, and traffic-related air pollution (Becerra, Wilhelm et al. 2013, Roberts, Lyall et al. 2013, Volk, Lurmann et al. 2013). Exposures to heavy metals and toxicants, for example lead and mercury, are well-recognized risk factors for a number of neurodevelopmental disorders, including autism (2000); however, current studies are correlative and do not show direct contributions to the ASD phenotype.

Human induced pluripotent stem cell (iPSC) technology provides us with a unique platform to study interactions between human genetics and environmental toxicants in autism patient-derived neural precursor cells (NPCs). Heavy metals
and traffic-related chemicals are neurotoxicants that can induce oxidative stress, which can disrupt biological processes or pathways that have been implicated in autism. Greater genetic vulnerability to ASD in conjunction with exposure to environmental toxicants in early development may cause some children to cross biological thresholds into ASD.

The goal of this chapter was to inquire into molecular and cellular interactions between environmental factors and ASD genetic components that could potentially manifest as relevant neurobiological phenotypes. I examined the effects of two environmental factors 1) a commonly used oxidative stressor, hydrogen peroxide (H$_2$O$_2$), and 2) a component of air pollution, methylmercury, (MeHg) on relevant NPC phenotypes in two families with idiopathic ASD.
7.1. Family 1 ASD NPCs exhibit greater ROS production after hydrogen peroxide exposure

Reactive oxygen species (ROS) are a biological double-edged sword. They are a natural by-product of cellular metabolism and play fundamental roles in signal transduction and cell homeostasis (Schieber and Chandel 2014). However, they can also form as a response to exogenous stressors and induce pathological conditions causing cellular damage and death (Schieber and Chandel 2014). If there are alterations or changes in levels of ROS, this may indicate a potential issue with cell health or metabolism which could also affect proliferation. For this reason, I chose to examine ROS levels in ASD and sibling NPCs. There are many different forms of ROS in the cell such as peroxides, superoxides, hydroxyl radicals, and singlet oxygen molecules. I decided as a first pass, it would be best to identify whether general levels of ROS were altered in ASD NPCs and if changes were observed in this broad measure, which could then be investigated more deeply at a later time.

To examine the production of general ROS, I used a cell permeable chemical probe, 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA). Once inside of the cell, intracellular esterases and oxidation cleave acetate groups of H$_2$DCFDA converting it to a highly fluorescent molecule, 2',7'-dichlorofluorescein (DCF). To account for potential differences in cell numbers I normalized DCFDA signal to the measurements obtained from fluorescence of the live nuclear stain Hoechst 33442. Due to time constraints, I was only able to examine ROS
production in Family 1 and was not able to look at Family 2 or additional patients described later in this dissertation. In Family 1, while I found no differences in baseline ROS production, after a 1h acute exposure to H$_2$O$_2$ (100 µM) sibling NPCs display no significant difference in ROS levels whereas ASD NPCs exhibited roughly 2-fold greater levels of ROS (**Figure 31**). I did not examine ROS production in Family 2 so this still needs to be explored.

**Figure 31:** Family 1 ASD NPCs have greater levels of ROS after hydrogen peroxide exposure than sibling NPCs

\[\text{ROS Levels (RFU)}\]

\[\begin{array}{cccc}
\text{H}_2\text{O}_2 \\
\text{0 µM} & \text{100 µM} \\
\end{array}\]

\[\begin{array}{c}
\text{SIB} \\
\text{ASD} \\
\end{array}\]

**Figure 31:** At 24h, Family 1 ASD NPCs display 2.5-fold increase in ROS in response to 1h H$_2$O$_2$ exposure (* p=0.02, SIB n= 3/3/5/31; ASD n=3/3/6/38; clone/NPC deriv/expt/wells).
7.2. Patient-specific sensitivity to hydrogen peroxide in DNA synthesis

To investigate if ASD NPCs had a differential response in DNA synthesis after \( \text{H}_2\text{O}_2 \) exposure, I employed a chronic model and exposed cells for 24h (Figure 32). I used this shorter time period, rather than the 48hs I had been using because it was unclear how long \( \text{H}_2\text{O}_2 \) remains active in culture and I did not want to allow for a recovery period from the stressor.

**Figure 32: Hydrogen peroxide exposure paradigm for DNA synthesis assay**

Interestingly, at 24h Family 1 sibling NPCs showed greater sensitivity to application of \( \text{H}_2\text{O}_2 \) at 30, 60, and 100 µM in DNA synthesis (Figure 33A). Due to differences in baseline DNA synthesis, as presented in Chapter 5 of this dissertation, I expressed the data as percent control, comparing each successive dose to the 0 µM condition for that individual. Conversely, in Family 2, ASD NPCs displayed a greater sensitivity to \( \text{H}_2\text{O}_2 \) from 10 to 100 µM (Figure 33B). Considering the patient-differences in proliferation, it is possible that this sensitivity to \( \text{H}_2\text{O}_2 \) may be related to magnitude of DNA synthesis rather than diagnosis dependent.
**Figure 33:** Patient-specific sensitivity to 24h hydrogen peroxide exposure displayed in DNA synthesis

A. **Family 1**

In DNA synthesis, after 24h exposure to H$_2$O$_2$, Family 1 A) SIB NPCs show greater sensitivity in DNA synthesis than ASD NPCs at 30 (** p<0.0001), 60 (**p<0.0001), and 100 mM (**p<0.0001, SIB n= 3/4/5/13; ASD n=3/4/7/21; clone/NPC deriv/expt/wells) and in Family 2 B) ASD NPCs show greater sensitivity in DNA synthesis than SIB NPCs at 10 (** p=0.007), 30 (** p=0.0003), 60 (** p=0.0002), and 100 mM (** p=0.0002, SIB n= 2/3/5/15; ASD n=3/3/5/15; clone/NPC deriv/expt/wells)
7.3. ASD NPCs display decreased vulnerability to MeHg exposure

Numerous studies have been performed on MeHg exposure in humans and rodent animal models both in culture and *in vivo* (Harada 1995, Grandjean, Weihe et al. 1997, Grandjean, Weihe et al. 1998, Burke, Cheng et al. 2006). Heavy metals such as mercury, inhaled from air pollution or ingested as MeHg in food, are neurotoxic and known to affect proliferation and synaptic function (Harada 1995, Castoldi, Coccini et al. 2001, Tamm, Duckworth et al. 2006, Xu, Yan et al. 2010). Indeed, the DiCicco-Bloom lab and others have shown that neuroprogenitors of many autism-related brain regions including cerebral cortex, hippocampus and cerebellum, exhibit G1 phase cell cycle arrest and cell death after MeHg exposure, effects which reduce brain growth and neuron numbers (Castoldi, Coccini et al. 2001, Faustman, Ponce et al. 2002, Burke, Cheng et al. 2006, Tamm, Duckworth et al. 2006, Falluel-Morel, Sokolowski et al. 2007, Xu, Yan et al. 2010, Sokolowski, Falluel-Morel et al. 2011). The neurotoxic effects of MeHg on proliferation could be amplified by ASD genetic vulnerability of dysregulated NPCs because they may converge on common signaling pathway(s). With idiopathic ASD NPCs and sex-matched sibling controls, I was able to directly examine whether autism NPCs may be more physiologically vulnerable than unaffected sibling control NPCs to environmental toxicant MeHg. In deciding the appropriate concentration of MeHg to use to determine whether there was a diagnosis-dependent sensitivity to oxidative stressors it is important to take into context the model system we are asking this question in. The culture
was a human patient-derived two-dimensional cellular model composed of a single, albeit heterogeneous, cell type—neural precursor cells. The developing human brain occurs in three-dimensional space and is composed of numerous cell types, including more mature neuron and glia. Taking these differences into consideration, while there are correlations with mercury and increased risk for autism, it is not appropriate to think of this as a model related to clinical exposure. Rather, we were thinking of this toxicant as a tool to identify if there were differences in response to an oxidative stressor. Many of the experiments related to MeHg were performed by undergraduate student Cristina Pinto and without her these findings would not be possible.

In examining the effects of MeHg on 48h DNA synthesis (Figure 34, exposure paradigm), ASD NPCs in both families exhibited a reduced sensitivity to the stressor than sibling NPCs (Figure 35). In Family 1, sibling NPCs have a 60% decrease at 0.3 µM MeHg exposure in DNA synthesis whereas ASD NPCs display only a 40% decrease to the same dose (Figure 35A). In Family 2, sibling NPCs have a 40% and 70% reduction in DNA synthesis to 0.2 and 0.3 µM MeHg exposure, respectively; ASD NPCs show greater resistance to negative effects of MeHg with a 20% and 40% reduction in DNA synthesis at these same respective doses (Figure 35B).
Figure 34: Methyl mercury exposure paradigm for DNA synthesis assay

![Diagram of methyl mercury exposure paradigm]

Figure 35: Family 1 and 2 ASD NPCs are less sensitive to MeHg exposure in comparison to sibling NPCs

A. **Family 1**

B. **Family 2**

Figure 35: ASD NPCs showed reduced sensitivity to 48h MeHg exposure in both A) Family 1 at 0.3mM (*** p=0.0001; SIB n=4/4/7/18, ASD n=4/5/14/41; clones/NPC deriv/expt/wells) and B) Family 2 at 0.2 mM (** p=0.0004), and 0.3 mM (** p<0.0001; SIB n=2/3/5/15, ASD n=3/3/4/12; clones/NPC deriv/expt/wells) compared to sibling NPCs in DNA synthesis.

The DiCicco-Bloom lab also has patient-derived NPCs from an individual with a genetic cause of ASD, 16p11.2 deletion. I was able to examine patient-derived 16p11.2 deletion NPCs response to MeHg on DNA synthesis and found that, similar to idiopathic ASD NPCs, they were significantly less sensitive to the negative effects on DNA synthesis than controls (Figure 36).
**Figure 36:** In 48h DNA synthesis, NPCs derived from two idiopathic ASD patients and one 16p11.2 ASD patient exhibit a reduced sensitivity to MeHg exposure compared to sibling controls.

![Graph showing DNA synthesis in response to MeHg exposure](image)

In examining cell numbers after 48h MeHg exposure, both sibling and ASD NPCs had a reduction in cell numbers at all doses examined (**Figure 37**). Interestingly, while ASD NPCs displayed less vulnerability to MeHg in DNA synthesis compared to sibling NPCs, they display no significant difference in numbers of cells. This suggests that despite apparent cell death, they are still continuing to synthesize DNA.
**Figure 37**: Family 1 and 2 ASD and sibling NPCs show no difference in sensitivity to MeHg exposure in cell numbers.

**Figure 37**: After 48h MeHg exposure, Family 1 and 2 idiopathic autism NPCs show no difference in cell numbers compared to sibling NPCs. (SIB n= 2/3/6/12; ASD n=2/3/4/9/18; individuals/clones/NPC deriv/expt/wells)
CHAPTER 8: DISCUSSION

8.1. Summary of results

Autism spectrum disorder is a pervasive set of developmental disorders whose pathogenesis remains largely unknown due to the heterogeneity of the disorder as well as the lack of representative mouse models. The advent and application of hiPSC technology has revolutionized scientific inquiry of neuropsychiatric diseases by providing access to live human patient-derived neural cells. A myriad of studies using hiPSCs have been able to shed light on disease-specific abnormalities in dendritic arborization, synaptogenesis, and neuronal function of neurodevelopmental disorders (Marchetto, Carromeu et al. 2010, Pasca, Portmann et al. 2011, Brennand, Savas et al. 2014, Doers, Musser et al. 2014, Marchetto, Belinson et al. 2017) as well as in neurodegenerative disorders (Nguyen, Byers et al. 2011, Cooper, Seo et al. 2012, Kondo, Asai et al. 2013). Evidence from autism genomic and post-mortem studies suggests no unifying theory but rather some basic disruption in normal brain development. Due to the complexity and dynamic nature of the developing human brain, disturbances during development create the potential for compensations, or even over-corrections, making identification of disease ontogenetic processes challenging to address, especially in heterogeneous disorders such as autism. There are many potential mechanisms or cellular processes that could contribute
to autism pathology, including but not limited to proliferation, migration, differentiation, early synaptogenesis, and dendrite pruning.

For my dissertation, I chose to examine differences in ASD and sibling neuronal precursor cell (NPC) proliferation. I created methods to rapidly and reproducibly study proliferation in human cells and applied these methods to identify differences in NPCs derived from two idiopathic autism patients in comparison to their sex-matched, sibling controls. Lastly, I examined whether there were differential responses or sensitivities to exogenous factors, namely growth factors or environmental toxicants, on these processes.

8.2. Develop rapid methods to study proliferation in human cells

The majority of studies using hiPSCs to study neuropsychiatric disease differentiate cells into neuronal subtypes or use a 3D culture system called ‘an organoid’ to recapitulate aspects of human brain development (Dolmetsch and Geschwind 2011, Pang, Yang et al. 2011, Lancaster and Knoblich 2014). These systems have been critical in studying and uncovering unique aspects of human development and disease; however, their more ‘mature’ nature precludes extensive study of proliferation. Thus, we chose to use neural precursor cells (NPCs), a highly proliferative cell population, as a model to ask questions about ontogenetic processes and disease initiation in idiopathic autism-patient derived cells. As previously mentioned, the DiCicco-Bloom Lab has extensive experience with development in rat and mouse cortical cell cultures, but had
never before studied human cells (DiCicco-Bloom and Black 1988, Lu and DiCicco-Bloom 1997, Mairet-Coello, Tury et al. 2009, Yan, Zhou et al. 2013). Given the lab history in cell culture, I spent my first year in the lab examining a multiplicity of media compositions, substrates, and cell densities to create a defined culture system for investigating differences in proliferation of autism and sibling NPCs.

As the main goal of my studies was to define differences in the proliferation of sibling and ASD NPCs, I executed this using a number of different approaches. The first approach at defining these differences was to assess DNA synthesis of the entire cell population using a radioactive tracer, tritiated thymidine. I performed this at 48h to quickly assess a number of different experimental groups and to indicate the sum total of DNA synthesis irrespective of duration of S-phase (10 min to 2h). The second confirmatory method was to enumerate the proportion of cells that enter S-phase. This was done by growing cells as a monolayer for 48hs, incubating them with a fluorescent thymidine analog for 2hs, and then dissociating them to analyze as single cells. The third approach was to count cell numbers via dissociation and using a hemocytometer at 2, 4, and 6 days.

Through these studies, I found that measures of DNA synthesis were often paralleled by measures of S-phase entry. Further, examining DNA synthesis at 48h allowed us to predict functional changes in cell numbers at 6 days. Synthesis of DNA occurs before mitosis and cell division and must occur
before cells increase in number. These processes take time and may not always be reflective of changes in cell numbers at this time point. Using these methods, I created a defined system that allowed us to identify responsiveness to growth factors and environmental factors. The methods designed were simple and relatively low cost suggesting they may be easily adapted for high throughput technology to examine potential therapies for other neuropsychiatric disorders.

The results of the methods I developed have been published:


8.3. Dysregulation of proliferation in ASD NPCs

In this case study of two families examining individuals with idiopathic autism in comparison to their siblings, I found a bidirectional dysregulation of ASD NPC proliferation. In Family 1, ASD NPCs exhibited a 65% reduction in 48h DNA synthesis that was paralleled by a 40% reduction in the proportion of cells entering S-phase and functionally resulted in 60% reductions in cell numbers at both 4 and 6 days. There was no significant difference in cell death which suggests the ASD NPCs from this family were indeed hypo-proliferative in comparison to their sibling due to reduction in the proportion of cells that entered S-phase. A number of different mechanisms could underlie this decrease in proliferation. As mentioned in the introduction, Cyclins and CKIs are the main
regulatory points in the cell cycle. Changes in levels or regulation of these molecules could elicit exit from the cell cycle and cause a reduction in S-phase entry. Cyclins and CKIs are further regulated by a number of complex and interconnected signaling pathways including MAPK/ERK, P13K/AKT, mitogen-stimulated Rac and NF-κβ signaling, cytokine signaling, signaling by receptors for ECM proteins, and the Wnt and Notch pathways. Changes at any point in these pathways could exert effects on proliferation.

Diametrically opposed, autism NPCs from Family 2 exhibited hyper-proliferation in comparison to their sibling NPCs. At 48h, ASD NPCs had a 60% increase in DNA synthesis that was accompanied by functional outcomes, a 40% increase in cell numbers at 6 days. As in the first family, though the opposite direction, increases in DNA synthesis and in cell numbers at 6 days could suggest alterations in levels of Cyclins and CKIs or alterations in the pathways that regulate them. While the differences observed in Family 2 DNA synthesis were not paralleled by a difference in S-phase entry, this is still a potential question. The number of experiments performed and clones examined were significantly less in the second family and more work needs to be done to confirm these results. There is also the possibility that there is a role for cell survival, perhaps the ASD NPCs in Family 2 have greater cell survival than the sibling. This would account for significant increases in 48h DNA synthesis and cell numbers at 6 days without affecting the proportion of cells in S-phase.

In both families, there was no significant difference between sibling and
autism proliferation of the hiPSCs which suggests that the proliferation changes occurred specifically in the neural precursor lineage (cell-type specific) and were not indicative of a systemic defect. A challenge in studying idiopathic autism and drawing conclusions is that, in the absence of a known genetic lesion or deeper genomic analysis of these families, it is not possible to conclude whether observed alterations are specific to the family, diagnosis, or individual. For example, Figure 26B shows the raw data for 48h DNA synthesis for both families plotted on the same graph. Though autism presents with differences in proliferation compared to their respective siblings, when comparing across families, Family 1 and 2 controls have widely variable rates of proliferation. Considering that this is a case study and examines only two families, it is impossible to define an average “normal” proliferation for a population. This absence of a known genetic cause along with the small sample size—inherent in these kinds of studies—makes it challenging to understand if these findings are representative of individual differences, interfamilial variability, or alternatively, if they represent differences in autism and sibling NPCs that reflect the basic mechanisms contributing to ASD. Future experiments utilizing more ASD-sibling pairs and interpretation of genomic data may indicate some effect of diagnosis.

Current hiPSC literature examining idiopathic autism have found evidence for an accelerated cell cycle and increases in proliferation of NPCs when compared to controls (Mariani, Coppola et al. 2015, Marchetto, Belinson et al. 2017). Importantly, unlike the cohort presented here, these studies have
selected ASD patients based on their presentation of an enlarged brain phenotype or macrocephaly. They suggest that this increase in NPC proliferation contributes to the enlarged brain phenotype and macrocephaly in their patient cohort. Though, as presented in the introduction, we do not necessarily presume that increased NPC proliferation is the only mechanism that results in a larger brain size, comparing our findings to available clinical data suggests some similarities with current literature. The Family 1 ASD subject, which exhibited a decrease in NPC proliferation in comparison to sibling, had a head circumference within the normal range (78th percentile). Interestingly, at the time head circumference was measured in the Family 2 ASD individual, which exhibited a significant increase in NPC proliferation in comparison to sibling, he was approaching macrocephalic (97th percentile). This information is compelling as it is in line with current literature and suggests that increases in NPC proliferation may result in an enlarged brain growth phenotype, one of the major consistent pathological models in ASD. However, given the range of NPC growth rates seen in siblings, comparing sibling NPC proliferation to their head circumference percentile would be useful information to understand this relationship. Unfortunately, head circumference data for the siblings is not available to us and this comparison is not possible.
8.4. Patient-specific responses to mitogenic stimulation via bFGF

In both families, sibling and autism pairs responded to bFGF with significant increases in 48h DNA synthesis and S-phase entry which were accompanied by functional increases in cell numbers at days 4 and 6. In Family 1, increases in the above measures reversed the proliferation defect in ASD NPCs which suggests that bFGF signaling was intact and ASD cells have the ability to proliferate more after growth factor stimulation. To put frankly, the cells remained competent to carry out this fundamental process, suggesting they are not grossly abnormal. This same conclusion is supported by initial characterization studies that indicate these ASD NPCs differentiate into all 3 neural lineages when incubated in proper inducing conditions for 7 to 10 days. That the cells are able to increase proliferation in responses to FGF could imply a number of potential mechanisms underlyling the proliferation defect. Perhaps, ASD NPCs have a reduction in endogenous levels of bFGF, alteration in levels or function of FGF receptors, or a reduction in activity of MAPK/ERK pathway? In Family 2, ASD NPCs had a significantly blunted response to a number of doses of bFGF in 48h DNA synthesis which could indicate a number of differences in ASD and sibling NPCs. Similar to Family 1, despite differences in direction, this baseline increased DNA synthesis and blunted response to bFGF could be reflective of an alteration in the levels of endogenous bFGF, levels of or function of FGF receptors, and are suggestive of an overactive MAPK/ERK pathway. If the cells have baseline increased MAPK/ERK activity then addition of bFGF,
which acts through this pathway, may not have as great of an effect. Also, remembering that many growth factors have different effects on different cell types and even on different maturation stages of a cell, this may indicate a difference in cell fate or cell differentiation potential.

8.5. Family 1 ASD NPCs exhibit increased ROS after addition of oxidative stressor hydrogen peroxide

In Family 1 ASD NPCs exhibited a significantly greater increase in ROS production after addition of oxidative stressor hydrogen peroxide in comparison to their sibling. There are a number of different endogenous sources of ROS within the cell and these different sources produce different ROS, for example peroxides, superoxide, hydroxyl radical, and singlet oxygen. I used a general marker for oxidative stress so an increased ROS in ASD NPCs could be produced from a number of different sources including NADPH complexes in the cell membrane, endoplasmic reticulum, peroxisomes, and the mitochondria. Dysfunction or over activity of these sources could underlie this phenotype. Conversely, catalase and dismutase are enzymes in the cell that convert ROS to benign molecules. If these enzymes are faulty than this could produce an increase in ROS, though this seems unlikely in this case.

The majority of evidence suggesting increased oxidative stress and mitochondrial dysfunction in ASD individuals have examined peripheral biomarkers in blood and urine, sources distinct and separate from the brain.
Some studies have shown direct evidence of oxidative stress and mitochondrial dysfunction in postmortem ASD brains; however, these studies are limited due to small sample sizes and availability of tissue (Anitha, Nakamura et al. 2013, Gu, Chauhan et al. 2013, Muratore, Hodgson et al. 2013, Tang, Gutierrez Rios et al. 2013, Golomb, Erickson et al. 2014). The work presented here corroborates findings from clinical and postmortem studies and also furthers this work by providing evidence for increased ROS in live ASD neural precursor cells.

8.6. Patient-specific responses after hydrogen peroxide exposure

In analyzing DNA synthesis after exposure to H$_2$O$_2$, I identified patient-specific responses. In Family 1, ASD NPCs demonstrate a resistance to H$_2$O$_2$ exposure whereas Family 2 ASD NPCs showed an increased sensitivity in comparison to their respective sibling controls. This could demonstrate a patient-specific difference in response to an environmental stressor or alternatively, considering the patient-specific differences in proliferation, this sensitivity to H$_2$O$_2$ may be related to the magnitude of DNA synthesis rather than diagnosis dependent. Cells that are actively dividing may be more vulnerable to insult or injury, so cells growing more slowly may be more ‘resistant’ to H$_2$O$_2$ as an oxidative stressor. Indeed, mouse and rat studies from our lab and others examining developmental consequences of oxidative stressors, such as MeHg, suggest that NPCs may be more vulnerable than more mature cell types (Faustman, Ponce et al. 2002, Falluel-Morel, Sokolowski et al. 2007, Sokolowski,
Obiorah et al. 2013). However, other studies examining levels of ROS and their role in proliferation suggest that stem cells and neural stem cells have high endogenous ROS levels and that hypoxic conditions can stimulate their proliferation (Zhu, Wu et al. 2005, Kryston, Georgiev et al. 2011, Le Belle, Orozco et al. 2011). Interestingly, many parallels can be drawn with cancer cells (Zhu, Wu et al. 2005, Kryston, Georgiev et al. 2011, Le Belle, Orozco et al. 2011). However, ROS signaling is complex and has diverse functions in normal cell development as well as in disease states. Similar to cell responses to growth factor signaling, responses to oxidative stressors and changes in ROS levels are often specific to cell maturation and cell type.

8.7. ASD NPCs exhibit a common decreased sensitivity to MeHg

Excitingly, in examining the effects of MeHg on DNA synthesis, a common ASD phenotype emerged. Regardless of magnitude of DNA synthesis, NPCs from 3 different ASD patients—two idiopathic patients and an individual with a genetically identified cause of autism, 16p11.2 deletion—display a significantly blunted response to negative consequences of MeHg on DNA synthesis. This decreased sensitivity to MeHg is irrespective of cell growth rate and thus, may be reflective of the ASD diagnosis. Further, when examining effects of MeHg on cell numbers between ASD and sibling controls in the two idiopathic families, there was no significant difference. This suggests that in the face of a toxicant, when cells are dying, ASD NPCs continue to synthesize DNA despite compromised
cell health and survival. This may be suggestive of a problem in DNA repair mechanisms. The molecular origin of cancer, a disease characterized by uncontrolled proliferation, derives from mutations in DNA sequence and dysfunction of DNA repair mechanisms and pathways. A few studies have estimated cancer risk in ASD and found significant increases compared to control individuals and even implicated genes involved in DNA repair (Crespi 2011, Hanahan and Weinberg 2011, Crawley, Heyer et al. 2016). Though there are many different DNA repair mechanisms, base excision repair (BER) is one of the most important ones in protecting the cell from frequently occurring small DNA base lesions and single-strand DNA breaks (Markkanen, Meyer et al. 2016). BER is a housekeeper system that is constantly working to clean and protect the cell from these genetic lesions (Markkanen, Meyer et al. 2016). Thus, any decrease in the capacity of this repair mechanism, from alterations in the protein sequence due to genetic polymorphisms, or changes in protein expression levels, can lead to genetic alterations that may not necessarily compromise any one cell but as a whole, may affect the entire system or individual.
9.1. Ongoing research

In thinking about the conclusions for this thesis, it is important to take into account the context of the larger NJACE project. First and foremost, the data presented here are a case study on proliferation of two families while the NJACE project has hiPSCs derived from 8 families with idiopathic autism and sex-matched sibling controls. Characterization of these remaining families will help elucidate and contextulize these current findings on proliferation. There has been some concern expressed that because the siblings share a genetic background with the idiopathic autism, they might have similar defects in cellular phenotypes. To begin addressing this issue, we have obtained unrelated control hiPSCs from NIMH and we are currently inducing them into NPCs to investigate cellular phenotypes. As a caveat, we do not know exactly how ‘normal’ these NIMH controls are because the blood to derive iPSCs was taken from de-identified CD34+ cord blood. They were checked for known gross genetic abnormalities but as we know, some disorders are not caused by a single genetic lesion and are much more complex. Additionally, we are not aware of their
ultimate neuropsychiatric outcomes as adults. This is not a criticism, merely an observation and something to keep in mind. Increasing the sample size by characterization of additional idiopathic autism individuals and related and unrelated controls will help us understand whether differences we have identified are person specific or diagnosis specific.

Secondly, while the work presented here represents my efforts to inquire into proliferation, other members of the NJACE project have also been working on these same cells and there is a much larger story to tell. Researchers in the group are working on a number of ‘-omic’ projects such as examining the genomics, proteomics, and metabolomics in these families. Findings from these big data studies will certainly shed light on some of the cellular phenotypes we have identified in culture. Additionally, my colleague Smrithi Prem has been examining neurites and their response to extracellular factors. In doing so, she has also examined signaling in these same ASD NPCs. Interestingly, in the ASD NPCs that have a reduction in proliferation, Family 1, she found a reduction in AKT signaling. Thinking this may underlie the defect in proliferation, I used an agonist of the AKT pathway, SC-79, to see if this rescued the proliferation defect. After trying multiple different conditions including varying cell density, time in culture, and substrates, SC-79 had no effect on proliferation in ASD NPCs. Perhaps a different AKT agonist would have a different result, but perhaps the difference in AKT signaling does not underlie the proliferation defect.
9.2. Variability in the iPSC model system, developing methods that are reproducible and robust.

The iPSC field has been extraordinarily challenged by the heterogeneity of the system, in differences in cellular and molecular phenotypes across different iPSC clones, different NPC inductions, as well as across passages of these different populations of cells. This extends into defining what constitutes a technical replicate versus what constitutes a biological replicate and contributes to successive difficulties in identifying the appropriate n-value to ensure results are rigorous and reproducible. These questions have been central to my studies in comparing sibling and autism NPCs. In my research, I used a minimum of 2 to 5 iPSC clones per an individual to derive NPCs from to study various measures of proliferation. Further, I restricted studies of NPC lines from passages 3 to 7 to further reduce variability. Due to differences across iPSC clones and NPC inductions, I believe it is necessary for future studies to examine a minimum 3 NPC lines derived from different individual iPSC clones in order to be confident in one’s scientific findings. These various NPC inductions should also be studied over multiple passages to ensure that results are not dependent on this variable.

9.3. Study potential role of cell cycle machinery and regulators in dysregulated proliferation in ASD NPCs

To understand molecular mechanisms underlying the significant differences in proliferation comparing idiopathic ASD and sibling NPCs, I propose
that we investigate protein levels of Cyclins and CKIs in both Families. Despite the evidence for a decreased proliferative population in Family 1 ASD NPCs, in preliminary studies I found no difference when examining cyclin D1, E, or a CKI from the cip/kip family, p27. However, there are other cyclins and CKIs that could be measured. Cyclin D has three homologues in humans, D1, D2, and D3. As I found no evidence for a difference in levels of cyclin D1, cyclin D2 or D3 could be examined. It may also be illuminating to measure levels of CKIs in the INK4a/ARF such as p15, p16, p18, or p19 given their role in promoting quiescence in stem and progenitor cells. I have not conducted any research on the levels of Cyclins or CKIs in Family 2 so this is an avenue that should be explored further.

9.4. Examine role of cell survival in increased proliferation of Family 2 ASD NPCs

In Family 2, given the increased number of ASD NPCs compared to sibling NPCs at 6 days in culture despite no difference in S-phase entry, I propose to examine whether cell survival might play a role in this outcome. An undergraduate honor’s thesis student in the lab, Anna Markhov, is currently enumerating numbers of cleaved caspase 3 + cells, a marker for cell death, to identify whether there might be a greater survival rate in the ASD NPCs.
9.5. Investigate signaling pathways that regulate proliferation

In preliminary results, Smrithi Prem found phosphor-S6 levels were reduced in Family 1 and increased in Family 2. This is interesting as it suggests a direct relationship with magnitude of proliferation with phosphor-S6 levels. Phospho-S6 is often used as a readout of activation of mTOR, a signaling pathway involved in cell proliferation and implicated in autism pathogenesis. We plan to investigate whether an inhibitor of phospho-S6 will normalize the hyper-proliferation and increase phospho-S6 in ASD NPCs from Family 2.

Another clear direction would be to examine protein levels of molecular players in the MAPK/ERK1/2 signaling cascade in both Family 1 and 2. Given the changes seen in NPC proliferation, either an underactive or overactive MAPK/ERK1/2 signaling could result in less or more NPC proliferation. In Family 2, the blunted response to bFGF, an activator of MAPK/ERK1/2, is potential further evidence for overactivation of this pathway. Examining levels of proteins involved in this signaling cascade could uncover a dysregulation in signaling related to these phenotypes. If we find evidence of dysregulation we could use specific activators or inhibitors to rescue respective proliferation defects.

9.6. Investigate cell energy metabolism

As a follow-up to increases in ROS production in Family 1, I propose that we investigate mitochondrial functioning and glycolysis. Dysfunction in either of these systems, which are integral in creating energy for the cell and play critical
roles in a variety of cellular processes, could cause increases in ROS. To examine mitochondrial functioning, the gold standard in the field is to measure the oxygen consumption rate (OCR) and its response to mitochondrial complex specific stressors. Using a Seahorse instrument that measures oxygen production and pH in live cells, we can serially inject oligomycin, FCCP, and a mixture rotenone/antimycin to identify a number of measures of mitochondrial functioning such as, basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. Indeed, I have begun preliminary experiments using this equipment. Another means to measure mitochondrial functioning would be to use a live fluorescent dye such as “Mitosox” to identify whether mitochondria are producing excess superoxide.

Another mechanism by which cell metabolism may be affected is in the other major energy producing pathway, glycolysis. We could examine glycolytic function of these cells through a number of different approaches. However, the first, and most readily available, would be to use the Seahorse instrument to measure the extracellular acidification rate (ECAR). Using the company’s glycolysis stress kit, we could serially inject glucose, oligomycin, and 2-deoxyglucose to measure basal glycolysis, maximal glycolytic capacity, and non-glycolytic acidification, respectively.

9.7. Study DNA repair mechanisms
The common phenotype I identified among ASD NPCs was their decreased sensitivity to MeHg. Continuing to synthesize DNA in the face of a stressor and abundant cell death suggests that there may be damage in DNA repair mechanisms. In both cancer and autism, genes involved in chromatin remodeling and genome maintenance, transcription factors, and signal transduction pathways that lead to nuclear changes (such as PI3K-AKT-mTOR signaling axis) have been associated with increased inherited risk (Crawley, Heyer et al. 2016). Mining the forthcoming genomic data to examine some of these risk genes and comparing them to sibling controls could identify various candidate genes to more closely scrutinize (Crawley, Heyer et al. 2016). If candidates are identified then we could measure transcripts or proteins of these and identify if there are differences in their levels. Potentially, if mutations of specific candidate molecules cause changes in amino acids of proteins of known structure, we could use molecular modeling software to identify whether this affects functioning of specific proteins/enzymes.

9.8. Conclusions

Ultimately, the system I have designed to examine proliferation in human NPCs has identified patient-specific and common patient phenotypes in examining autism NPCs in comparison to sibling controls. Though this system has a number of advantages, such as enabling identification of human molecular and cellular phenotypes, it is important to remember that it is a useful tool and
not synonymous with what occurs in the individual from which the cells were obtained. There are some limitations to keep in mind. This is an artificial in vitro system which is far removed from the growing, live human brain. A brain is composed of multiple cell types, including both neurons and glia, and has a 3D structure. These factors are essential in normal brain development; however, they are not present in this model system. While we are able to investigate the consequences of a genetic background on cellular phenotypes, more and more evidence suggests hiPSCs and their differentiated progeny acquire mutations and CNVs with increased culture length (Merkle, Ghosh et al. 2017). This requires careful genetic characterization of cells and could be a particular challenge in studying idiopathic disorders where a number of genes are thought to be involved. Despite limitations, studies employing hiPSCs have begun to uncover human specific aspects of development and disease (Chambers, Fasano et al. 2009, Marchetto, Carromeu et al. 2010, Pasca, Portmann et al. 2011, Cooper, Seo et al. 2012, Shcheglovitov, Shcheglovitova et al. 2013, Brennand, Landek-Salgado et al. 2014).

Something I have learned in my time working in the lab and from my scientific studies is that it is very important to stop and take a pause and ask yourself, “What is the question?” before moving forward. There are a number of different possible directions that would undoubtedly deepen our understanding of human development and disease. However, it is our responsibility to stop chasing the white rabbit down the hole of unending future experimental could dos
and would haves. These studies began as exploratory and now that we have successfully been able to identify patient-specific phenotypes and responses as well as a phenotype common in NPCs derived from different ASD patients, what next? Previous research spent a considerable amount of time attempting to categorize ASDs via different clinical manifestations of the disorder. With current technology where it is, this phenotypic classification is getting pushed out and we are moving towards grouping based on similarities in pathogenetic pathways or dysregulated cellular processes. In order to do this will require identifying specific biomarkers that can reliably predict ASD diagnosis. Hopefully, through increasing sample size and deeper analysis of these systems we will be able to do just that.
## Appendix

### Appendix 1: Master table of family and clone codes

<table>
<thead>
<tr>
<th>Family</th>
<th>Family Code</th>
<th>Diagnosis</th>
<th>iPSC clone</th>
<th># of NPC derivations</th>
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<tbody>
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<td>1072</td>
<td>SIB</td>
<td>2139</td>
<td>2</td>
</tr>
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<td></td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2135</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ASD</td>
<td>2004</td>
<td>3</td>
</tr>
<tr>
<td></td>
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<td>1993</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2002</td>
<td>1</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>2009</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>1851</td>
<td>1</td>
</tr>
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</table>
Appendix 2: DNA synthesis in response to fibroblast growth factor (bFGF)

2.1. Family 1072 bFGF dose response, 10K, 2 Days

A)

Raw Data, 1072 SIB after FGF application at 0.3, 1, 3 ng/mL (**p<0.0001) and

B) %CTRL, 1072 ASD 0.3, 1, 3, 10 ng/mL (**p<0.0001); (1072 SIB n= 1/1/1/2; 1072
ASD n=1/1/1/3; clones/NPC deriv/expt/wells)
2.2. Family 1072 bFGF dose response, 10K, 4 Days

A. Raw Data, 1072 SIB after FGF application at 0.3, 1, 3, and 10 ng/mL (**p<0.0001); 1072 ASD after FGF application at 1 (* p=0.01) and 3 ng/mL (* p=0.03); and B) %CTRL, 1072 SIB 1 (* p=0.02), 3(* p=0.01), 10 ng/mL (* p= 0.05); 1072 ASD 0.3, 1, 3, and 10 ng/mL (** p<0.0001); (1072 SIB n= 1/1/1/2; 1072 ASD n=1/1/1/3; clones/NPC deriv/expt/wells)
2.3. Family 1072 bFGF 10 ng/mL, 10K, 2 Days

A. Raw Data, 1072 SIB and ASD NPCs after FGF application 0 ng/mL (**** p<0.0001) and 10 ng/mL (*** p=0.003) and B) %CTRL, after 10 ng/mL FGF (*** p=0.0008); (1072 SIB n=1/1/1/2; 1072 ASD n=1/1/3/9; clones/NPC deriv/expt/wells)
2.4. Family 1072 bFGF 10 ng/mL, 10K, 4 Days

A. Raw Data, 1072 SIB and ASD NPCs after FGF application 10 ng/mL (* p=0.02) and

B. %CTRL; (1072 SIB n= 2/2/6; 1072 ASD n=2/3/9; clones/NPC deriv/expt/wells)
Appendix 3: DNA synthesis in response to epidermal growth factor (EGF)

3.1. Family 1072 EGF dose response, 100K, 48h

A.

B.

A) 1072 Raw Data and B) 1072 %CTRL, SIB 0.3, 3, 10 ng/mL (** p<0.0001) and ASD 3 ng/mL (** p=0.008) (SIB n= 3/3/4/17; ASD n=2/3/6/27; clones/NPC deriv/expt/wells)
3.2. Family 1077 EGF dose response, 100K, 48h

A. DNA Synthesis (CPMs, $10^4)$

<table>
<thead>
<tr>
<th>EGF (ng/mL)</th>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>0.3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

B) 1077 %CTRL, (SIB n= 2/3/4/12; ASD n=2/3/4/12; clones/NPC deriv/expt/wells)

D. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th>EGF (ng/mL)</th>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>3</td>
<td>124</td>
<td>111</td>
</tr>
<tr>
<td>10</td>
<td>109</td>
<td>109</td>
</tr>
</tbody>
</table>

A) 1077 Raw Data, and B) 1077 %CTRL, (SIB n= 2/3/4/12; ASD n=2/3/4/12; clones/NPC deriv/expt/wells)
3.3. Family 1072 EGF, 10K, 2 days

A. Raw Data, 1072 SIB after EGF application 0.3 (* p=0.01), 3 ng/mL (** p=0.003);

B) %CTRL, 1072 ASD 3 ng/mL (* p=0.01) (1072 SIB n= 1/1/1/2; 1072 ASD n=2/2/2/6;
clones/NPC deriv/expt/wells)
3.4. Family 1072 EGF, 10K, 4 days

A) Raw Data, 1072 SIB after EGF application 0.3 (** p=0.0008), 3 ng/mL (** p=0.0004); B) %CTRL, 1072 SIB 0.3 ng/mL (* p=0.05) and ASD 0.3 (** p=0.003), 3 ng/mL (** p=0.0001) (1072 SIB n= 1/1/1/2; 1072 ASD n=1/1/3; clones/NPC deriv/expt/wells)
3.5. EGF + FGF, 100K, 48h

A. DNA Synthesis (CPMs, 1x10^4)

<table>
<thead>
<tr>
<th>Expansion (%)</th>
<th>FGF (ng/mL)</th>
<th>FGF + EGF (ng/mL)</th>
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<tbody>
<tr>
<td>30</td>
<td>0.66</td>
<td>0.84</td>
</tr>
<tr>
<td>3</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.59</td>
</tr>
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</table>

B. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th>Expansion (%)</th>
<th>FGF (ng/mL)</th>
<th>FGF + EGF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
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<tr>
<td>10, 10</td>
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</table>

A) Raw Data, 1993(1), P7 after FGF application 3 ng/mL (* p=0.032) and B) %CTRL, FGF 3 ng/mL (* p=0.049); (ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 4: DNA synthesis in response to serotonin (5-HT)

4.1. Family 1072 5-HT dose response, 100K, 48h

A) Raw Data and B) %CTRL, 1072 SIB 100 (* p=0.02), 200 (**** p<0.0001), and 300 ug/mL (**** p<0.0001); 1072 ASD 100 (** p=0.002), 200 (**** p<0.0001), 300 ug/mL (**** p<0.0001); (SIB n= 1/1/2/13; ASD n=1/1/2/14; clones/NPC deriv/expt/wells)
4.2. All families’ response to 200 µg/mL 5-HT, 100K, 48h

A.

![Graph showing DNA Synthesis with control and experimental conditions](image)

B.

![Graph showing DNA Synthesis percentage control](image)

A) Raw Data and B) %CTRL, **** p<0.0001 (1072 SIB n= 4/4/8/34; 1072 ASD n=3/4/12/46; 1077 SIB n=2/2/10/29 clones/NPC deriv/expt/wells)
4.3. 5-HT + FGF, 100K, 48h

A. DNA Synthesis (CPMs, $1 \times 10^4$)

<table>
<thead>
<tr>
<th></th>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10, 300</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Expansion (%), FGF (ng/mL), 5-HT (ug/mL), FGF, 5-HT (ng/mL, ug/mL)

B. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th></th>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>100</td>
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</tr>
<tr>
<td>10</td>
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<td>5</td>
<td>11</td>
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<tr>
<td>10, 300</td>
<td>25</td>
<td>38</td>
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</table>

Expansion (%), FGF (ng/mL), 5-HT (ug/mL), FGF, 5-HT (ng/mL, ug/mL)

A) Raw Data and B) %CTRL (1072 SIB n= 1/1/2/6; 1072 ASD n=1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 5: DNA synthesis in response to pituitary adenylate cyclase activating peptide (PACAP)

5.1. Family 1072 PACAP, 100K, 48h

A. 

B. 

A) Raw Data and B) %CTRL ASD 3 (* p=0.015), 10 nM (* p=0.015); (1072 SIB n= 3/4/8/27; 1072 ASD n=3/6/14/46; clones/NPC deriv/expt/wells)
5.2. Family 1077 PACAP, 100K, 48h

A. DNA Synthesis (CPMs, 1x10^4)

B. DNA Synthesis (%CTRL)

A) Family 1077 Raw Data and B) %CTRL; (1077 SIB n= 2/2/4/12; 1077 ASD n=1/1/3/9; clones/NPC deriv/expt/wells)
5.3. PACAP + bFGF, 100K, 48h

A. Raw Data, 2139(1), P8, SIB FGF (** p=0.001) and FGF + PACAP (**** p<0.0001); %CTRL, 2139(1), P8, FGF (*** p=0.0001) and FGF + PACAP (**** p<0.0001); (SIB n= 1/1/1/3; clones/NPC deriv/expt/wells)

B. DNA Synthesis (CPMs, 1X10^6)

<table>
<thead>
<tr>
<th>Expansion (%)</th>
<th>FGF (ng/mL)</th>
<th>PACAP (nM)</th>
<th>FGF + PACAP (ng/mL, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
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<td>10</td>
<td>2.0</td>
<td>1.1</td>
<td>2.2</td>
</tr>
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</table>

A) Raw Data, 2139(1), P8, SIB FGF (** p=0.0001) and FGF + PACAP (**** p<0.0001); %CTRL, 2139(1), P8, FGF (*** p=0.0001) and FGF + PACAP (**** p<0.0001); (SIB n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 6: DNA synthesis in response to insulin

6.1. Insulin, 100K, 48h

A. Raw Data, 2139(1), P8 Expansion 1% (**** p<0.0001) and Expansion 1% + Insulin (**** p<0.0001) and B) % CTRL, 2139(1), P8 Expansion 1% (**** p<0.0001) and Expansion 1% + Insulin (**** p<0.0001), (n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 7: DNA synthesis in response to brain-derived neurotrophic factor (BDNF)

7.1. BDNF, 100K, 48h

A.

B.

A) Raw Data, 1072 ASD and B) %CTRL, BDNF 10ng/mL (** p=0.0002); (ASD n= 2/2/2/4; clones/NPC deriv/expt/wells)
Appendix 8: DNA synthesis in response to nerve growth factor (NGF)

8.1. Family 1077 SIB and 1072 ASD NGF, 100K, 48h

A.

![Graph showing DNA Synthesis (CPMs, 1X10^4) vs. NGF (ng/mL)]

- SIB 1077: 1.2, 1.3
- ASD 1072: 4.1, 4.4

B.

![Graph showing DNA Synthesis (%CTRL) vs. NGF (ng/mL)]

- SIB 1077: 100, 106
- ASD 1072: 100, 107

A) Raw Data, 1077 SIB, 07c16, P5 and 1072 ASD, 1993(1), P5 and B) %CTRL (1077 SIB n= 1/1/1/3; 1072 ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 9: DNA synthesis in response to Neurotrophin-3 (NT-3)

9.1. NT-3, 100K, 48h

A. DNA Synthesis (CPMs, 1x10^4)

<table>
<thead>
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<th>10</th>
<th>30</th>
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</thead>
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<tr>
<td>Raw Data</td>
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<td>4.7</td>
<td>4.7</td>
<td>4.3</td>
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</table>

B. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th>NT3 (ng/mL)</th>
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<th>10</th>
<th>30</th>
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<td>Raw Data</td>
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<td>94</td>
<td>86</td>
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</table>

A) Raw Data, 2139(2), P7, NT-3 30 ng/mL (* p=0.03) and B) %CTRL, (SIB n= 1/1/1/4; clones/NPC deriv/expt/wells)
Appendix 10: DNA synthesis in response to Oxytocin (OT)

10.1. Family 1072 OT, 100K, 48h

A. 1072 Raw Data, and B) %CTRL, (SIB n= 1/1/2/13; ASD n=2/2/3/20; clones/NPC deriv/expt/wells)
Appendix 11: DNA synthesis in response to ciliary neurotrophic factor (CNTF)

11.1. CNTF, 100K, 48h

A. DNA Synthesis (CPM, 1x10^4)

<table>
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<tr>
<th>CNTF (ng/mL)</th>
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<tbody>
<tr>
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<td>2.9</td>
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</tbody>
</table>

B. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th>CNTF (ng/mL)</th>
<th>0</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Synthesis</td>
<td>100</td>
<td>124</td>
</tr>
</tbody>
</table>

A) Raw Data, 2139(1), P8 after CNTF application 20 ng/mL (* p=0.023); B) %CTRL, 2139(1), P8 after CNTF application 20 ng/mL (* p=0.023); (SIB n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 12: DNA synthesis in response to leukemia Inhibitory factor (LIF)

12.1. FGF, LIF, LIF + FGF, 100K, 48h

A. Raw Data, SIB 2139(1), P8 FGF (** p<0.0001) and FGF + LIF (**** p<0.0001); B) %CTRL, SIB 2139(1), P8, SIB FGF (**** p<0.0001) and FGF + LIF (**** p<0.0001); (SIB n= 1/1/1/3; clones/NPC deriv/expt/wells)
Appendix 13: DNA synthesis in response to valproic acid (VPA)

13.1. VPA, 50K, 48h

A. DNA Synthesis (CPMs, 1x10^4)

<table>
<thead>
<tr>
<th>VPA (mM)</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.1</td>
<td>5.1</td>
<td>4.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

B. DNA Synthesis (%CTRL)

<table>
<thead>
<tr>
<th>VPA (mM)</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>104</td>
<td>86</td>
<td>64</td>
</tr>
</tbody>
</table>

A) Raw Data, JM cells and B) %CTRL, 2 mM (** p<0.0005); (n= 1/1/2/6; clones/NPC deriv/expt/wells)
Appendix 14: DNA synthesis at low density (0.5X10^4 cells/cm^2) in response to growth factor panel

14.1. Growth factor panel, 10K, 2 days

A) Raw Data, 1072 ASD 2009(1), P4 FGF (** p=0.001); (1072 ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
14.2. Growth factor panel, 10K, 4 days

A) Raw Data, 4 days, 1072 ASD 2009(1), P4 FGF (**** p<0.0001), EGF 0.3 (*) p=0.01), 3 ng/mL (** p=0.003); (1072 ASD n= 1/1/3; clones/NPC deriv/expt/wells)
Appendix 15: DNA synthesis in response to AKT agonist, SC-79

15.1. Family 1072 SC-79 dose response, 100K, 2 days

A) Raw Data, 2 days, 100K, 1072 SIB SC-79 10 (** p<0.002), 30 ug/mL (***/p<0.0001); 1072 ASD SC-79 10 (* p<0.03), 30 ug/mL (***/p<0.0001); B) %CTRL, 1072 SIB 10 (** p= 0.0082), 30 ug/mL SC-79 (***/p<0.0001); 1072 ASD SC-79 10 (** p<0.0082), 30 ug/mL SC-79(***/p<0.0001); (1072 SIB n= 1/1/3; ASD n= 1/1/3; clones/NPC deriv/expt/wells)
15.2. **Family 1072 SC-79, 100K, 2 days**

A) **Raw Data, 2 days, 100K**; B) **%CTRL**; (1072 SIB n= 1/1/3/9; ASD n= 2/2/2/6; clones/NPC deriv/expt/wells)

**SC-79 (µg/mL)**
- 0
- 0.1
- 0.3

**DNA synthesis**
- **CPMs (1x10^4)**
  - SIB
  - ASD

**DNA synthesis (%CTRL)**
- SIB
- ASD
A) Raw Data, 4 days, 100K; B) %CTRL; (1072 SIB n= 1/1/3/9; ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
15.4. **ASD 1072 SC-79 dose response, 10K, 2 days**

A. 

![Bar chart showing DNA synthesis (1x10^6) for different SC-79 concentrations (μg/mL).]

B. 

![Bar chart showing DNA synthesis (%CTRL) for different SC-79 concentrations (μg/mL).]

A) Raw Data, 1072 ASD, 4 days, 100K; B) %CTRL; (1072 ASD n= 1/1/2/7; clones/NPC deriv/expt/wells)
15.5. ASD 1072 SC-79 dose response, 10K, 4 days

A. 

![Graph showing DNA synthesis (CPMs, 1x10^3) vs. SC-79 (µg/mL)]

- DNA synthesis (CPMs, 1x10^3)
  - 0: 0.37
  - 0.1: 0.51
  - 0.3: 0.52
  - 1: 0.48
  - 2: 0.47

B. 

![Graph showing DNA synthesis (%CTRL) vs. SC-79 (µg/mL)]

- DNA synthesis (%CTRL)
  - 0: 100
  - 0.1: 129
  - 0.3: 134
  - 1: 125
  - 2: 123

A) Raw Data, 1072 ASD, 4 days, 100K; B) %CTRL; 1072 ASD 0.1 (* p=0.02), 0.3 µg/mL (**) (p=0.005); (1072 ASD n= 1/1/2/7; clones/NPC deriv/expt/wells)
A) Raw Data, Family 1072, 2 days, 10K; B) %CTRL; (1072 SIB n= 1/1/3/9; ASD n= 2/2/4/13; clones/NPC deriv/expt/wells)
15.7. Family 1072 SC-79, 10K, 4 days

A. DNA Synthesis (CPMs, 1x10^3)

![Bar chart showing DNA synthesis with SC-79 concentrations of 0, 0.1, and 0.3 μg/mL.]

B. DNA synthesis (%CTRL)

![Bar chart showing DNA synthesis with SC-79 concentrations of 0, 0.1, and 0.3 μg/mL.]

A) Raw Data, 1072, 4 days, 10K; B) %CTRL; (1072 SIB n= 1/1/3/9; ASD n= 2/2/4/13; clones/NPC deriv/expt/wells)
Appendix 16: DNA synthesis in response to AKT antagonist, MK2206

16.1. Family 1072 MK2206, 100K, 2 days

A. MK2206 (nM)

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DNA synthesis (CPMs, 1x10^4)

- SIB: 5, 4, 4, 4, 4
- ASD: 0, 0, 0, 0, 0

B. MK2206 (nM)

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>30</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

DNA Synthesis (%CTRL)

- SIB: 100, 96, 93, 93, 88
- ASD: 100, 92, 89, 89, 89

A) Raw Data, Family 1072 SIB, 2 days, 100K, MK2206; B) %CTRL; (1072 SIB n= 1/1/3/8; ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
16.2. Family 1072 MK2206, 100K, 4 days

A.

B.

A) Raw Data, Family 1072 SIB, 4 days, 100K, MK2206; B) %CTRL; (1072 SIB n= 1/1/3/8; ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
16.3. Family 1072 MK2206, 10K, 2 days

A. DNA synthesis (CPMs, 1x10^6)

<table>
<thead>
<tr>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

B. DNA synthesis (%CTRL)

<table>
<thead>
<tr>
<th>SIB</th>
<th>ASD</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>101</td>
<td>107</td>
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<td>104</td>
<td>99</td>
</tr>
<tr>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>94</td>
<td>94</td>
</tr>
</tbody>
</table>

A) Raw Data, Family 1072 SIB, 2 days, 10K, MK2206; B) %CTRL; (1072 SIB n= 1/1/3/8; ASD n= 1/1/1/3; clones/NPC deriv/expt/wells)
16.4. Family 1072 MK2206, 10K, 4 days

A. Raw Data, Family 1072 SIB, 4 days, 10K, MK2206; B) %CTRL; (1072 SIB n= 1/1/3/8; clones/NPC deriv/expt/wells)

B. DNA synthesis (%CTRL) vs. MK2206 (nM)
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