AUTISM HUMAN NEURAL PRECURSOR CELLS EXHIBIT COMMON DEFECTS IN NEURITE OUTGROWTH, CELL MIGRATION, AND mTOR SIGNALING

by

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III. Abstract of the Dissertation

Autism Human Neural Precursor Cells Exhibit Common Defects in Neurite Outgrowth, Cell Migration, and mTOR Signaling

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Autism spectrum disorders (ASD) are a group of developmental disorders characterized by deficits in social interaction and communication and the presence of repetitive and restrictive behaviors. Despite high prevalence (1:68), and large social and economic impacts, uncovering the mechanisms that contribute to ASD and finding therapeutics for treatment of the disorder has been thwarted by our inability to directly study human neurons, the limitations of animal models, and disorder heterogeneity.

Recent studies have found that ASD risk genes converge onto the cerebral cortex of the developing mid-fetal brain (8-24 weeks old). During this time, neural precursor cells (NPCs) in the developing brain are undergoing proliferation, migration, and differentiation to form neurons and the normal cytoarchitecture of the brain. Indeed, post-mortem and imaging studies in humans with ASD have uncovered structural changes that are suggestive of alterations in these basic developmental processes. However, genetic, imaging, and post-mortem analyses cannot give mechanistic insight into the alterations found in the ASD brain. For the past few decades, studies to uncover the mechanisms underpinning ASD have primarily been conducted in rodent models. However, rodent models, due to differences in physiology and genetics, are unable to truly recapitulate human

neurodevelopment. Moreover, rodent models cannot be utilized to study the vast majority of cases of ASD which are idiopathic or polygenic. Therefore, to really understand ASD, we need to study developmental processes like proliferation, migration, and differentiation in human neural cells derived from individuals with idiopathic ASD. Yet, until the discovery of induced pluripotent stem cell (iPSC) technology such studies were impossible.

iPSCs are stem cells reprogrammed from mature somatic tissues (like white blood cells) that have the capability of forming almost any cell in the body including neurons. As iPSCs retain the genetic signature of the individuals from whom they are derived, we can for the first time, study the development and function of neural cells of people with genetically complex neuropsychiatric disorders. The three iPSC studies of idiopathic ASD published thus far have uncovered common defects in synapse formation, dendritic spines, and neuronal activity in ASD neurons. Yet, despite studies indicating the importance of early neurodevelopment in ASD pathogenesis, most iPSC studies have focused on post-mitotic differentiated neurons and have largely neglected study of early developmental processes in NPCs. Therefore, the goals of my studies were to assess neurite outgrowth, migration, and signaling pathways in neural precursor cells derived from 6 individuals with ASD. Our cohort consists of three individuals with idiopathic ASD (I-ASD) and their unaffected brothers (Sib) as controls, 3 individuals with 16p11.2 deletion (16pDel) and ASD and unaffected controls from the NIMH.

Fascinatingly, despite the heterogeneity of ASD, all 6 patients in our cohort had reductions in neurite outgrowth and cell migration when compared to unaffected individuals. On the other hand, we were able to define distinct autism NPC "subgroups" by using developmentally relevant extracellular factors (EFs) such as serotonin (5-HT),

PACAP, and nerve growth factor (NGF). Specifically, treatment with EFs stimulated neurite outgrowth and cell migration in both unaffected patients and 16pdel NPCs, whereas they failed to elicit neurite or migration responses in I-ASD. Further studies revealed that NPCs derived from all the ASD NPCs, both idiopathic and 16pDel, showed dysregulations in the mTOR pathway. Two individuals (I-ASD-1 and I-ASD-3) had lower mTOR pathway activity characterized by reductions in P-AKT and P-S6 while the other four individuals (I-ASD-2 & the 3 16pDel patients) showed higher mTOR pathway activity as characterized by higher levels of P-S6. Thus, molecular subtyping of patients was accomplished by characterizing the levels of mTOR pathway components. As signaling pathways have been shown to regulate neurodevelopmental processes, I wanted to manipulate and "normalize" mTOR pathway activity in these patients to see if neurodevelopmental phenotypes could be rescued. Thus, I selected 1 patient from each group (under vs overactive mTOR) and applied agonist and antagonist drugs. In I-ASD-1, where mTOR pathways were underactive, the use of AKT agonist sc-79 rescued the neurite outgrowth, migration, and EF response defects seen in these NPCs. Conversely, application of AKT inhibitor MK-2206 to Sib-1 NPCs, led to reductions in neurite outgrowth and migration and abolished EF responses in this unaffected individual! In I-ASD-2, where mTOR pathway activity was higher, application of MK-2206 successfully increased neurite outgrowth and migration to the level of Sib NPCs. These studies show that the mTOR pathway is a critically important regulator of neurodevelopmental processes and that targeting this altered pathway could rescue developmental defects seen in our ASD patient NPCs.

In conclusion, by studying human neural precursor cells derived from patients with ASD, I discovered that alterations in early developmental processes that are essential to

building the brain are commonly altered. Moreover, by utilizing EFs and studying mTOR signaling, I was able to determine different subtypes of ASD. Lastly, common aberrations in the mTOR pathway were also observed in our ASD patients. By targeting these mTOR abnormalities, I successfully reversed the phenotypes seen in our NPCs. Thus, by using patient derived NPCs, I have demonstrated the utility of this model in the study, categorization, and potentially even treatment of autism spectrum disorders. Future studies can help elucidate how alterations in NPCs correlate with patient phenotypes and whether drugs that can rescue phenotypes *in vitro* could ultimately be made into targeted therapeutics for patients.

IV: Acknowledgements

There is an often-erroneous image of science being conducted alone in a lab by a semi-crazed man with shockingly untidy white hair. Of course, in addition to pointing out that not all scientists are men with bad hair, it is important to note that science is rarely done in isolation and that collaboration and support are essential to the scientific endeavor. Thus, I wish to express my eternal gratitude to all the individuals who made this dissertation possible including individuals who have encouraged me and supported me through life and others who have provided important guidance and mentorship.

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blossom as an individual and a scientist. Manny has also consistently and empathetically supported me throughout my many illnesses in grad school and through periods of stress. He was also always there to encourage me to take a break and relax once in a while too! I truly cherish his mentorship and look forward to future collaborations

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To all my other mentors, advisors, and pillars including Dr. Vassie Ware, Dr. Michael Burger, and Dr. Michael Gill at Lehigh. To my 8th grade teacher Mrs. Sicca. Lastly to all my other friends and people who were there for me when I needed them most. Thank you. I hope I have made you all proud and I am forever grateful for your support

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Chapter 1: Introduction

<u>Foreword:</u>

According to the World Health Organization (WHO), by 2020, neuropsychiatric diseases will surpass cancer, cardiovascular disease, and motor vehicle accidents to become the 2nd leading cause of disability and disease burden globally. Data from the WHO shows that individuals with neuropsychiatric disorders have a 40- 60% increased risk of mortality and morbidity – which persists even in high income countries. Yet, even in 2018, mental illnesses are not treated with the gravitas or importance of cancer or heart disease. Moreover, there remains a social stigma in the public and even among health care providers regarding the treatment and needs of mentally ill or developmentally disabled individuals.

In addition to the social barriers, neuropsychiatric disorders ranging from bipolar disorder to schizophrenia to autism are genetically complex, highly heterogeneous, and often have unknown etiology. Until recently, these disorders were studied in animals which could not adequately capture the genetic and phenotypic heterogeneity of human neuropsychiatric illnesses. Thus, mechanistic understanding of these disorders and development of therapeutics for these disorders have been limited. In many cases, particularly in the treatment of disorders Hence, a new approach needs to be taken to better understand and treat neuropsychiatric disorders.

While many of these disorders are vast and diverse, accumulating evidence suggests that many neuropsychiatric disorders have developmental origins. To build the brain, a set of well-orchestrated and precisely timed processes must occur. It's easy to imagine then, that even slight perturbations in these processes could change the structure,

function, and integrity of the brain. Thus, a useful way to study neuropsychiatric disorders would be to assess neurodevelopmental and molecular differences in the neuronal cells of individuals with these disorders. In fact, the current categorization of neuropsychiatric disorders by behavioral symptoms rather than by molecular or neuropathophysiological commonalities, may be an impediment to the study and treatment of these disorders. Indeed, a review of the progress in the cancer field over the past few decades truly highlights the value of categorizing diseases by molecular pathology instead of by organ of origin or cluster of symptoms. With advances in molecular and biochemical analyses along with "big data" approaches the cancer field ultimately found that tumors that originate in totally different tissues- like breast and liver, may have common underlying molecular mechanisms. By targeting these molecular defects, survival rates and prognosis have been vastly improved in the past few years. Likewise, in the field of neuropsychiatry, categorization of diseases by molecular origin rather than behavioral symptoms could greatly advance understanding and treatment of these disorders.

Now, for the first time, induced pluripotent (iPSC) technology allows us to derive and study live neurons from individuals with neuropsychiatric disorders. With this technology, we can now study the neurodevelopmental and molecular pathology of human neural cells in the context of complex disease genetics! Thus, we could use iPSCs and "big data" approaches to uncover common molecular pathology amongst different neuropsychiatric disorders. This approach could also pave the way for the development of targeted therapeutics that could revolutionize the way neuropsychiatric illnesses are treated. In my work, iPSCs have been utilized to perform a comprehensive analysis of developmental and molecular abnormalities in multiple individuals with different subtypes

of autism. My aim is to understand the commonalities and differences amongst these patients in order to distinguish/characterize different cellular and molecular forms of autism. This approach could ultimately help us better categorize developmental disorders and lead to better therapeutics. Indeed, while the cohort I am studying has ASD, it is likely that defects seen in this disorder are also commonly found in other neurodevelopmental and neuropsychiatric disorder. Thus, in the following chapters, I will review the clinical and genetic aspects of ASD, explore the developmental and molecular aberrations seen in ASD models, and compare some of these aspects to other developmental disorders.

Autism: Features, Diagnosis, Prevalence, Management & Disorder Impact

Autism spectrum disorders (ASD) are a set of neurodevelopmental disorders characterized by deficits in social interaction and communication and the presence of repetitive restrictive behaviors [1]. Though these common impairments define ASD, there is marked heterogeneity in onset, expression, and severity of symptoms amongst affected individuals [2-5]. For example, those with severe ASD may be non-verbal and require extensive support for survival while high functioning individuals develop language and can achieve independence. Further, up to 70% of those with ASD suffer comorbid conditions including intellectual disabilities, epilepsy, anxiety, and depression [6-8]. Thus, the clinical presentation of autism can be varied and complex which makes study and treatment of the disorder challenging. Moreover, ASDs have many overlapping symptoms with other developmental disorders such as schizophrenia and ADHD which further complicates understanding the disorder [9, 10] Indeed, despite decades of studies, the underlying etiology of ASD is unknown. As with many human diseases, it is unlikely that there is one single underlying factor that contributes to ASD. Thus, numerous avenues of research are

necessary to uncover and understand the pathogenesis of ASD.

There are no standardized medical tests (like a blood test) to detect ASD, making the identification and diagnosis of the disorder more complex and difficult. Generally, diagnoses are made in two steps- an initial developmental screen (at the pediatrician's office) followed by a comprehensive diagnostic evaluation with an expert clinician [11-13]. Experienced clinicians can diagnose ASD as early as age two using standardized assessment tools such as the Diagnostic and Statistics Manual (DSM), the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) [14, 15]. Diagnosis of ASD can be delayed because individuals can be diagnosed with other disorders like ADHD, mild forms of ASD can be missed on an initial screen, or repetitive behaviors often do not manifest during a short pediatrician's visit [16, 17] . Certainly, while ASD can be diagnosed as early as 2, average age of diagnosis is between 4 and 6 years and for mild cases diagnosis can be delayed to age 8 or can even be missed until adolescence or adulthood [17, 18]. Research has shown that delayed diagnosis is detrimental to behavioral and social outcomes and leads to children missing important early intervention programs and failing to get adequate support in schools [16,17]. The importance of early diagnosis has led to a slew of studies which are attempting to find an objective method to diagnose ASD. While the search for blood and biological markers have encountered barriers, some fMRI/MRI and eye-tracking studies have made progress in early diagnosis [19-28]. Lastly, as evidence has accumulated that autism risk is higher in siblings, baby siblings of kids with autism are also being screened at younger ages [29-32].

While progress has been made towards reducing the mean age of diagnosis for ASD, the gold standard of treatment for ASD is still behavioral intervention. There are

currently two objectively confirmed comprehensive models of early behavioral intervention including the Applied Behavior Analysis (ABA) and the Early Start Denver Model (ESDM) [33-37]. Often, with these behavioral interventions parent training is included for the best outcomes. A randomized controlled trial with toddlers has found that the ESDM is efficacious for improving behaviors and assuring growth in normative adaptive behavior [38]. Further studies have also found that higher intensity and increased frequency of interventions along with earlier age of intervention also lead to better outcomes in children [39-46]. Yet, these behavioral therapies are often costly and require intensive familial involvement and extensive time commitments. In some cases, years of behavioral therapy are necessary to gain small improvements. Furthermore, for some individuals, behavioral therapy must be administered life-long for optimal functioning. However, there are currently no therapeutics or medical interventions available that treat the core symptoms of ASD. In fact, behavioral therapy is the only treatment that improves and targets core symptoms while medical of children with ASD largely revolves around treating co-morbid conditions like depression, sleep issues, and seizures [47-50]. However, management of co-morbid conditions does greatly improve moodiness, attention issues, aggression, and self-harm [48, 50-52]. Thus, while behavioral intervention should always be a key part of the treatment of ASD, it would be highly effective to have therapeutic drugs to treat the core symptoms of ASD to better enhance outcomes.

Current estimates from the CDC suggest that 1 in 68 individuals in the United States have autism. Reported prevalence rates have increased over time-for example, in 2000 the prevalence of autism was 1 in 150. A large body of research suggests that these increases in prevalence are at least partly due to better identification and diagnosis of the disorder

along with changing diagnostic criteria that have occurred with new versions of the DSM
[53]. There is also some concern that children who have other developmental disorders such as intellectual impairment, anxiety disorders, and specific language impairment may be misdiagnosed with ASD thereby further inflating prevalence rates [54]. Males are thought to be disproportionately affected by autism- at a 4:1 ratio. There are multiple hypotheses for the disparity of ASD diagnoses between sexes. Some studies suggest that male fetuses are more susceptible to developmental injury, others show fetal testosterone levels may increase susceptibility while fetal estrogen is protective, and finally, other studies suggest that females need a higher genetic load to manifest disease [55-62] However, while males may be more susceptible to ASD, newer literature suggests that the 4:1 ratio is inflated and that females with ASD often go undiagnosed. Studies have found clinician bias, differential socialization of male and female children, and diagnostic tests that have been developed based on male symptoms of ASD are significant barriers to female diagnosis [55-65]. Indeed, studies have found that most females diagnosed with ASD are either severely impacted or show externalizing behaviors such as aggressiveness or hyperactivity. Further studies find that as cognitive functioning (IQ in particular) decreases, the sex ratio in children with autism approaches 1:1 while with increases in IQ the sex ratio can be as high as 8:1 male to female [56, 66]. Thus, current diagnostic criteria do not consider the potential for differential manifestations of autism in females and potential compensation in girls with higher IQ. Therefore, girls and woman who would greatly benefit from behavioral intervention are often left to fend on their own.

With rising prevalence, the economic costs of ASD have become a concern in the United States and globally. In 2015, autism cost the US economy approximately 265 billion

dollars. By 2025, autism is estimated to cost the US economy 461 billion dollars which will likely exceed the cost of stroke, hypertension, and diabetes. These costs come from an aggregate of money spent on medications, health care, special needs services, family services, as well as care giver time and reduced care giver productivity in the work force [67, 68]. On average, families with kids who have autism are more likely to report financial problems, reduce or stop work because of their child's condition, and pay more than $1000 a year for their child's care out of pocket [68]. In addition to the monetary burden, families that have kids with ASD face additional personal and social burdens in their lifetime. Caregivers for individuals with ASD, often report high levels of stress, depression, and low quality of life [69-71]. Likewise, "outcomes" for individuals with ASD are also often considered poor. For example, studies have found that even individuals on the milder end of the spectrum had limited independence, often failed to maintain permanent employment, and remained socially isolated [72, 73]. Moreover, higher mortality and morbidity are also found in individuals with ASD. Studies suggest that comorbid conditions contribute to higher mortality and morbidity, however, impaired communication with health care providers or reduced access to health care are also contributory [74-79]. Not all outcomes are bleak, however, as a recent prospective study which followed up 17 years later with children diagnosed with ASD at age 2 found that 9% of the participants had largely overcome core difficulties associated with ASD and no longer retained a diagnosis [46]. The children who "outgrew" their ASD diagnoses were more likely to have participated in early intervention treatments, started treatment at a younger age, and generally had IQ over 70. This suggests, that at least in higher IQ patients, appropriate intervention and better quality of care can mitigate the negative outcomes associated with ASD. In children with low IQ,

benefits of early intervention are not as large as those seen with higher IQ children. However, earlier intervention and intensive behavioral therapy still lead to behavioral and IQ improvements in children with low-functioning autism [80-82].

In short, ASDs are a complex and heterogeneous set of neurodevelopmental disorders with a high prevalence and a large social and economic impact on society. Yet, despite being a large public health problem, there are no objective measures to diagnose ASDs nor are there any therapeutics that treat the core symptoms of this disorder. To better diagnose and treat ASD, it is important to understand the molecular pathways that are aberrant in ASD and how these aberrations have changed brain development. This will allow for the development of more targeted therapeutics and may facilitated discovery of biomarkers that would allow earlier diagnosis. It is also important to realize that ASDs are classified by behavioral symptoms- many of which are found in other neuropsychiatric disorders. Thus, comparing and contrasting these disorders and characterizing them by molecular defect may make diagnosis and treatment more effective. In next two sections, I will review the genetics of ASD and the neuropathological abnormalities seen in the disorder as well as other disorders. These studies will help elucidate the various factors that may cause ASD and help shed light on what is different about the ASD brain.

The Genetics of Autism:

In the 1950s to 1970s, some psychiatrists believed that "refrigerator" mothers who had less affectionate "colder" mothering styles caused autism [83-85]. However, twin studies spanning from the late 70s to the early 90s largely abolished this "refrigerator mom" theory. Twin studies that use both identical (monozygotic) and fraternal (dizygotic) pairs generally help parse the contributions of genetics vs environment for a disorder. In identical twins,

who share 100% of their DNA, higher likelihood of both twins having a disease would indicate high heritability. On the other hand, fraternal twins only share 50% of their DNA like typical siblings. Thus, if fraternal twins have a higher rate of disease than regular siblings, this indicates environmental risks. In 1977 the first twin study in autism found that if one identical twin had autism, there was a 90% chance that the other twin also had autism [86]. Subsequent studies in the 1980s and 90s and meta-analyses of these studies also reported 80-90% concordance rates suggesting that autism is highly heritable [87-91]. Fraternal twins had about a 0-10% concordance rate according to these early studies. These studies, however, had small sample sizes (less than 50 pairs), did not sample from the general population, often used narrow diagnostic criteria to define ASD, and either relied on diagnosis records or parental reports to determine diagnosis rather than administering tests themselves. In 2011, using rigorous methods and a larger sample size, Hallmayer et al challenged the large difference in ASD concordance rates between identical and fraternal twins [92]. Hallmayer et al found that the concordance rate in identical twins was 77% while fraternal twins had a 31% concordance rate. As the fraternal twin concordance rate was higher than previously reported, this suggested that environmental risk factors played a larger role in ASDs. However, as seen with the identical twins, genetics does still play a large role. Indeed, even typical siblings and 1° relatives of individuals with ASD have a higher risk for autism than the general population [29, 93, 94]. For example, a study by Oznoff et al found that younger siblings of children with autism had a 20% chance of developing ASD when compared to the 1-2% found in the general population [29]. Thus, we see that autism is a highly heritable disorder. However, high heritability does not necessarily imply that all cases of ASD have the same genetic underpinning.

While genetics play a large role in autism susceptibility, almost 70-80% of cases of autism are idiopathic, meaning they are genetically undefined [95, 96]. In about 10-15% cases of autism, the precise genetic alterations that contribute to disease have been identified. These cases include syndromic disorders such Rett syndrome, Fragile-X Syndrome, and Tuberous Sclerosis, which are caused by alterations in a single gene. Moreover, newer studies have identified alterations in copy number variants which also contribute to ASD such as deletions and duplications in 16p11.2 and 22q13. Interestingly, the genes associated with the monogenic forms of autism have not been shown to contribute to risk of idiopathic ASD [96, 97]. Moreover, there are very few studies that have looked at how similar or different syndromic and idiopathic ASDs are in phenotype, transcriptome, or neuropathology, though, these studies are becoming more common [98]. Thus, genetic knowledge from syndromic ASDs, may not necessarily help understand idiopathic ASD. About 40-50% of ASD risk is conferred by commonly inherited genetic variants [96]. These variants are present widely in the population and their presence only mildly elevates disease risk. Researchers postulate that the presence of many disease associated common variants along with environmental insults could be the cause of some ASD cases. Indeed, a recent study by Weiner et al in more than 6,500 families found that children with ASD inherit a higher burden of common genetic variants associated with autism than would be expected by chance alone [99]. Yet, these common variants have been more difficult to study, as large genome wide association studies are necessary to identify these variations. On the other hand, rare genetic variants with high penetrance/impact have a much stronger causal effect on ASD risk and are thought to contribute to 10-20% of ASD [100, 101]. However, only a very tiny percentage of people carry a particular rare mutation. Moreover, these rare

mutations often occur de novo, meaning that parental genomes do not carry the mutation seen in the proband. Thus, rare de novo variants do not really help account for the increased ASD risk seen in siblings or nor do they explain why ASD sometimes "runs in families". In addition, newer studies are suggesting that rare variant de novo mutations are often associated with more severe ASD with impactful comorbidities such as low IQ and seizures [99, 102]. More studies are finding that de novo genetic variants are higher in individuals with ASD [103-105]. For example, de novo CNVs are four times more common in individuals with ASD when compared to unaffected siblings [106]. However, presence of a de novo CNV does not always necessary correlate with disease. Moreover, polygenic background can increase or decrease the risk that a de novo mutation would lead to disease. Thus, ASD is further complicated by incomplete penetrance of variants along with variable expressivity of these variants in individuals. Overall, these studies show both the etiological and genetic heterogeneity of ASDs which mirror the clinical heterogeneity of the disorder. Moreover, we see that a majority of cases of ASD have undefined genetic etiology and a significant percentage of ASD may be due to polygenic causes.

While genome wide association studies and other studies have uncovered a multitude of genes associated with ASD, newer studies have used pathway analysis techniques to uncover whether these ASD risk genes could be involved in regulating common process. Reviews that have analyzed numerous genetic studies to find points of convergence have uncovered 4-8 categories that ASD risk genes can fall into [107-114]. Alterations have been observed in genes and proteins that are in the following category 1) proteins that can alter neural activity or have activity dependent expression such as ion channels 2) regulators of post-synaptic translation such as FMR1 3) proteins involved in

cell adhesion such as CNTNAP2, neurexins, neuroligins, and cadherins 4) genes that specify or determine the ratio of excitatory to inhibitory neurons like neuroligin-2 5) Cytoskeletal proteins 6) members of signaling pathways such as MAPK and P13K-mTOR, 7) chromatin regulators such as CHD8, and 8) immune-associated molecules. These genetic convergence studies are largely bioinformatic and largely use results from GWAS studies. To definitely demonstrate convergence of genes, experimental testing in model systems is required. Moreover, while GWAS studies can identify polymorphism and alterations in genes, these studies are not usually designed to correlate genetic alterations with changes in gene expression or protein production. However, the expression patterns of some ASD genes have bene annotated using whole-genome transcriptome profiling in blood cells and sometimes brain tissue from ASD and control participants. Efforts have also been made to build proteomic interactomes of ASD risk gens to understand how gene products functionally interact [109]. However, it is important to note that the effects of genetic polymorphisms on genes are often tissue specific- only about 60% of genes expressed in the brain are also expressed in blood. Thus, transcriptomic profiling of blood cells alone may not truly reflect gene expression in the ASD brain [115]. However, study of brain transcriptomics is largely limited by the availability of post-mortem brain samples.

Convergent pathways can help us narrow down what processes may be altered in ASD. However, convergence alone does not necessarily indicate causality. These common pathways that are implicated in ASD could be confounds or a non-causal consequence of ASD. For example, excess microglia (or immune activation) is commonly observed in ASD [116]. The excess microglia could be causal, that is higher levels of microglia could lead to autism or it could be a confound- a change in chromatin modifiers could lead to

both ASD and excess microglia. Alternatively, the excess microglial phenotype could be seen as a consequence of ASD, where having the disorder leads to the activation of microglia. Thus, while convergence studies help narrow down potential etiologies of ASD, it is important to remain cautious about the results of these studies. Furthermore, even though pathway analyses have helped focus attention to processes which are altered in ASD, the vast range of functions these gene regulate has not helped with providing further insight into what developmental processes are altered in the brain to cause ASD. Moreover, the "categorization" of many of these genes are artificial or limiting. For example, an ASD risk gene categorized as a cell adhesion molecule may also play roles in synapse formation. Additionally, if we look at an earlier time point in development, the cell adhesion molecule that regulates synapse formation in the postnatal brain may be important for promoting cell migration in embryonic development! Interestingly, a recent paper has suggested that 70% of genes found on the SFARI list of syndromic developmental disorders converged onto the regulation of two basic developmental cellular processes: proliferation and differentiation of neural stem cells [117]. Indeed, many other reviews of genetic literature are suggesting that psychiatric illnesses including ASD begin with abnormal specification, growth, expansion, and differentiation of embryonic neural precursor cells [118, 119]. Thus, while pathway analyses are showing that ASD genes can be categorized by their putative functions in adults, it is important to look at where in development these genes are expressed and what roles in development these genes play.

Newer genetic studies have begun to focus on the expression of ASD risk genes in development. A study by Willsey et al (2013) explored convergence of 9 ASD associated mutations in relation to their expression by brain region, cell type, and time points in human

development [120]. The study found that the risk genes converged onto glutamatergic projection neurons in layer 5 and 6 of the human mid-fetal (12-26 weeks post conception) prefrontal and primary motor-somatosensory cortex. This study and others have also found a temporal convergence to early development, including in the mid-fetal striatum as well as perinatal thalamus and cerebellum, which are regions that are implicated in ASD. Since the small number of genes analyzed in the Willsey study limited the points of convergence uncovered, larger studies will be necessary to fully understand the spatiotemporal pattern of ASD risk genes. Another study by Parikshak et al (2013), which used a much larger set of ASD risk genes, found that ASD genes often converged onto pathways related to translational and transcriptional regulation in glutamatergic projection neurons in the cortex [121]. Expression of these genes ranged from fetal to early neonatal developmental period. During development, genes are often activated and inactivated spatially and temporally. Thus, understanding the developmental spatiotemporal expression of these genes could help us understand how alterations in the same gene, depending upon location and time of expression, could contribute to differential phenotypes.

In conclusion, genetic studies illustrate that autism is highly heritable and that the genes associated with ASD ultimately seem to converge onto the regulation of neurodevelopmental processes during embryonic/fetal and early postnatal development. While genetic studies can show us that ASD risk genes are expressed in certain areas of the brain during development, these studies alone cannot show us what functions these genes may play in development. Moreover, genetic models alone cannot elucidate how gene alterations can lead to the changes in brain structure and behavior that cause ASD. To truly understand what these genes do and to establish causality, gain and loss of function

experiments must be conducted in a model system. Yet, single genetic alterations alone are rarely causative in ASD. In fact, genetic background and polygenic factors often contribute to disease pathogenesis. Thus, not only is it important to study the importance of ASD risk genes in a model system, it is important to consider these alterations in the context of genetic background of an individual. Of course, genetic studies cannot show us what alterations are present in the ASD brain. Thus, it is important to study and review the neuropatho-logical features of autism in the human brain. Post mortem and imaging studies can help us understand if the processes implicated by genetic studies are leading to expected alterations in the brains of individuals with ASD.

The Autism Brain: Neuropathology and Imaging Studies:

The behavioral symptoms associated with ASD are suggestive of changes in the function and perhaps structure of the brain. Repetitive behavior, for example, could be due to alterations in motor systems such as the striatum and pre-motor cortex. Post-mortem studies are incredibly important for taking an in depth microscopic and macroscopic look at the brain [122]. However, unlike disorders such as schizophrenia and Alzheimer's, the availability of post-mortem tissue has been severely limited for ASD. In fact, from 1980 to 2010 only about 100 post-mortem ASD brains were analyzed [123]. The first post-mortem studies of ASD was published in 1980s by Williams et al and was followed by seminal work conducted by Bauman and Kemper, and Bailey et al [124-127]. These studies found abnormalities such as reduced neuronal soma size and increased cell packing density in many regions including the hippocampus, entorhinal cortex, amygdala, and anterior cingulate gyrus. Studies by Bauman and Kemper and Williams et al did not find any alterations in the cerebral cortex while studies by Bailey et al observed increased cell

number and size of the cerebral cortex and regional disorganization in ASD individuals. The most consistent finding across all these initial studies were reductions in Purkinje neurons in the cerebellum. However, the power and generalizability of these early post-mortem studies were limited due to the extremely small sample sizes that ranged between 1 to 6 brains. Moreover, nearly all the patients from these early studies had intellectual impairments, seizure disorders, or other co-morbid conditions. Thus, it was unclear whether the pathological findings were due to ASD or due the comorbid condition.

Newer post-mortem studies often have larger sample sizes (7-20 patients) and include patients that do not have seizures or intellectual impairments. Over the years, many studies have observed alterations in the brains of individuals with ASD. The most consistent differences have been observed in the cerebellum, where almost 60% of the brains studied to date have shown some sort of pathology such as reduced cell numbers, altered cell size, or altered dendrites [123, 128-133]. Changes in regions of the brain such as the amygdala, hippocampus, striatum, and brainstem have also been observed in ASD. However, alterations in these regions are not always replicated or consistent across groups [134]. Some groups have also found altered cell numbers, presence of disorganized patches and changes in cell packing density in the cerebral cortex of individuals with ASD. about half of ASD brains analyzed to date do not have cortical alterations [130, 134, 135]. Much like the early post-mortem studies, the newer studies, even with larger samples sizes have not been able to detect one common unifying pathological feature that contributes to ASD.. All these studies are showing that the defects seen in ASD may be caused by alterations in basic developmental processes. For example, the changes in brain size and cell numbers observed in ASD can be caused by changes/abnormalities/alterations in cell proliferation,

cell death, or synapse formation. In the brain, the proliferation of progenitor cells that make neurons, the appropriate death of these cells, and the bulk of early synapse formation occurs embryonically or perinatally. Thus, an abnormality in brain size is suggestive of an alteration that occurred in one of the basic processes that builds the brain. Indeed, a study by Wegiel et al 2010 found subcortical, periventricular, hippocampal, and cerebellar heterotopias in 4 out of 13 brains studied, suggesting abnormal neuronal migration. Likewise, cerebral dysplasias were found in the neocortex of 4 brains while cerebellar dysplasias were observed in 12 out of 13 of the ASD brains studied which reflects multi-regional dysregulation of neurogenesis, neuronal migration, and maturation in ASD [136].

While post-mortem studies have been invaluable for showing neurobiological substrates of autism, the dearth of post-mortem tissue has limited analysis of autism neuropathology. In addition, post-mortem studies are hampered by factors such as the presence of co-morbid conditions, alterations in brain structure caused by medications, lack of rigor/stereological analyses, and influences of cause-of-death or preservation on tissue integrity. Moreover, post-mortem studies are showing us the "end-point" of a disorder. Thus, for example, findings such as gliosis or elevations in neuroimmune molecules could be a consequence of having ASD rather than causal to ASD. Finally, samples obtained are usually from older children and adults and therefore the developmental progression ASD cannot be studied. On the other hand, imaging techniques give us insight into the live brain and do not involve invasive techniques. Imaging also allows us to collect functional data on the brain and monitor and compare developmental progression of disorders. Newest advances in imaging have allowed for the imaging of brains of neonates and even fetal brains which is an incredibly powerful mechanism to study developmental disorders.

Structural data on ASD brains has largely been derived from MRI. Again, much like the post-mortem studies, there seems to be no consistent defect that has been uncovered in all cases of ASD. Rather, some common patterns are observed in ASD brains, though none are pathognomonic. One commonly reported finding is increased brain volume from ages 2-4 that seems to occur in both grey and white matter [137-140]. This increased brain volume has been reflected in clinical data that suggests individuals with ASD often have larger heads (proxy for brain size) in early childhood [132, 141, 142]. Newer studies however, have found that larger head size may be overestimated in children with due to the use of flawed head size charts [143]. Moreover, larger head sizes can also be found in unaffected family members of children with ASD, suggesting that larger heads are not necessarily tied to ASD pathology [144, 145]. However, current data suggests that about 20% of individuals with ASD are macrocephalic, meaning their head sizes are greater than two standard deviations above the mean [146]. Yet, about 10-15% of individuals with ASD also have the opposite phenotype of microcephaly [141]. Another commonly reported finding is decreased volume in the corpus callosum suggesting reduced hemisphere to hemisphere connections in ASD [147-150]. Indeed, diffusion tensor imaging studies which give insight into myelinated axon bundles in the brain have also reported changes in the corpus callosum [148, 150-152]. However, it is unclear how common corpus callosum defects are in ASD patients. Studies in individuals with agenesis of the corpus callosum, a disorder where the corpus callosum fails to form or is severely reduced, shows about 1/3 of individuals with this disorder have ASD [153, 154]. This suggests that pathology of white matter tracts is associated with autism. While some studies have observed changes such as decreased volumes in cerebellar vermis or alterations in volume of the amygdala, these findings often were not confirmed by other

groups. Moreover, reports of altered cortical thickness, volume, and surface area have also been inconsistent [147]. In addition to ASD heterogeneity, inconsistencies between imaging studies can be due to differences in sample population demographics, image acquisition settings, data algorithms used and even the machines utilized the process the data [150, 155-157]. Indeed, one study applied 3 commonly used analysis methods to the same brain size data acquired from individuals with ASD and typical individuals. Strikingly, whether significant differences in brain size were found depended upon which analysis method was utilized [158]. This highlights the need for rigorous methodology applied across imaging studies. In addition to issues with methodology, the MRI studies reviewed above were largely conducted in children older than 4 or in adolescents and adults. It is important to note that brain volumes can expand due to behavior, for example, musicians often have enlargements in certain areas of the auditory cortex. Thus, in these studies it is sometimes unclear if brain regions are enlarged due to "practice" of certain behaviors or whether the enlargement is contributing to these behaviors. Moreover, these imaging studies cannot tell us why there are changes in brain size or axon bundles in the ASD brain.

In addition to volumetric and structural studies, fMRI studies have provided insight into the activity of brain regions and brain circuits in ASD [147, 159, 160]. This allows us to understand if structures commonly altered in ASD also show differential function which could lead to the altered behavior seen in ASD. Much like structural studies of ASD brains, there is considerable heterogeneity in results across fMRI studies. Again, there are some common themes that have emerged. Generally, fMRI studies have observed alterations in the activity of regions of the brain associated with the core deficits of ASD including social, language, and motor areas. For example, during social processing, studies have found

hypoactivation in the amygdala and prefrontal cortex of individuals with ASD [161, 162]. Hypoactivity has also commonly been noted in the fusiform gyrus, a facial processing area, when autistic individuals look at pictures of faces or facial expression. In addition to alterations in structures associated with ASD core symptoms, some studies have noted that there are changes in connectivity in the ASD brain. Connectivity reflects how functionally coupled two brain regions are during a task and thus alterations in connectivity could reflect changes in synapses or axons and dendrites between regions. Many studies have found reduced functional connectivity between frontal and parietal and frontal and temporal regions [163-165]. However, connectivity studies have also sometimes reported increased connectivity between cortical regions but decreased connectivity from cortical to subcortical regions. Overall, functional studies have shown that brain regions that are typically associated with ASD "function" differently in autistic individuals in comparison with typical individuals. However, fMRI is measuring blood flow as a proxy for brain activity which may not necessarily reflect neuronal activity. Yet, fMRI studies suggest the function or connectivity of brain regions are altered in ASD.

Recently, studies have begun focusing on children of younger ages and conducting longitudinal trajectory studies. Such studies are incredibly valuable as they allow us to compare brain development in typical and ASD individuals. Thus, these studies can give us clues as to what developmental processes may be going awry to lead to disease. Furthermore, results from these studies can help us uncover biomarkers which would aid in early diagnosis of the disorder. As ASD is usually not diagnosed until age 2, it is often difficult to study children younger than this age. Thus, recent studies have focused on prospectively following children who are at high risk for developing autism such as baby

siblings of kids with ASD. One of the earliest MRI studies in baby sibs was conducted by Shen et al (2013) where MRI scans were taken at three-time points: 6-9 months, 12-15 months, and 18-24 months of age. 55 infants were recruited, 33 were high risk and 22 were low risk and intensive behavioral assessments were also conducted at 24 months to determine if infants had ASD, other developmental delays, or were typically developing [166]. More recently, this same study paradigm was expanded to 343 infants (222 high risk, 122 low risk) by the same group in conjunction with a large consortium of universities. Much like the initial smaller studies, they found that infants who developed ASD had significantly greater extra-axial CSF volumes at 6 months compared to children who were typically developing or who had other developmental delays [167]. By the end of the 2017 study, 47 children were diagnosed with ASD and the extra-axial CSF volume predicted this outcome with an accuracy of 69% and sensitivity and specificity of 66% and 68% respectively. The authors postulated that increased extra-axial CSF was due to disruption in CSF flow. Studies suggest that reduced CSF flow would alter the transportation of growth factors and inflammatory molecules [168-170]. These molecules have important effects on regulating developmental processes and thus alteration of their clearance could alter development leading to ASD. Another study utilized MRI to look at brain volumes in 106 infants with high ASD risk in comparison to 42 low risk infants [26]. The study found that hyperexpansion of cortical surface area between 6 and 12 months of age preceded the brain volume overgrowth that was seen between 12 and 24 months in 15 infants diagnosed with ASD at 24 months. The brain volume was linked to severity of social defects in these children. The study had an 81% positive predictive value and a sensitivity of 88%. Thus, understanding the mechanism by which cortical surface area expansion occurs can

potentially provide insights into autism pathogenesis. Other studies conducted in high risk infants have observed increased thickness in corpus callosum and altered functional connectivity (fMRI) in children who were later diagnosed with ASD [27, 171]. Interestingly, the functional studies by Emerson et al had a specificity of 100% and a sensitivity of 81%, indicating functional networks are a potentially valuable tool for early diagnosis [27]. In sum, these studies in very young high-risk infants show that the development of the brain is altered in children who are later diagnosed with ASD. Now, some researchers are even beginning to use fetal MRI to understand the development of ASD even before birth. These studies, are currently unpublished and will provide fascinating insight into brain development and could help better elucidate what mechanisms are contributing to ASD. One caveat, however, is all these studies have currently been conducted exclusively in children who have a high risk of developing ASD. Thus, it is unclear whether these observations are specific to high risk populations or generalizable to ASD as whole. Moreover, it is also unclear how specific these studies are to ASD. For example, it is unknown whether cortical hyper-expansion or altered functional connectivity are also seen in schizophrenia or in individuals with intellectual impairments.

In summary, a look at the brain of individuals with ASD through both post-mortem and imaging studies has shown us that, in general, individuals with ASD have alterations in brain structure and function. However, there is no single defining pathological or functional change that is seen in ASD brain. Yet, the changes seen in the ASD brain suggest aberrant neurodevelopment. Neither imaging nor post-mortem studies are able to show us the mechanism(s) by which structural and functional alterations are occurring in the ASD brain. For the past few decades, studies to uncover the underpinning neurodevelopmental

mechanisms have largely been conducted in rodent models. Through these model systems, we can observe the consequences of altering known genetic and environmental factors on brain structure, brain function, and behavior. In the following sections, I will review these rodent studies to garner more insight into the neurodevelopmental aberrations that may lead to autism. First, however, it is important to deviate for a moment to discuss some other neurodevelopmental disorders and their similarities to ASD

Other Neurodevelopmental Disorders (NDDs) and ASD:

There are a vast variety of NDDs that affect the structure and function of the brain and lead to abnormal behavior. Some of these disorders such as Tuberous Sclerosis, Rett, Prader-Willi, Angelmann, Fragile-X and Down Syndrome have established genetic causes. Yet, even in these cases, there is often vast heterogeneity in the clinical presentation and severity of these disorders. Like ASDs, other NDDs such as intellectual impairments, ADHD, bipolar disorder and schizophrenia are largely idiopathic. A plethora of studies have found that many NDDs share common clinical characteristics [172]. For example, alterations in social behavior are found commonly amongst many NDDs including autism, Prader-Willi, schizophrenia, and Down Syndrome. Moreover, intellectual impairments and ADHD co-occur with numerous neurodevelopmental disorders such as Down Syndrome and autism [173-175]. Thus, often the clinical "distinctness" of some of these disorders is blurry and questionable [176-181]. In addition to clinical similarities, NDDs also share common genetic and environmental etiological factors. For example, perinatal immune activation has been implicated in the pathology of autism, schizophrenia and bipolar disorders [182-184] . Similarly, newer studies are finding that many genetic factors associated with ASD, such as genes involved in synapse formation, mitochondrial function, signal

transduction and astrocyte differentiation, are shared by other disorders including schizophrenia and bipolar disorder [172, 185]. In addition to genetic similarities, imaging and post-mortem studies also show brain pathology that is similar in numerous NDDs. For example, altered cerebellum and insula are found in Down Syndrome, autism and schizophrenia [186-189]. Thus, since many NDDs seem to have shared genetic risk factors, similar brain pathological and functional features, it is important to contrast and compare these disorders. Furthermore, results unearthed from studying a model of ASD could be applicable to the study of schizophrenia and vice versa. Such similarities between NDDs also necessitates that we develop better molecular understanding of these disorders. Thus, categorization and treatment of these disorders can be more objective. In the following sections, I will review rodent studies regarding aberrant neurodevelopment and ASD with occasional review of other NDDs that have commonalities with ASD.

Rodent Models of Autism and other NDDs:

For the last few decades, rodent models have been invaluable to help understand both normal neurodevelopment and developmental disorders [190]. Unlike human genetic, imaging, and post-mortem studies, rodent models allow us to conduct mechanistic causal studies on disorders such as ASD. As mammals, rodents share many commonalities with humans including genes, biological processes, brain circuitries, and to some extent, behaviors. Thus, study of rodent circuitry gives some insight into human neurobiology and neuropathology. With rodent models, we can carefully alter genes and environmental conditions and then observe the effects of these alteration on brain development, brain structure, and behavior. The effects of experimental manipulation can be studied both in vitro and in vivo in the organism. In vitro culture studies allow researchers to take a

reductionist approach and study the effects of a single variable or learn more about cellular biology. On the other hand, in vivo models allow us to understand a phenotype in the context of a living organism. With mice and rats, cells can be harvested from the brain and cultured (known as primary neural cultures) to study a specific phenotype and then these results can be confirmed (or not) in an in vivo system providing important perspectives on biology. For the last few decades, rodent models were also the primary means to study embryonic development. Access to fetal brains and the ability to dissect and study these brains across multiple time points in development have provided invaluable knowledge. For disorders like ASD, embryonic and perinatal developmental aberrations are thought to play key roles in disease development. Thus, mouse models are an important system to study how neurodevelopmental aberrations can lead to disease.

There are a multitude of mouse models of ASD. In general, effective models of disease have face validity, meaning they have visible symptoms that are associated with the disorder, construct validity, meaning the animals should show similar biological dysfunction to humans with the disease (genetic or neuropathological), and predictive validity meaning that treatments or manipulations that are effective in the mouse should also be useful for humans [191-193]. A majority of autism models are made by knocking out or overexpressing genes associated with syndromic forms of ASD such as Fragile-X Syndrome (FMR1), Cowden's Syndrome (PTEN), and Rett Syndrome (MECP2). These models have shown many features related to ASD such as changes in behaviors, brain regional sizes, neural circuits, and synaptic structures and functions [191, 194-196]. These genes are usually highly penetrant and thus produce animal models that have face validity and construct validity. Moreover, these genes often cause large changes in the brain that are

easier to visualize and study than would be changes caused by subtle genetic alterations like common genetic variants or SNPs. While alteration of these syndromic genes provides valuable insight into how the brain can be altered in ASD, syndromic genes are not commonly associated with a majority of cases of ASD that are idiopathic. More recently, genetic models of ASD have also focused on non-syndromic rare variants in genes such as neurexins, neuroligins, and CNTNAP2. Again, these genes are highly penetrant, but these alterations are very rarely found in the population and often run in families.

As reviewed, 50% of ASD risk is attributed to common variant genes. There are mouse models with alterations in genes that are common variants for ASD risk, such as the Engrailed-2 model studied in our lab [197, 198]. While these models have been important for illustrating the function of genes like EN-2 in brain development, the models do not necessarily reflect the minor changes that are occurring in humans with these disorders. For example, the A/C haplotype of EN-2 is associated with increased risk of ASD in humans and leads to higher promoter activity of the EN-2 gene [199]. However, the mouse model used in our lab has a complete knockout for the EN-2 gene - a more exaggerated/drastic alteration than what is seen in the humans. Yet, this more drastic genetic change is often needed to observe altered behavior and brain pathology that can be reliably studied. ASD can also be due to polygenic interactions and human genetic background can determine the penetrance of certain ASD associated genes. However, these subtle genetic alterations and the effects of human genetic background cannot be studied in mice. Indeed, it is nearly impossible to make a mouse model of idiopathic ASD which represents the overwhelming number of ASD cases. However, there is one mouse model known as the BTBR mouse that is considered a model of idiopathic ASD. The BTBR

mouse was not made by genetic alteration of ASD risk genes, rather, BTBR mice are highly inbred and exhibit many of the symptoms associated with ASD [200, 201]. Lastly, another mechanism to model ASD is through environmental exposure. These models include embryonic exposure to teratogens like thalidomide and valproic acid (VPA) or maternal exposure to infection leading to an immune response (maternal immune activation). Yet, the connection between ASD and these environmental exposures is not as well established as the genetic causes of ASD. Moreover, some exposure models only pertain to a small subsection of the population (such as children with mothers who took VPA). Thus, while mouse models of ASD have been useful for understanding the consequences of genetic alterations and environmental exposure on brain structure and behavior, it is important to keep in mind that these models are not really representing the majority of ASD cases.

Interestingly, while genetic and post-mortem studies have indicated that embryonic and fetal development are incredibly important in ASD pathogenesis, an overwhelming number of ASD studies have focused on developmental processes that largely occur in the post-natal and adult brain such as synapse formation. The surprising paucity of embryonic neurodevelopmental studies unfortunately limits the amount of supporting evidence I can provide for some of the sections discussed. Yet, I will begin by briefly reviewing brain development and then discuss embryonic developmental processes that may go awry in ASD. Much of the information presented will be derived from the rodent models discussed above, however, I will draw parallels to studies in humans too.

Brief Overview of Embryonic Neurodevelopment

A developing embryo starts out as a fertilized egg which divides to form a mass of pluripotent cells. These pluripotent cells ultimately divide and differentiate to form 3

"germ" layers known as the endoderm, mesoderm, and ectoderm [202]. The ectoderm, which is the outer most layer of the embryo, gives rise to the entire nervous system of a vertebrate organism. During the process of neurulation, the ectoderm thickens to form the neural plate. The neural plate then invaginates and forms the neural tube which ultimately develops into the brain (rostral) and the spinal cord (caudal). Likewise, the cavity of the neural tube goes on to form the fluid filled ventricles of the brain. A portion of the neural plate that is not incorporated into the neural tube, becomes neural crest cells that migrate across the developing embryo to ultimately form the PNS and a multitude of other cells including melanocytes. The rostral portion of the neural tube then rapidly expands to form three primary brain vesicles or pouches. These pouches ultimately expand and segment to form the forebrain, hindbrain, and midbrain and all the structures within these brain regions. The formation of the neural tube occurs approximately mid-gestation in rodents (E10.5-11 in rats and E 9-9.5 mice) and during early gestation in humans (3-4 weeks) [203-205]. In addition to these gross patterning events, there are very intricately orchestrated cellular and molecular changes occurring in the brain during development [206].

Ectoderm thickening and neural tube formation involve a tightly orchestrated series of events regulated by extracellular factors and appropriate expression of genes in ectodermal cells [202]. For example, the ectodermal layer which gives rise to the nervous system also forms the epidermal cells of skin. Thus, early ectodermal cells either can become neural or epidermal cells. What cells they become is largely determined by inductive extracellular signaling factors that either promote differentiation into one cell type, repressive factors that prevent differentiation into a cell type, and the ability of the cell to respond to these signals. A cell's ability to respond to extracellular factors, or EFs,

is determined by the receptors on the cells, the type of signaling pathways/signaling molecules active in the cell, and the transcription factors expressed by the cell. Studies in the 1980s and 90s uncovered that early ectodermal cells released bone morphogenetic proteins (BMPs) which repress the ability of ectodermal cells to become neural and promoted epidermal fate [207]. Thus, the presence or absence of BMP is an essential signal for cell differentiation and cell fate. Likewise, other extracellular factors such as WNT and FGF are essential molecules that aid in the patterning of the brain and allow for the generation of vast diverse neuronal subtypes [208, 209]. These factors act through signaling transduction pathways to ultimately turn on or off genes that will allow the cell to adopt a new fate or conduct a behavior (like migrate).

From this summary of early brain development, we see that cells of the early embryo are undergoing processes such as proliferation, migration, and differentiation to form the nervous system. Moreover, these processes are tightly regulated by extracellular factors, signal transduction systems, and gene transcription. Alterations in these cellular processes, due to aberrant action of EFs, signaling transduction systems, or genes could easily lead to abnormal brain structure- or if early enough can be lethal to the embryo. Indeed, mouse model studies have shown that neural tube defects such as exencephaly (brain develops outside skull) or anencephaly (lack of brain formation) can arise from alterations in genes that regulate signaling pathways like WNT. Likewise, the failure to create normal bilateral cerebral ventricles, or holoprosencephaly, can result from disturbed signaling by neuropeptides like pituitary adenylate cyclase activity polypeptide (PACAP) or sonichedgehog [210]. Interestingly, in these models, alterations in basic developmental processes like proliferation, migration, and cell death are observed [211, 212].

As indicated by human post-mortem studies, changes in architecture are seen in multiple regions of the ASD brain including the cortex, hippocampus, and cerebellum. The cerebral cortex has a central role in cognitive and emotional processing and is associated with regulating many behaviors that are abnormal in neuropsychiatric and neurodevelopmental disorders. Thus, understanding development and its regulation in the cortex will help better elucidate the basis of neuropsychiatric and neurodevelopmental disorders. In the following section, I will review cortical development with a specific focus on neurite outgrowth/differentiation and migration which are central to my studies.

Cortical Neurodevelopment:

The cerebral cortex is composed of an olfactory "paleocortex," the hippocampal "archicortex," and the neocortex. In human beings, the neocortex comprises 90% of the cortical surface and is the region of the brain that has undergone the largest evolutionary expansion when compared to rodents and even other apes [213]. The neocortex is characterized by a complex 6-layered architecture containing projection neurons and interneurons [214]. In the adult brain, layers of neurons can be distinguished by specific molecular markers and distinct axonal projections. The deep layers of the cortex (V, VI) have axons that project subcortically while the superficial layers (IV to II) are composed of intracortical neurons that project within the hemisphere or to the contralateral cortical hemisphere. As the neural tube is closing during development, the layer of neuroepithelial cells (NECs) that is closest to the hollow part of the tube (which ultimately becomes the ventricles) forms a proliferative zone known as the ventricular zone (VZ). In the anterior most region (which becomes the cortex), these NECs divide symmetrically to form more NECs. This symmetrical division of NECs allows for production of an adequate pool of

progenitor cells that will ultimately form the billions of neurons and glia that comprise the brain [215, 216]. Studies have shown that limiting division of these NECs greatly reduces the size and thickness of the cortex [217]. Around E10.5-E11.5 in the mouse, the NECs differentiate into another progenitor cell type known as apical radial glial cells [218]. Radial glial cells are distinguished by the expression of the transcription factor PAX-6. Unlike NECs, radial glia divide asymmetrically to produce a radial glia and an immature neuron or a radial glia and an intermediate progenitor cell (IPC). The newly produced IPCs move into the subventricular zone while the earliest neurons migrate further to form the 6 layers in an inside-out manner. That is, deeper layers of the cortex form first and then newly formed neurons migrate through the already established layers to form the superficial layers of the cortex. While migrating, immature neurons extend and retract processes known as neurites which ultimately become axons and dendrites. These axons and dendrites allow for neurons to connect and communicate cortically and subcortically. Once the neurons have reached their destination, they undergo further morphological and molecular differentiation to make the neuronal subtypes specific to their layer. The neurons then continue to mature, form synapses, and become electrically active to ultimately produce the pyramidal excitatory neurons of the neocortex. While early formation of neurons occurs in utero, many steps of cortical development such as the production of glia and synapse formation and pruning occur postnatally. Interneuron production occurs in a different region of the forebrain; however, these cells also migrate to reach the cortex.

The literature reviewed above pertains to the rodent neocortex. While rodent and human neurodevelopment are similar, human neurodevelopment is more complicated and lengthy [219, 220]. For example, in addition to the VZ/SVZ, humans and other great apes have

an additional neurogenic layer in the cortex known as the outer subventricular zone (OSVZ) which contains another distinct set of radial glial cells. This OVZ is thought to be responsible for the expanded cortical surface area in primates [221-223]. In addition, there are differences in timing between rodent and human development as seen in Figure 1 [224]. For example, migration of cortical neurons in the rat occurs between E19 and E22. However, in humans, neuronal migration begins at 18 weeks post conception and can continue up till week 36 (though a most cortical neurons are in place by 24 weeks). Moreover some processes like myelination continue to occur well past the age of 20 in humans whereas myelination is complete by P20 in rats. Thus, while the study of rodent neurodevelopment and its alterations inform us of the basic processes involved in building the brain, these processes, their regulation, and their timing are not analogous to human development.
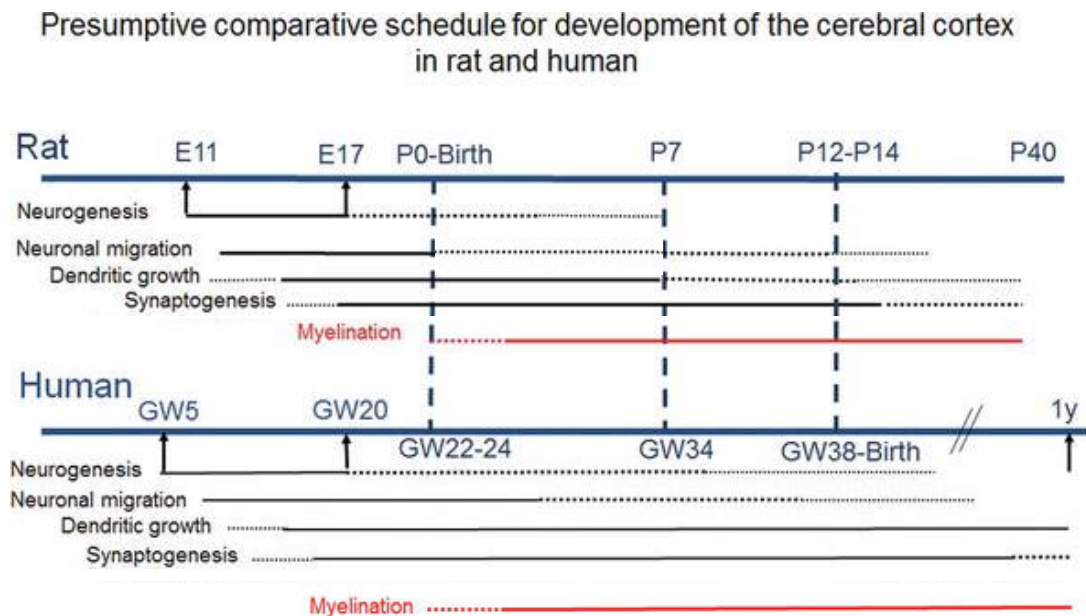
Figure 1: Time windows of important developmental processes in human and rat [224].

Cell Migration and Neurite Outgrowth in the Cerebral Cortex:

The proper layered structure of the cortex is dependent upon migration of immature neurons from the ventricular and subventricular zones to the appropriate layers. Moreover,

the elaboration and retraction of early neurites is occurring while neurons are migrating. Studies have found that there are multiple modes of migration and multiple morphologies of neurons in the developing cortex [225-228]. One mode of migration, known as somal translocation occurs during early corticogenesis to form the deeper layers of the cortex. During somal translocation, neurons extend a leading process (neurite) into a region of the developing cortex known as the marginal zone above the VZ/SVZ. Shortening and lengthening of the leading processes allows the soma of the new neurons to move into the deep layers of the cortex. Later, as the neocortex becomes thicker, neurons migrate by locomotion. In this process, as newborn neurons start to move out of the VZ, they begin to extend and retract multiple processes dynamically while their soma "wander" around. These multipolar cells then become bipolar and use fibers of radial glia to migrate past the deep layer cells to form the superficial layer. While migration is occurring, cells also determine their neurite polarization- that is specification of the axon and dendrite occur. Interestingly, we see that alterations in neurite outgrowth are occurring while cells are migrating showing the intimate connection between migration and differentiation. Indeed, mutations that alter migration often change the morphology or neurite complexity of neurons. For example, alterations in the cytoskeletal regulator, doublecortin (DCX) leads to improper formation of cortical layers and the accumulation of multipolar cells in the cortex of rats. Likewise, overexpression of DCX leads to an overproduction of cells with bipolar morphology along with altered lamination of the cortex [229, 230]. Thus, early neurite outgrowth and migration are closely coupled processes that are both important for the normal morphology and placement of neurons in the cortex.

Both migration and neurite outgrowth are regulated by numerous extracellular

factors, upregulation and downregulation of adhesion molecules like cadherins, and by regulation of the actin and tubulin cytoskeleton [231]. For example, the EF reelin is a large secreted extracellular matrix glycoprotein that is thought to regulate neuronal migration [232-234]. Reeler mice, which have mutations in the reelin gene, show multiple defects such as inverted cortical laminar structure, abnormal orientation of cell bodies, and changes in axonal and dendrite projection. In these mice, the "inside-out" formation of the cortex does not occur because newborn neurons fail to migrate past older neurons and therefore stay in the deeper layers of the cortex. In addition to changes in laminar structure, alterations in reelin changes polarization of migrating neurons which ultimately leads to dendritic abnormalities in the brain [234, 235]. Similar abnormalities are found in numerous other mouse models including those with mutations in T-box genes, APOE2, and Dab1. Some of these molecules such as Dab1 and APOE2 are downstream mediators of reelin signaling to the nucleus [236-238]. Thus, we see that alterations in the morphological changes and specification of early neurites can alter dendrites and axons in mature neurons. We also see that both migration and neurite outgrowth are often regulated in conjunction and alterations in either process can alter cortical cytoarchitecture.

There are some very severe neurodevelopmental disorders that are caused by mutations of genes that regulate neurites and migration during early development [239, 240]. One such disorder, lissencephaly, leads to a brain that is "smooth" and devoid of sulci and gyri. It is caused by a mutation in the tubulin associated protein LIS1 which is thought to regulate dynein [241, 242]. RNAi experiments targeting LIS1 prevents or slows down the migration of neurons from the VZ and SVZ leading to accumulation of neurons in these compartments [243]. Knockdown of LIS1 also led to the accumulation of multipolar neurons

[244]. As early neurite outgrowth and migration are integral to the structure and function of the brain, it is possible that these alterations are found in disorders like ASD. However, post-mortem analyses of ASD brains hardly show the striking changes in cortical cytoarchitecture that are seen in disorders like lissencephaly. Yet, there is evidence of more minor disruptions in migration and neurite outgrowth in the ASD brain such as altered cortical mini-columns, the presence of ectopic neuronal patches, and mis-oriented neuronal somas [122, 132, 134, 136, 245]. Moreover, genetic studies have shown that many autism risk genes code for molecules involved in the cytoskeleton which as shown is incredibly important for regulating neurites and migration. Thus, it is important to study neurite outgrowth and migration phenotypes in rodent models of ASD.

Alteration in Migration and Neurite Outgrowth in ASD:

Developmental studies of ASD models have largely focused on processes such as synapse formation and neuronal activity in the postnatal brain. The limited studies that have focused on embryonic brain development have emphasized dysregulation of proliferation during early neurogenesis. Focus on proliferation had largely stemmed from pathological studies in humans showing that altered brain size is commonly seen in ASD. These same pathological studies have shown alterations in neuronal architecture in the ASD brain. Yet, study of cell migration and early neurite outgrowth in the embryonic cortex are very limited. However, there are multiple models of ASD that show defects in both developmental processes [231]. For example, TBR1 is a transcription factor that is expressed after cortical progenitors begin to differentiate and is highly expressed in early born neurons of the deep layers of the cortex [231, 246, 247]. De novo mutations have in TBR1 have been found across some individuals with ASD. Moreover, mouse models with either

deletions or alterations in TBR1 often show behavioral symptoms reminiscent of ASD and disorders likes schizophrenia. Even though TBR1 is thought to be expressed after the developmental process of migration has occurred, mice that are knockouts for TBR1 displayed vast change in cortical laminar structure [248, 249]. Moreover, the alterations were varied across different parts of the cortex (instead of uniform alterations like reeler mice). A study by Hevener et al 2001, used BrdU pulses at E105, 11.5, 13.5 to track the survival and location of neurons across multiple embryonic and post-natal time points in wildtype (WT) and TBR1 knockout mice. In mutant mice, early born neurons largely occupied superficial positions when injected at E10.5 and analyzed at E18.5 [237]. There were also clusters of ectopic cells born at different times accumulating in the caudal cortex. Thus, by tracing neurons across time, Hevener et al were able to show abnormal migration in this mouse model. Likewise, TBR1 KO mice had alterations in embryonic axon growth and targeting. In another ASD model with a mutation in Ankrd11, there was mislocalization of cortical neurons with excess cells accumulating in the VZ and SVZ during embryonic development and alterations in the positioning of upper and lower layer neurons in the post-natal brain of mice [250]. Defects in cortical lamination which are suggestive of aberrant migration have also been seen in other mouse models including those with altered PTEN, CNTNAP2, bcl11a and Baf170 [249, 251-255]. Humans with defects in PTEN and CNTNAP2, for example, have shown altered cortical lamination and symptoms such as seizures, autism, and hyperactivity, which are paralleled in the mice [256-259]. Yet, while these studies have found alterations in the brain of mice that parallel human defects, very few of these studies have actually looked into the mechanism by which these defects are occurring in the mouse brain. For example, CNTNAP2 mouse have often shown alterations in cortical

lamination [260]. While many studies have postulated that migration defects are central to this alteration almost none of the studies have actually traced the migration of these cells or looked at the movement of early cortical cells to see if this is the case. Thus, while these mouse models give us the ability to probe mechanism, many studies are not performing an in-depth analysis as to why brain alteration are present in ASD models.

Mouse mutants of many genes that show migration defects also exhibit alterations in early neurite outgrowth, specifically the early transit of neurons from a multipolar to bipolar morphology. This include genes such as Bcl11a, Auts2, Foxg1, Foxp1, LDS1, and FMR1, as well as members of the WNT pathway [249, 251-253, 261-265]. In particular, AUTS2 is associated with ASD and numerous other disorders such as intellectual disability, ADHD, schizophrenia epilepsy, and developmental delay [266]. This indicates that AUTS2 has important roles in development and its alteration can change brain and behavior. A study by Hori et al 2014 used shRNA (with GFP) techniques to knockdown AUTS2 levels embryonically at E15.5 via electroporation and studied the cortical cells in vitro at E16.5 [267]. In these cultures, immature neurons (TUJ1+) showed reduced number, length and branching of axons and dendrites. In cortices that were electroporated with Aut2s shRNA and GFP at E14.5, cells with GFP expression failed to migrate to the appropriate layers and ectopic cells were found across the cortex. During early migration to the cortex, the shRNA-expressing neurons also displayed twisted irregular leading processes with abnormal branching. In aggregate, these observations indicate that early neurite outgrowth and migration defects often are commonly dysregulated.

In humans with ASD, post-mortem studies showed abnormalities in the cortex, hippocampus, and cerebellum which were suggestive of alterations in neurodevelopment.

Likewise, mouse models of ASD, often have parallel changes in brain structure that mirror those of the human. Using these mouse models, we find that the altered brain structures we see are caused by alterations in developmental processes. In particular, we find that alterations in migration and early neurite outgrowth are commonly associated with ASD and other neurodevelopmental disorders. Of course, as mentioned, these developmental processes are heavily regulated by extracellular factors and by signaling pathways. In the following section, I will review the roles of some important EFs that have been the focus of my study and their relation to neuropsychiatric disorders. I will also review the regulation of development by signaling pathways and the roles of these pathway in ASD and other neurodevelopmental disorders

Extracellular Factors in Brain Development and NDDs

As reviewed above, brain development in general is characterized by a well-orchestrated set of processes including proliferation, migration, and differentiation/ maturation. These processes are highly regulated by extracellular factors (EFs) which are growth factors, neuropeptides, or neurotransmitters which influence cells through activating signaling pathways and ultimately changing gene expression. Indeed, some EFs that are integral for neurodevelopment were briefly discussed above including WNT, BMPs, Reelin and FGF. In the following sections, I will review some EFs that have been the focus of my studies and that are integral players in influencing neurodevelopmental processes or are associated with ASD pathology. Of course, as my work has largely focused on migration and differentiation, my review of EFs will largely relate to these developmental processes. Moreover, I will also review the putative role or importance of these EFs in regulating behavior or influencing the pathogenesis of ASD and other

neurodevelopmental disorders. It is important to note that in general, studies of EF regulation of development (particularly early embryonic development) are highly limited. Likewise, while there are studies hinting the roles of many of these EFs in behavior and neuropsychiatric disorders, these studies are also limited.

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP):

PACAP, coded by the ADCYAP gene, is a neuropeptide that is part of a superfamily of peptide hormones including vasoactive intestinal peptide (VIP), secretin, and glucagon. In the brain, PACAP is mostly present as a 38-amino acid peptide, which as suggested by the name, activates adenylate cyclase via the PKA pathway to make cAMP [268, 269]. PACAP can also signal through the PKC and ERK pathways. PACAP exerts its actions through binding to three receptors, VPAC1, VPAC2, and PAC1 which are all g-protein coupled. Both VPAC1 and VPAC2 bind to VIP and PACAP with high affinity, however PAC1 receptors are selective for PACAP binding. PACAP is known to have vast, pleiotropic function in many regions of the CNS and in the embryonic development of the nervous system. Studies in the 1990s found high expression of PACAP or PAC1 receptor mRNA in rat brains as early as E14 and in mouse brains by E 9.5 [270-273]. Further in situ studies found that PACAP mRNA was identified at E13 in the rat developing cortex, hippocampus, amygdala, cerebellum, spinal cord, and dorsal root ganglion [274]. Moreover, in developing systems, PAC1 is highly expressed on neural precursor cells in important developmental areas such as the SVZ and VZ [275]. This high expression of PACAP and PAC1 throughout embryonic brain development in multiple brain regions suggests that PACAP may have interesting roles in shaping brain development. PACAP is also found in the early post-natal brain further showing its important in brain development.

A review of developmental studies of PACAP show a wide range of processes that PACAP regulates. For example, PACAP is seen to have both pro- and anti-mitogenic effects in the developing cortex depending upon developmental stage [276-282]. Studies in our lab found that PACAP acts as a pro-mitotic signal during early neurodevelopment in mice (E 9.5) by acting on the PKC pathway [281]. However, from E13.5 onward both in vivo and in vitro, PACAP reduces proliferation through PKA signaling. In addition to halting proliferation, in later stages of embryonic development, PACAP also allows cortical precursor cells to exit the cell cycle and begin differentiation. A study by Lu and DiCicco-Bloom 1997 showed that PACAP exposure reduced [3H] thymidine incorporation by 43% and increase neurite outgrowth by 2-fold in cortical precursor cells after E13.5 [283]. This study along with a few others show that PACAP plays important roles in regulating differentiation and neurite outgrowth in the developing cortex and other regions like the hippocampus and cerebellum [198, 278, 284]. In addition to regulating proliferat-ion and differentiation, PACAP has also been shown to have important roles in regulating migration and cortical cytoarchitecture [285, 286]. Some studies have shown that PACAP halts migration in cerebellar granule cells in the early postnatal brain to allow for the precise laminar structure of the cerebellum [287-289]. More recent data has shown that PAC1 receptors are found in DCX positive neuroblasts migrating from the SVZ suggesting the influence of PACAP on migration in the cortex [290, 291]. A study by Ohtsuka et al 2008 found that PACAP administration into the telencephalon of E13.5 mice, altered the layer position of newly generated neurons in the cerebral cortex [292]. Thus, while there are only a few studies of PACAP on brain development these studies together suggest that PACAP has wide-spread function across both embryonic and post-natal development.

Given the numerous functions that PACAP regulates, we would expect that alterations in PACAP or it's receptors could lead to altered neurodevelopment and behavior. Indeed, clinical evidence has shown that gain-of-function in chromosomal regions that include PACAP lead to neurodevelopmental disorders such as Trisomy 18. Individuals with trisomy 18 often have microcephaly and spina bifida. Alterations in the 18p11.3 region (where PACAP is located) is also associated with holoprosencephaly a defect of forebrain development where the double lobes of the cerebrum fail to form [210, 293]. However, these larger chromosomal locations include a multitude of other genes in addition to PACAP which could be contributing to these neurodevelopmental disorders. A study by Lang et al 2006 created transgenic mice that overexpressed the human form of the PAC1 receptor [294]. These mice had larger ventricles, smaller cerebral cortex and corpus callosum. These altered brain structures were correlated with massive increases in neuronal apoptosis and significantly reduced proliferation in the developing cortex of the transgenic embryos. Thus, we see that alterations in PACAP lead to altered brain structure and therefore could contribute to the pathogenesis of developmental disorders.

In addition to being associated with altered brain structure, there are some genetic and animal behavior studies that associate PACAP with neuropsychiatric disorders and abnormal behavior. The 18p11 region, which contains the PACAP gene, is associated with increased susceptibility to schizophrenia and bipolar disorder [295-297]. Two independent groups also found that increased copy numbers of VPAC2 also increased schizophrenia risk [298, 299]. The rs1557299 SNP in the 18p11.32 region (19 kb downstream of PACAP gene) was found to be linked to repetitive restrictive behavior in children with ASD [300]. However, it is unclear whether polymorphism of this SNP alters PACAP gene expression.

Thus, we see that PACAP may play a role in the pathogenesis of neuropsychiatric disorders. However, most genetic studies are just correlational. A look at the few rodent model studies of PACAP modulation show that PACAP and PAC1 knockout mice show abnormalities in behavior such as alterations in social and locomotor behavior that were reminiscent of autistic and schizophrenic symptoms [301-304]. Thus, it is possible that alterations in PACAP alter the development of the brain thereby changing behavior.

In summary, we see that PACAP is a neuropeptide that has important functions regulating multiple developmental processes. We have seen that alterations in PACAP or it's receptor can change brain structure and animal behavior. However, despite the promising studies showing the effects of PACAP on brain development and behavior, studies on the effects of PACAP or the important of PACAP have been limited in ASD and other developmental disorders. Thus, it would be valuable and useful to utilize and study PACAP in the context of human neurodevelopment and neurodevelopmental disorders.

Nerve Growth Factor (NGF) & Other Neurotrophins:

Neurotrophins are a class of growth factors that are important regulators of neural survival, development, function, and plasticity both in the CNS and PNS [305]. The most well-known and studied neurotrophins in mammals include Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin (NT) 3 and 4. Neutrophins are generally synthesized as a large protein in a "pro" form which is then cleaved to make the mature neurotrophin (Pro-BDNF vs BDNF). All neurotrophins are thought to act through tyrosine kinase receptors to activate signaling transduction and ultimately transcription of genes to exert their influence. Specifically, NGF, BDNF and NT3 act on the trkA, trkB, and trkC receptors respectively. Neurotrophins also bind to a p75NTR receptor which is

particularly important during development and is thought to have the ability to bind the "pro" form of neurotorphins. NGF was the first neurotrophin to be isolated and characterized and its discovery played important roles in the understanding of cellular interactions in development [306-309]. Early experiments with NGF anti-serum in neonatal rodents showed that blocking NGF led to almost a total disappearance of sympathetic para- and pre-vertebral ganglia [309-314]. Moreover, cell cultures of sympathetic and sensory cells derived from chick embryos failed to survive and grow unless NGF was added to the culture medium [306-314]. Specifically, in the sympathetic nervous system NGF seems to be essential for the survival of catecholaminergic (norepinephrine) neurons and could even induce the upregulation of tyrosine hydroxylase, the enzyme that synthesizes catecholamines. In addition to promoting survival, these early studies showed that NGF allowed for the growth and guidance of neurites and axon bundles of sensory and sympathetic neurons. These experiments cemented NGF's vital role in the development, survival, and differentiation of sensory and sympathetic neurons [306-314]. Early studies in the CNS, however, showed that neither NGF nor its antiserum had any effects on brain norepinephrine or survival of cells in the brain of newborn animals, adult animals, and even in embryos exposed through maternal injection. Moreover, early binding studies failed to show NGF binding in the CNS which ultimately led to the erroneous conclusion that NGF was not involved in brain development, structure, or function. This, along with the discovery of BDNF, which has wide CNS distribution, led to limited study of NGFs role in the CNS and its development [306-314].

By 1971, autoradiography studies showed that NGF was present in the normal adult brain [315]. However, unlike catecholaminergic neurons in the PNS, brain

catecholaminergic neurons were not responsive to NGF. Yet, by the 1980s, multiple studies

began to show that the cholinergic neurons in the basal forebrain were responsive to NGF

[310, 314, 316, 317]. Moreover, NGF expression was also seen in the regions innervated by

cholinergic basal forebrain cells including the hippocampus and neocortex. NGF was also

shown to be able to increase levels of the enzyme that synthesized acetylcholine, choline-

acetyl transferase, ChAT, in vivo in the septum, hippocampus, and cortex of newborn rats

and in vitro in embryonic rat striatal cultures [318-322]. This suggested that NGF was indeed

found in the brain and that it was regulating cholinergic neurons in both the adult and

developing brain. In the embryonic rat brain, dramatic increases are seen in the expression

of BDNF, NT3 and NGF between E11 and E12 which coincides with the onset of

neurogenesis [323]. At this early time point, NT3 expression is the highest followed by NGF

and BDNF, which has lowest levels of expression. NGF receptor transcript began to appear

at E10 preceding the increase of NGF. Over time, NT3 expression decreases while BDNF

expression increases. NGF expression however remains fairly constant throughout

development. Thus, the presence of high levels of NGF and NT3 during early development

suggest that these neurotrophins (and not so much BDNF) may play roles in regulating

basic developmental processes such as proliferation, migration, and differentiation.

However, unlike the well-established knowledge of NGF's ability to regulate all these

processes in the PNS and in PC12 (adrenergic pheochromocytoma tumor cells), studies in

embryonic forebrain cells are very limited. A few studies have looked at the role of NGF

in forebrain primary cultures. One study found that NGF stimulated the proliferation of

E13.5-E14.5 primordial striatal cells that expressed Nestin after FGF priming [324].

Withdrawal of the NGF from these cultures also prompted differentiation of these cells

Another study, which looked at E17 hippocampal cultures, found that NGF controlled dendrite development [325]. NGF receptors are also present in both embryonic cortical and hippocampal cultures from the rat as early as E14 [326]. In vivo models of NGF's effects on the cortex are limited. More recent studies have found that infusion of NGF into the medial part of the developing neonatal frontal cortex of rats led to reduced behavioral flexibility, increased perseverance, and alterations in pre-pulse inhibition [327-329]. These behavioral changes are all phenotypic symptoms that are typically associated with ASD and schizophrenia and thus are potentially suggestive for the roles of NGF in disease pathology. However, as a caveat, these studies were using high doses of NGF that were able to bind to p75R and lead to cell death in the cortical subplate. Alterations of the subplate have commonly been seen in schizophrenia and thus these studies are mostly showing that high doses of NGF can elicit cell death in important CNS areas. Thus, developmentally it could be involved in pruning and apoptotic processes necessary to have a functioning brain. Thus, while NGF is abundant during development and has diverse effects on developing cells, further studies are needed to learn about its effects in the developing cortex and hippocampus and whether it can contribute to disease pathology.

As neurotrophins have important roles in regulating synaptic plasticity and brain function, many studies have sought to explore the role of these growth factors in neuropsychiatric disorders. Of the neurotrophins, BDNF has been studied most widely for its association with multiple neuropsychiatric disorders. In humans, BDNF and trkB receptors levels were found to be decreased in the hippocampus of post-mortem tissue of individuals with major depressive disorders (MDD) or suicide victims [305]. Likewise, alterations in SNPs in the BDNF gene, alterations in serum BDNF level, or alterations in

BDNF and trkB receptor levels have also been seen in bipolar disorder, anxiety disorders, PTSD, schizophrenia, and autism [330-332]. On the other hand, studies of NGF expression and levels in human neuropsychiatric disorders have been limited. This is partially due to the view that NGF's primary roles are in the PNS or sympathetic nervous system. However, a few studies have assessed blood and brain levels of NGF in blood and brain of individuals with ASD, schizophrenia and ADHD. In schizophrenia analysis of blood samples have indicated lower levels of serum NGF, though post-mortem analyses of brain tissue have been inconclusive [332-336]. In addition to serum levels, there have also been genetic studies that show that SNPs in the NGF and NGFR being associated with higher likelihood of schizophrenia [337]. Likewise, in ADHD, Guney et al (2013) found higher serum levels of NGF and another study associated the rs6330 SNP in the NGF gene with higher ADHD risk [338]. With ASD, studies of peripheral NGF levels have been inconsistent. A study by Nelson et al (2001) found no alteration of NGF in serum of children with ASD or intellectual impairment when compared to controls [339] Likewise, Riikonen et al found no change in NGF levels in children with non-syndromic ASD but did see alterations in girls with Rett syndrome [340]. A more recent study by Dincel et al (2013) found elevated NGF levels in Turkish children with ASD [341]. In general, these studies have smaller sample sizes (under 50) and none to date have looked at CSF or post-mortem levels of NGF. Moreover, no studies have associated ASD with genetic alterations in NGF or its receptor. Even in schizophrenia and ADHD, it is unclear whether NGF is contributing to the pathogenesis of the disorder. Specifically, the lack of rodent model studies in the role of NGF on brain development make it currently impossible to draw causative conclusions. Interestingly, one study of perinatal maternal immune activation (MIA) in rodents did note

higher levels of NGF in the cortex of exposed P0 pups [342, 343]. MIA has been correlated with the pathogenesis of ASD and schizophrenia. Thus, it is possible that cortical alterations caused by MIA could be mediated partially by NGF. However, these studies are just preliminary indications that NGF has roles in neuropsychiatric disease. Further deep investigation is required before NGFs role in disease and development can be confirmed.

Serotonin (5-HT)

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter well known for regulating behavior and for its role in the pathogenesis and treatment of major depressive disorders. In the adult brain, 5-HT is mainly produced in the raphe nucleus in the hindbrain. It is synthesized from the amino acid tryptophan by the enzyme tryptophan hydroxylase (TPH). Axons of the serotonergic neurons in the raphe project to almost all regions of the CNS including the frontal cortex, hippocampus, nucleus accumbens, and hypothalamus. There are 7 main types of serotonin receptors ($5-H_1-5HT_7$), though some serotonin receptors have multiple subtypes ($5HT1_{A, 1B}$) with distinct distribution and functions. The serotonin receptors are mainly G-protein coupled and act through Gs, Gi, or Gc signaling. Research on 5-HT has uncovered its diverse roles in mood regulation, emotional processing, memory, social interaction, pain, sleep, appetite, and regulation of the hypothalamic-pituitary-adrenal axis[344-346]. In addition to its very important roles in the adult brain, serotonin is also an important regulator in neurodevelopment. New research suggests that disruption in the 5-HT system during development, by either genetic or environmental causes, can produce small changes in brain structures involved in emotional regulation. Alterations in these circuits, in turn, can predispose an individual to a wide variety of neuropsychiatric disorders [347-351]. Thus, understanding the role of 5-HT in brain

development could shed light on the pathogenesis of many disorders including ASD.

In rodents, the first neurons containing 5-HT are detectable as early as E10-E12 in the hindbrain region that ultimately develops into the raphe nucleus. These serotonin neurons begin to grow and extend fibers in to the spinal cord and forebrain within 2-5 days of initial serotonin neuron production [347, 352-354]. In fact, by E17, axons from the raphe to all major regions in the frontal cortex have been established [355]. Likewise, in humans 5-HT is detectable as early as 5 weeks in the hindbrain and by 8 weeks fibers of these serotonergic neurons are found in the spinal cord and the forebrain [356, 357]. Yet, even before the birth of these raphe neurons, 5-HT is present in the fetal environment as early as E10.5 in rodent and 3 weeks in humans due to placental production [358, 359]. These early maternal levels of 5-HT are thought to be important for regulating fetal neurodevelopment. In addition to the presence of 5-HT, in the rodent, the serotonin transporter (SERT) and $5HT_{1A}$, $5HT_{2A}$ and $5HT_7$ receptors are all present before birth and some of these proteins are expressed as early as E12 [360-364]. Even in humans, studies have shown that SERT and 5HT1A are present at 8pcw and 12 pcw respectively [357, 365]. Moreover, $5HT_{1A}$ densities peak between 16 and 22 weeks of gestation with highest expression levels in the hippocampus and frontal cortex of the human developing brain. Thus, we see that 5-HT, SERT, and 5HT receptors are all present in early development in the forebrain when NPCs are proliferating, migrating, and differentiating.

Of course, the mere presence of serotonergic neurons, their projections, and their signaling components in the brain does not necessarily mean that serotonin has any roles in regulating neurodevelopment. While there are no gross morphological differences in the brains of rodents with altered 5-HT levels, more subtle changes in cytoarchitecture, cellular

morphology, and axon bundles have been seen in the forebrain of these rodents [347, 354, 366, 367]. This suggested that 5-HT is more likely "fine-tuning" developmental processes and may not be necessary for gross brain structuring. Studies in many model organisms from C. elegans to mouse to rat have found that 5-HT regulates and modulates neural precursor cell proliferation, migration, neurite outgrowth, axonal guidance, synapse formation, and wiring [368]. In 1978, Lauder and Krebs showed that administering 5-HT depleting drug pCPA to pregnant rats slowed the onset of neuronal differentiation in the cortex and the hippocampus in embryonic rats [369]. This suggested that 5-HT is an important regulator of the switch from proliferation to differentiation which was later confirmed in many model organisms [370]. 5-HT has also shown to have trophic and neurogenic roles in the hippocampus both during development and in adulthood [371]. 5-HT has been shown to be important in the regulation of neural migration as initially in murine neural crest cells and neurons in C. elegans [372, 373]. In the developing rodent cortex, newer studies have also uncovered roles for 5-HT in migration of both pyramidal cells and interneurons. A study by O Ricccio et al 2011 used in utero electroporation and time lapse imaging to find that application of 5-HT during migration of pyramidal neurons slowed the migration rate of these cells. Moreover, animals lacking SERT (which leads to higher brain 5-HT) had changes in the positioning of superficial layer cortical projection neurons in the cortex [374]. Another study by the same group showed that 5-HT also regulated the migration of cortical interneurons arising from the ganglion eminence a finding that was confirmed in another independent paper [375, 376]. Thus, in addition to regulating early proliferation of precursors, 5-HT is also integral for proper migration of cortical cells. As reviewed 5-HT promotes differentiation but it is also responsible for enhancing neurite outgrowth and guiding axonal

fibers [377]. In murine neuroblastoma lines addition of 5-HT has increased both neurite number and length [378]. In mice, embryonic depletion of serotonin alters the maturation of pyramidal neurons in layers III and V by reducing dendritic arborization and complexity [376]. Likewise, in cultures of both E16 hippocampal neurons and E15 thalamic neurons, addition of 5-HT increased the number and length of neurites [379, 380]. In rodents, serotonin is essential for the normal development of the sensory barrel cortex which is organized in "barrel"-like modules that corresponds to the arrangement of whiskers on rodent face[381, 382]. In 5-HT knockouts, barrels are not formed. Serotonin is also essential for the guidance of thalamocortical axons to sensory regions in rodents [383]. In particular, SERT is found on thalamic afferents and deletion of SERT alters thalamocortical axonal growth into the barrel fields of the mouse cortex. In humans, alterations of the somatosensory cortex are observed in ASD. Interestingly, genetic studies in humans find that ASD risk genes converge onto glutamatergic neurons in the somatosensory cortex (and other regions) of the mid-fetal brain [120]. This mid-fetal period is analogous to the developmental time-point in rodents when serotonin is facilitating barrel cortex development. Thus, serotonin could be involved in sensory map development in humans. Ultimately, we see that 5-HT is involved in the modulation and regulation of almost all early developmental processes. 5-HT also regulates later developmental events such as synapse formation and modulation of neuronal firing. Thus, alterations in 5-HT could perturb brain development which could ultimately increase vulnerability or contribute to neuropsychiatric diseases

Over 50 years ago, elevated blood levels of 5-HT were found in children with ASD, making it one of the first biomarkers in ASD [384]. Meta-analyses and further studies of children with autism have revealed that almost 30% of autistic children studied have

elevations in blood 5-HT [385]. Blood levels of 5-HT are largely due to platelet uptake and storage of the factor; thus blood levels may not necessarily be indicative of brain serotonin. A recent study in 248 individuals with ASD found that 98 individuals had high blood levels of 5-HT but low CSF levels of 5-HT indicating blood levels are not reflective of CSF levels [386, 387]. Moreover, PET studies show altered 5-HT synthesis rates in autistic children when compared to unaffected siblings and epileptic children. All of this suggests that both brain and peripheral serotonin may be commonly altered in ASD. However, it is unclear what is causing these alterations, whether these alterations contribute to the symptoms associated with ASD, and whether altered 5-HT actually has any role in disease pathogenesis. In addition to alterations in blood and CSF serotonin, genetic studies have found some associations with 5-HT and ASD. However, considering the pervasive roles that 5-HT plays during development, altered 5-HT related genes are not commonly associated with ASD. For example, polymorphisms in SERT have been associated with ASD, though the studies are largely inconsistent [388, 389]. Interestingly, one study showed that SERT polymorphisms were equally transmitted between unaffected sibs and probands. However, probands with the "s" allele transmission had more severe impairments in social interactions and communication than those who got the "l" allele, indicating that SERT gene polymorphism, instead of contributing to the pathogenesis of ASD, may be important in defining levels of impairment. Of course, genetic associations and levels of 5-HT in the blood and CSF may not necessarily mean 5-HT is contributing to ASD pathogenesis. However, mouse model studies of 5-HT alteration, as reviewed above, show changes in cortical cytoarchitecture and cell morphology. These animals also show behavioral symptoms that are often associated with ASD. For example, mice with ASD associated

SERT SNP displayed abnormalities in social interaction, communication, and had repetitive behaviors suggesting that serotonin status can contribute to ASD pathogenesis [390]. These mice, much like humans with ASD, also showed elevated blood levels of 5-HT when compared to wild-type controls. However, the ability of the SNP to cause ASD like symptoms was dependent upon the genetic background of the mice [391]. Thus, the inconsistent association of SERT with ASD in humans could also be dependent upon genetic background. Limited studies in humans have found that depletion of tryptophan, the precursor to 5HT, worsens repetitive behaviors [392, 393]. More recently, Daly et al conducted an fMRI based double-blind placebo-controlled crossover study of acute tryptophan depletion in 14 individuals with ASD (and normal IQ) in comparison to unaffected controls. Specifically, this study assessed the role of serotonin on inhibitory brain function during a Go/No-go task. Altered inhibitory functions are thought to contribute to repetitive restrictive behaviors in ASD. In autistic individuals, depletion of 5-HT upregulated fronto-thalamic activation and down-regulated striato-cerebellar activation thereby "normalizing" these circuits to resemble those of untreated unaffected individuals. This suggests that serotonin may be involved in the pathology of repetitive behaviors or inhibitory circuits in ASD. Moreover, depletion of serotonin could potentially "normalize" these altered circuits (Daly 2014). Yet, in other patients, SSRIs (which increase serotonin) have been efficacious in reducing rituals and repetitive behaviors in ASD patients. Overall, it seems that that 5-HT can modulate the aberrant behaviors seen in ASD [394]. However, different individuals with ASD may have different "serotonin profiles" which determine whether increasing or decreasing 5-HT is more beneficial. While these studies show 5-HT is important in ASD behavior, these drug studies were conducted

in adults and thus does not necessarily imply that 5-HT is involved in disease pathogenesis. More recent studies have begun to show that SSRI treatment in pregnant mothers is associated with increased ASD risk in children, though, these studies are preliminary [395-397]. Interestingly, SSRIs in rodents alter both placental and fetal 5-HT which could be changing brain cytoarchitecture thereby predisposing children to ASD. Indeed, studies in rats show that perinatal SSRI exposure increases anxiety behaviors, reduced social exploration, and altered cytoarchitecture of the cortex, thalamus, hippocampus and raphe [398-402]. Thus, in rodent models, we see that SSRI can alter neurodevelopment and lead to brain structure and behavioral phenotypes that are reminiscent of ASD and other neuropsychiatric disorders. However, some studies have suggested that maternal depression in general may increase the risk of ASD and altered neurodevelopment in children [398, 399]. Regardless, we see that 5-HT is an incredibly important modulator of pre- and post-natal development. Therefore, alterations in 5-HT homeostasis during development can contribute to altered brain structure and ultimately abnormal behavior.

In conclusion, we find that 5-HT in addition to its roles as a neurotransmitter is an important modulator of neurodevelopment. Alterations in the levels of 5-HT have commonly been observed in multiple neuropsychiatric disorders including autism. Interestingly, in animal models, experimental alteration of 5-HT levels during development can lead to behavioral symptoms that are reminiscent of autism, schizophrenia, and OCD. Moreover, these animal models of 5-HT alteration also show subtle neurodevelopmental changes. Thus, it seems that 5-HT may contribute to disease pathogenesis or at least predispose individuals to developing ASD and other neuropsychiatric disorders. Thus, due to the importance of serotonin in both development and behavior it is an excellent EF to

study in the context of human brain development and disease pathogenesis.

Oxytocin (OXT)

Oxytocin is a neuropeptide colloquially known as "the love hormone" with well-established roles in pair-bonding, lactation, and parturition in mammals [403-405]. Additionally, OXT has been implicated in the regulation of complex social behaviors such as empathy, affiliative behavior, and response to social stress [405-407]. In 1992, the Insel lab published a paper showing that chronic central oxytocin infusion increased the non-sexual interactions of male rats, which sparked great interest in OXT's contributions to social behavior [408]. Since then, there have been an abundance of studies in rats, voles, and mice on the effects with OXT injection, intranasal administration, and on deletion of OXT or it's receptor CD38 [405, 409-413]. Knockouts generally have reduced social learning, reduced maternal behaviors, and reductions in partner preference. In the new millennium, the large body of rodent literature showing the importance of OXT in social behavior prompted studies on the effects of OXT in humans [414]. In humans, intranasal OXT stimulates increased interpersonal trust in economic games, ensures continual trust after betrayal, increases the accuracy of judging emotions on faces, and improves facial recognition memory [405, 406, 414-417]. Due to the importance of oxytocin in regulating such behaviors in both rodents and humans, it has garnered great attention in disorders with social defects like ASD and schizophrenia. Research on OXT in ASD and other neurodevelopmental disorders has largely focused on two aspects 1) Does OXT dysfunction contribute to ASD pathology and 2) Can OXT treat the social defects associated with ASD?

In 1998, Modahl et al found that plasma oxytocin levels were lower in 29 children with ASD when compared to typical children [418]. However, there was considerable

variability and overlap between the levels of OXT studied in these children. Subsequent studies on plasma OXT levels have been variable with some studies failing to replicate the lower OXT levels and several studies that did find similar decreases in plasma OXT in ASD [419-421] Lower levels of plasma oxytocin have also been reported in schizophrenia [422]. The authors of these studies suggested that this data could indicate that OXT abnormalities may exist in ASD or schizophrenia, though peripheral levels may not necessarily be indicative of brain levels of OXT. Currently, there are no published studies of OXT levels in the CSF of individuals with ASD, though Karen Parker's lab at Stanford are currently recruiting and studying patients for CSF OXT and Vasopressin levels. However, the Parker lab has shown that there is a significant correlation between plasma and CSF OXT levels (r= 0.56) in a study of anxiety in children [423].

Genetic studies have also focused on the contribution of OXT and its receptor to neurodevelopmental disorders [420, 424, 425]. One of the largest and most comprehensive meta-analytic studies OXTR SNPS in 3941 individuals with ASD from 11 independent samples found that there were significant associations between ASD and 4 SNPs in the OXTR [426]. Again, different studies have found variable association with ASD and OXT SNPs (86-92). One interesting study by Parker et al (2014) found that SNPs in OXTR and plasma oxytocin were highly heritable in families. For example, even unaffected Sibs of individuals with autism have lower levels of OXT and ASD-associated SNPs in OXTR [427] . This indicates that dysregulations in OXT may not be uniquely associated with ASD social phenotypes. Rather, variations in OXT may contribute to individual differences in social functioning. Thus, while OXT may be important for regulating social functioning, it's role in ASD pathogenesis is unclear in humans.

Many mouse models of ASD show changes in the levels of OXT or its' receptor and often show rescue of behavioral defects with OXT administration [428, 429]. For example, the heterozygous Reeler mouse had significantly lower expression of OXTR in several brain areas such as the cortex and hippocampus [430]. Likewise, CNTNAP2 knockout model of OXT showed reduced expression of oxytocin in the PVN of the hypothalamus. In these mice, intranasal administration of OXT rescued social behavioral defects, indicating OXT was contributing to ASD associated behavior defects in these mice [431] . In FMR1 mouse models, treatment of pregnant dams with OXT the day before birth restored normal GABAergic function in these models and abolished social communication defects [432]. FMR1 mice also showed lower OXT immunoreactive neurons in the PVN [429]. In humans, studies of intranasal OXT administration have been done in individuals with ASD. Papers that review the overall data of OXT administration in ASD find that the effects of OXT are often complicated and inconsistent [420, 433-436]. As shown in Young et al (2015), two-thirds of the 12 studies on oxytocin and social cognition reviewed reported non-significant effects of OXT administration [433]. However, in 1/3 of the studies OXT did indeed improve social cognition. Many researchers in the field have suggested that OXT may only improve social cognition under certain contexts or situations (98-100). Some studies suggest that OXT may be beneficial for some subtypes of ASD whereas it has no effects in other subtypes. For example, on average, OXT seems to be beneficial for individuals with milder social defects whereas those with more severe social defects have less benefits with OXT treatment (86, 98-101). Thus, OXT may be a useful treatment for just a subset of ASD and its social benefits may be highly context dependent.

While OXT has been extensively studied in social behavior and as a potential

treatment for ASD, there are very few studies on OXT's effects on neurodevelopment and developmental processes. Studies by Alstein et al (1988) in rats found that the levels of the oxytocin precursor peptide could be detected in the rat brain as early as E18 [437]. qRTPCR studies in the developing rat also detected expression of the oxytocin receptor as early as E12 [438]. A more recent study checked for dynamic ligand binding distribution of OXTR in the developing mouse brain. Using male and female C57BL/6J, this study found that OXTR binding started at E18.5 and continued to rise till it peaked around adolescence at P21 [439] . At these time points in the mouse and rat brain the early processes of proliferation, migration, and initial neurite outgrowth are largely complete. Thus, it is unlikely that OXT is regulating any of these processes in the rodent brain. However, as mentioned OXT receptors do develop before OXT synthesis, and thus, external sources of OXT (maternal) could still bind and regulate development, similar to 5-HT. Though there are currently no studies suggesting roles for OXT in early development, OXT is believed to play important roles in early post-natal brain development [440, 441]. For example, OXT is important for regulating the switch of GABAergic neurons from excitatory to inhibitory [442]. Moreover, neonatal administration of OXT alters the serotonergic and dopaminergic systems, suggesting roles in the regulation and development of these neurotransmitter systems [392, 443-446] . Thus, while OXT may not regulate neurogenesis, migration, or early neurite outgrowth, it has important developmental roles in the rodent brain.

Developmentally, the E18.5 rat/mouse are thought to be equivalent to a 2nd trimester human fetus. In the human, in this 2nd trimester stage, neurogenesis is still actively occurring to form the cortex. Thus, if OXT expression increases around this time in human fetuses, it is possible that OXT could regulate early development. A look at the developing

human brain on the Allen Brain Atlas shows that in humans OXT receptor mRNA has a small peak in expression in the cortex, striatum, and hypothalamus at pcw 17-21 (2nd trimester). Immunocytochemical staining suggests that oxytocin positive cells begin to emerge in the PVN as early as the 14/15th week of gestation [447, 448]. However, there are no studies on the role of OXT in the human brain at these early developmental stages. Interestingly, a recent cell culture study in human neuroblastoma culture line SH-S75Y showed that treatment with OXT lead to increased number of neurites and increased neurite length. OXT treatment also led to changes in the expression levels of cytoskeletal proteins and proteins associated with the growth of neuronal cones [449]. A more recent study replicated the neurite outgrowth studies of OXT and found that OXT treatment also increased the expression of SHANK1 and SHANK3 genes [450]. The SHANK3 gene has been commonly associated with ASD. Thus, based on developmental expression and the ability of OXT to alter the expression of neurites, cytoskeletal proteins, and scaffolding proteins associated with ASD, it is possible that OXT plays interesting roles in the regulation of neurodevelopment, particularly in humans. Yet, at present such studies are limited, despite wide-spread interest in oxytocin in the ASD field.

Thus, in summary, oxytocin is a neuropeptide that has important roles in regulating several diverse social behaviors. However, whether dysregulation of OXT contributes to disorders with social defects is unclear. OXT has also been considered a therapeutic in the case of disorders like ASD. However, evidence is currently limited on its efficacy and further studies need to be done to see if OXT is more beneficial for some individuals than others. Finally, studies of OXT's roles in development are limited despite studies showing OXT is capable of inducing proliferation and neurite outgrowth.

Fibroblast Growth Factor-2 (FGF2)

The FGF family of growth factors has been studied for their integral role in regulating everything from early neurulation and anteroposterior axis determination, to cortical patterning, to postnatal development [213, 451-455]. There are more than 22 known secreted FGF ligands and 4 membrane-bound FGF receptors. Thirteen of these FGF ligands are known to be expressed in the brain during embryonic development along with FGFR1, 2, and 3. FGF2 is one of the earliest discovered FGF ligands and has well-established roles in development. In the rat, FGF2 is detected as early as E9.5 in the telencephalon and has continued expression into adulthood indicating potential wide-spread roles in development and brain function [456-458]. FGFs were first discovered as a mitogenic factor in fibroblast cells. However, it's mitogenic properties extend to multiple tissue types including the brain. As early as E9.5 FGF-2 has roles in increasing the number of neuroepithelial cells of the early neural tube. In 1999 Vaccarino et al demonstrated that injection of FGF2 into the ventricles of embryonic rat brains at E15.5 increased the cerebral cortex volume by 18% and increased the number of neurons by 87% whereas postnatal injections increased both hippocampal and cerebellar neuron numbers [459-461]. On the other hand, mice lacking FGF2 gene had fewer cortical neurons and glia by adulthood [462-464], as well as diminished neurons in the hippocampus [461]. FGF-2 also stimulated glial progenitor proliferation and aided in the expansion of astrocytes in the brain. Thus, FGF-2 seems to be essential for early neuroepithelial progenitor pool expansion and is capable of increasing proliferation of VZ progenitors which are essential for regulating brain size. In the humans, FGF2 and its receptor mRNA were found in both neural and glial precursor cells derived from 2nd trimester fetuses [465]. Moreover, in cultured human fetal brain cells FGF-2 enhances long-term survival, suggesting that FGF2 could also be critical in human neurodevelopment [466].

In addition to regulation of proliferation, some studies suggest that FGF-2 is also important for the proper organization of the cortex, regulation of differentiation, and regulation of migration [451, 455]. In addition to having thinner cortices, mice lacking FGF-2 have less distinct cortical layers, lower numbers of differentiated pyramidal neurons, and had ectopic neurons in the corpus callosum and deep layers of the cortex [467]. These defects were suggestive of abnormal migration and differentiation and indicated that FGF-2 may be necessary for the regulation of these processes. Likewise, a study by Abejon et al 1995 showed that in E16 cultures of rat hippocampus, administration of FGF-2 stimulated proliferation in Nestin positive cells but enhanced differentiation and neurite outgrowth in Nestin negative cells [468]. This indicates that FGF may have differential functions on different cell populations in the brain thereby giving it the ability to regulate a diverse set of developmental processes. However, studies on FGF2's roles in non-proliferative processes were limited. On the other hand, other FGFs such as FGF8 and FGF10 have been shown to have roles in both migration and differentiation in the cortex. Overall, cortical expression of FGF2 and its receptors, the ability of FGF2 to either stimulate proliferation or differentiation depending upon cell type, and the aberrant cortical phenotypes seen in FGF2 knockout mice suggests that FGF2 has important roles in regulating multiple steps of development throughout embryonic and postnatal life. Thus, embryonic and post-natal alterations in FGF could contribute to the pathogenesis of neuropsychiatric disorders.

In the last few years, evidence has accumulated that links many neuropsychiatric disorders to altered regulation of proliferation and differentiation both in the developing and adult brain. As FGF regulates many of these processes in the brain, more studies have begun to focus on potential roles of FGF in disease pathogenesis [469-475]. In post-mortem

samples of individuals with depression and schizophrenia, studies have found reductions in FGF expression in the cortex and hippocampus [470, 476]. A recent study by Esnagoglu et al found that children with ASD on average had lower serum levels of FGF-2 than typically developing children (n= 60) [477]. In children with autism, brain size alterations are commonly found. For example, approximate 20% of children with ASD have macrocephaly while around 10% exhibit microcephaly. These altered brain growth phenotypes have led some researchers to hypothesize that FGF abnormalities could exist in ASD, though currently no genetic studies have found this association [478, 479]. Moreover, it is unclear whether rodent models of increased or decreased FGF function exhibit autism-like behaviors. On the other hand, SNPs in FGF and its receptors have been associated with increased schizophrenia risk [480-484]. Interestingly, these SNPs were associated with changes in brain volumes of certain regions of the schizophrenic brain such as the hippocampus [483-485]. Studies have also found that patients with schizophrenia have increased FGF2 protein in the serum [486]. In addition to these associations in humans, certain rodent models of altered FGF do show "schizophrenia-like" behaviors. In FGFR1 knockout mice, embryonic loss of the receptor leads to decreased cortical thickness, impairments in sensorimotor processing, motor hyperactivity, reduced pre-pulse inhibition, and enhanced startle response-all phenotypes which are commonly observed in humans with schizophrenia [487-489]. Another study found that mutation in the NPAS3 gene led to an 80% reduction in FGFR1 receptors in mice. This reduction was associated with altered reelin expression in the cortex, altered cortical proliferation and migration, altered social behaviors, and repetitive restrictive behaviors [490]. Mutation in the NPAS3 gene in humans was seen in a rare familial case of schizophrenia[491]. Thus, it is possible that alterations in

genes that regulate FGF expression could lead to neuropsychiatric disorders via altered FGF signaling. Thus, while current studies do not show definitive links between ASD and FGF, FGF does seem to be associated with schizophrenia risk. However, behavioral and neuropsychiatric consequences of altered FGF in development are very limited. Moreover, genetic association studies looking into alterations of trophic factors is also limited. Yet, as an important regulator of development, it would be valuable to assess and study the effects of FGF in the pathogenesis and neuropathology of neuropsychiatric disorders.

Signaling Pathways in Neurodevelopment and Autism:

In the section above, we saw the many extracellular factors are involved in the regulation of neurodevelopment. In vivo, multiple factors, each with their own temporal expression and developmental function, come together to influence neural precursor cells and immature neurons to build the brain. As discussed in the review of neurodevelopment section, response to an EF is largely determined by the presence of the appropriate receptor on a cell membrane. When an EF binds to the receptor in many cases, it leads to a chemical change in receptor confirmation which ultimately leads to the activation or inhibition of downstream proteins. Often a chain of proteins is altered by receptor binding and this cascade of molecular changes is known as a signal transduction pathway or a signaling pathway. Ultimately, activation of a signaling pathway leads to the transcription of genes or modification of proteins that will allow the cell to conduct a behavior or change its state. Activation and timing of a signaling pathway in a cell often represents the amalgamation of signaling coming from multiple receptors on the cell. Thus, the activation of a signaling pathway is the cell's way of integrating numerous environmental signals to to respond appropriately to environmental cues. In the developing brain, precursor cells must undergo

numerous different morphological and functional changes. Moreover, these cells are being bombarded with a plethora signals such as EFs, nutrients, and metabolites that need to be integrated in order to determine appropriate behavior. Thus, not only are signaling pathways critical for regulation of development, aberrations in signaling pathways can easily alter cellular behavior and cellular responses to the environment. Altered cellular responses of course would change neurodevelopment and could lead to neuropsychiatric disorders. Indeed, as reviewed in the genetic section, pathway analyses of ASD risk genes and risk genes for other neurodevelopmental disorders have uncovered that proteins involved in signaling pathways are highly associated with these disorders. Thus, in the following sections, I will review 3 signaling pathways that have commonly been implicated in ASD and other NDDs including the P13K-mTOR pathway, the MAP-K (ERK) pathway, and the PKA pathway. I will discuss the roles of these pathways in normal development along with the consequences of their aberration with regards to neuropsychiatric diseases. It is important to note that many of the EFs discussed in the prior section do signal through these pathways which I will also discuss in the upcoming section.

The PI3K-mTOR Pathway:

The mTOR signaling pathway acts as a molecular integrator that processes the messages received from numerous environmental signals to regulate and orchestrate appropriate cellular and organismal behavior. In response to EFs, metabolites, nutrients, and cell energy status, the mTOR pathway exerts its influence by regulating a host of diverse cellular processes including protein synthesis, transcription, lipid synthesis, autophagy, metabolism, and organelle biogenesis, maintenance, and destruction [492-494]. With such important roles in cellular function, changes in the mTOR pathway have been

implicated in a wide array of diseases such as cancer, diabetes, obesity, autism, epilepsy, and other neurodevelopmental disorders. In the developing brain, mTOR is implicated in orchestrating many of the basic processes necessary to build the brain and in regulating the proper function of the adult brain. mTOR is known to regulate proliferation of neural precursor cells, promote differentiation, migration, and neurite outgrowth in early neuroblasts, regulate the assembly and maintenance of early neural circuits, facilitate experience dependent plasticity in the postnatal and adult brain, and regulate complex behaviors like feeding, sleep, and circadian rhythms [495-497]. In the cancer field, the number of molecules targeting the mTOR pathway has exploded in the last few years, and this drug class has been successful in restricting tumor growth [498]. Likewise, its importance in both brain development and adult brain function make it both an appealing pathway to study in the context of neuropathology and an important molecular target for potential therapeutics.

The mTOR Signaling Cascade:

EFs like NGF, BDNF, NT3, PACAP, insulin and IGF-1 are well known to activate the mTOR pathway [499]. Binding of these EFs to receptors usually activates a kinase known as PI3K (Figure 2). Activation of the canonical mTOR pathway starts with the activation of PI3 kinase which recruits and phosphorylates AKT at the plasma membrane. AKT is a central node of the mTOR pathway and on its own can regulate numerous downstream effectors and is a positive regulator of multiple cellular functions such as proliferation, growth, survival, and metabolism in multiple species. Phosphorylated AKT then phosphorylates and inactivates the tuberous sclerosis complex (TSC1/TSC2) which is an important inhibitor of the mTOR complex. Inactivation of TSC then allows mTOR to become active and phosphorylate many downstream molecules including S6-Kinase and

4EBP which are regulators of translation (Figure 2). In addition to the cascade described, there are numerous other pathways and molecules that can regulate and activate mTOR. One important molecule, PTEN, is important for de-phosphorylating and deactivating P13K which therefore reduces mTOR pathway activity. Molecules downstream of mTOR such as S6K are also known to create a feedback loop with upstream molecules such as AKT to establish regulation of this pathway [493, 496]. Activation of other signaling pathways can also influence mTOR (Figure 2). Mutations in many members of the mTOR pathway have been shown to alter pathway activity and ultimately cell behavior.
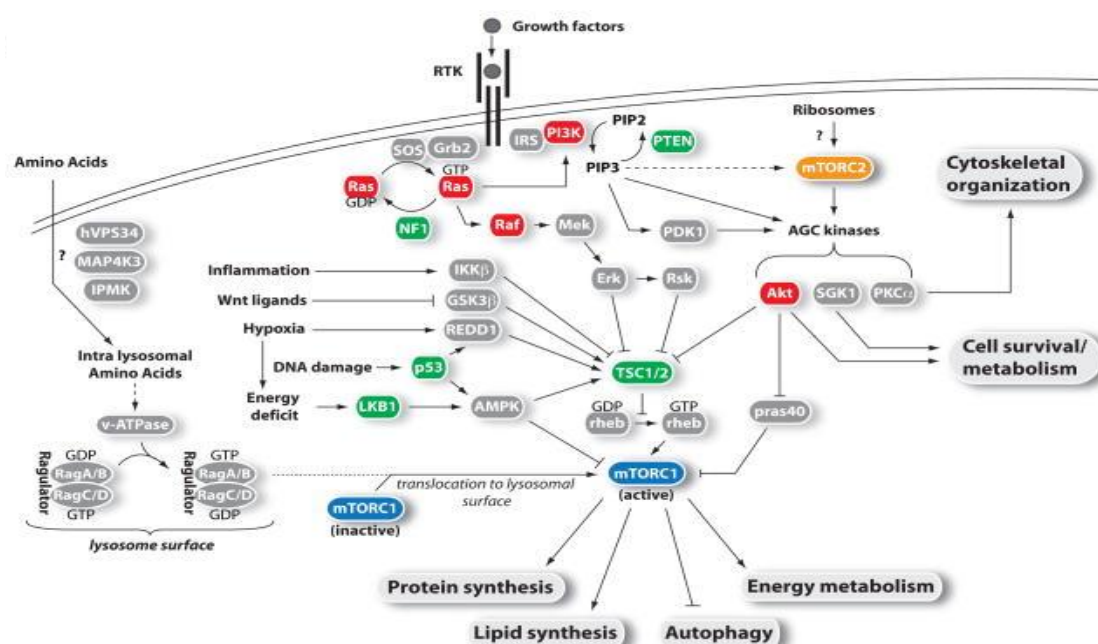
Figure 2: The various players, roles and interactions of the mTOR pathway [494, 500]

mTOR Regulation of Neurodevelopment:

Studies in yeast models and tumor lines illustrated that mTOR has critical roles in proliferation and survival of cells [492, 493, 501]. Thus, it is logical to suppose that the mTOR pathway regulates similar cellular processes in the developing brain. In rodents, whole-

body knockout of certain members of the mTOR pathway such as mTOR and PTEN, both led to embryonic lethality as early as E5.5 showing that this pathway is essential in the development of the embryo, even before formation of the neural tube [502, 503]. Other members of the pathway like AKT and S6 kinase have multiple isoforms, therefore, loss of one isoform can be compensated for by the other isoforms and so mice null for one isoform are largely viable. Thus, initial exploration of the mTOR pathway's roles in development were limited to study of AKT or S6K knockout models, focused on animals with minor mutations PTEN or mTOR, or utilized drugs such a rapamycin which inhibited mTOR activity. Later, development of conditional knockout animals allowed for the selective deletion of these and other genes in specific cell types or developmental periods which allowed for specific exploration of the role of mTOR in brain development.

One of the earliest model systems to study the impact of mTOR alteration on brain development was the "flat-top" mouse. This mouse strain was generated by random chemical mutagenesis which led to a single nucleotide mutation in an intron of the mTOR gene leading to reduced kinase activity. These mutant embryos failed to develop a telencephalon due to an extreme lack of proliferation in the early progenitor cells that expand to form the telencephalic vesicles [504]. A severe lack of proliferation and absent telencephalon were also observed in embryonic mice exposed to mTOR antagonist rapamycin (via maternal injection) between E5.5 and E8.5 [505]. In another study, mTOR regulator raptor was selectively deleted in neural precursor cells by placing Cre recombinase under the Nestin promoter. These animals began to show smaller brain sizes beginning at E15.5 due to a reduction in cell numbers and cell size. The size difference between mutant and WT mice increased progressively until birth [506]. Further analysis of

neural precursor cells derived from conditional knockout (cKO) animals showed reduced proliferation at E16.5 and increased cell cycle length at E15.5. There was also increased cell death of TUJ1+ neurons at E19.5. mTOR's role in regulating cell death were also observed in a study by Kassai et al 2014 that showed transgenic mice with overexpression of mTOR under Emx1 promoter (specifies dorsal telencephalic precursors) had atrophied cerebral cortex due to increased cell death at E12 [507]. In sum, we see that overall brain growth, survival, and proliferation of precursors along with regulation of cell-cycle are all important to neurodevelopment and are regulated by mTOR.

Other members of the mTOR pathway such as AKT, PTEN, P13K, and S6K are also important in neurodevelopment. In the developing mouse cortex, immunostaining has shown the presence of phosphorylated (active) AKT throughout the cortex with particularly high enrichment in the NPCs of the VZ suggesting a role in development [508, 509]. Indeed, loss of the AKT1 and AKT3 isoforms in mice leads to smaller brains. In AKT3 null mice, smaller brain size is due to smaller and fewer cells while in AKT1 null mice fewer cells were observed but changes in cell size were not [510, 511]. Likewise, mutations that reduce function of PTEN or TSC1/TSC2 (thereby increasing mTOR activity) are also associated with altered brain size, specifically larger brains (macrocephaly), in rodents [512, 513]. For example, mice with conditional Nestin-PTEN loss have larger brains with abnormal layering in the cortex, hippocampus, and cerebellum. Moreover, NPCs from all these regions at E14.5 showed increased proliferation and reduced apoptosis [514]. Similarly, rats lacking TSC2, which die at E11.5, have huge expansion in the number and volume of neuroepithelial cells of the neural tube prior to death. Interestingly, in humans, deletion of the 1q42-q44 region which encompasses AKT3 leads to a variety of developmental

aberrations in the brain including agenesis of the corpus callosum and microcephaly while overactivation of this region leads to macrocephaly and epilepsy [515]. Likewise, mutations in P13K, PTEN, TSC2 and mTOR have all been associated with altered brain size in people. Thus, mTOR pathway members seem to be essential for the regulation of brain size by controlling proliferation and cell death in early embryonic development.

While neurodevelopmental studies of the mTOR pathway have largely focused on its role in regulating proliferation and survival, this pathway is also important for regulating migration, differentiation, maturation and neurite outgrowth, and lineage specification. However, studies of migration, despite its incredible importance in building the brain, are limited. Yet, altered cortical lamination, changes in cortical cytoarchitecture and ectopic neurons (suggestive of migration defects) have been observed in animal models of mTOR alteration. For example, a study by Kassai et al 2014 found that transfection of E14 cortical precursors with constitutively active mTOR prevented proper migration of developing cortical neurons to the pial surface and led these neurons to remain in the intermediate zone [507]. Moreover, transgenic mice expressing this constitutively active mTOR in Emx-1 dorsal telencephalic precursors had abnormal distribution of upper layer Cux-1 positive neurons at P0 (505). Knockdown of mTOR regulator raptor via shRNA rescued the neuronal migration abnormalities in both the electroporation and transgenic models showing that normal mTOR pathway activity was essential for normal migration and cortical cytoarchitecture. Likewise, mice with conditional deletion of Tsc1 under Emx-1 showed altered cortical and hippocampal lamination, ectopic neurons, changes in organization of the olfactory bulb, and heterotopias suggesting abnormal migration [516]. Similarly, expression of a constitutively active form of the mTOR activator Rheb (coupled

to GFP), in neural progenitors of the cortex at E15.5 led to a striking change in the lamination of the cerebral cortex at P0 all the way till P28 (last time point analyzed) . Specifically, in mutant mice, only 35% of GFP+ neurons reached layer 2/3 compared to the 93% in controls [517]. Another interesting study by Jossin et al 2007, labeled the brains of E14 wild-type mice with Cell Tracker Green and then treated these mice with a selective P13K inhibitor [518]. After 18 h of treatment with the inhibitor migration speed was significantly reduced from 17.96 um/hr to 1.94 um/hr. The studies reviewed above all show that embryonically, members of the mTOR pathway are essential for the regulating neuronal migration to ensure proper development and cytoarchitecture of the cortex. Yet these studies reviewed represent almost all studies that have to date explored the roles of mTOR in migration in embryonic neural systems. There are a few other studies of the role of mTOR's role in neural migration, however, these studies are conducted in postnatal animals in regions such as the SVZ and dentate gyrus of the hippocampus where neurogenesis and migration are still occurring. Even in these postnatal systems, mTOR plays an important role in ensuring normal migration of newly generated neuroblasts.

mTOR also plays roles in the regulation of neuronal differentiation during development. A study by Fischwick et al (2010) created a mouse model with a mutation in PDK1, the kinase that phosphorylates AKT, leading to an 80-90% reduction in PDK1 levels and "underactivity" of the mTOR pathway [519]. Unlike the PDK1 null embryos, these PDK mutant embryos survive until E11.5. In the embryonic hindbrain of mice, at this point, neurogenesis has led to the production of early neurons. Analysis of PDK1 mutant embryos at E9.5 revealed that there were fewer neurons and axons than heterozygous littermates yet no differences in cell death. Moreover, there was a huge reduction in the

expression of proneural differentiation gene Neurogenin2 (Ngn2) in mutant mice suggesting that neuronal differentiation was reduced by aberrations in mTOR signaling. In mouse models with overactive mTOR, such as the EMX-1 TSC1 cKO animals, premature differentiation of neurons was observed in the E14.5 cortex [516, 520, 521]. Other studies have found that activation of the mTOR pathway via EFs such as IGF-1, Insulin and TGF-B are essential for the differentiation of mouse and rat cortical cells between E13.5 and E16.5 Indeed, the ability of these EFs to stimulate cortical precursor differentiation are abolished by administration of mTOR antagonist rapamycin [522]. In aggregate, these studies show that mTOR activity is essential for regulating the timing and onset of neuronal differentiation in both the forebrain and hindbrain of developing embryos.

In addition to regulating the switch from proliferation to differentiation, the mTOR pathway is also important for the growth of early axons and dendrites, known as neurites. The proper specification of axon-dendrite polarity occurs in embryonic development and is essential for neural information flow. In most neurons, there is a single axon and multiple dendrites. Dendrites usually receive signals while axons transmit signals. In E18 rat cortical and hippocampal cultures, reduction of mTOR activity via shRNA reduced the number and length of dendrites whereas upregulation of mTOR increased dendrite length and arborization [523, 524]. Similarly, treatment of E15 mouse cortical precursor cells with mTOR antagonist rapamycin, led to reductions in the percentages of cells with neurites in culture [525]. In addition to mTOR, TSC1/2 and GSK-3β (a downstream molecular target of AKT) also have shown to regulate neurite outgrowth and complexity. Overexpression of TSC1/2, which leads to reduced mTOR pathway activity, suppressed axon formation, while the knockdown of TSC1/2 led to ectopic axon formation in hippocampal cultures derived from

both E18 rat and E17 mouse [526]. Likewise, constitutive activity of GSK-3β in E18 hippocampal pyramidal neurons led to the inhibition of axon formation . On the other hand, reduction of GSK-3β with a peptide inhibitor and siRNA led to the formation of multiple axons in one neuron [527-531]. Thus, again, we see both over and underactivity of the mTOR pathway lead to alterations in important neurodevelopmental processes.

Overall, we see that mTOR plays wide spread roles in the regulation of embryonic neurodevelopment from control of proliferation to cell survival to neurite outgrowth. Moreover, both under and overactivity in the pathway lead to aberrant brain structure in rodents. Of course, in addition its importance in embryonic brain development, mTOR regulates synapse formation, synaptic plasticity, myelination, and even neuronal function in the postnatal and adult brain [495, 532, 533]. These postnatal and adult roles of mTOR in the brain have been much more extensively studied as synapse formation is one of the critical steps involved in many behaviors in adult animals. As ASD and other neurodevelopmental disorders show alterations in the many developmental processes regulated by mTOR, it is likely that abnormalities in this pathway may contribute to ASD.

Dysfunctions in mTOR in Neurodevelopmental Disorders:

Dysfunction in mTOR signaling is found in several genetic syndromic disorders that have high rates of ASD including Tuberous Sclerosis (TSC), PTEN-associated ASD, Neurofibromatosis-1, Fragile-X syndrome, Rett Syndrome, Phelan-McDermid Syndrome, and Angelman's Syndrome [534-536]. Thus, it seems that mTOR abnormalities and ASD are tightly linked. Indeed, as reviewed above mouse models with mutations in TSC and PTEN showed abnormal brain pathology that is commonly seen in patients with ASD. Moreover, both humans and mice with these defects share common phenotypes like repetitive

behaviors, impaired social interaction, and seizures which are common symptoms of ASD. Interestingly, in some cases, treatment with inhibitors of mTOR pathway in adult mouse models of overactive mTOR can even ameliorate ASD symptoms like impaired social interaction and repetitive behaviors [537]. This suggests that not only does mTOR contribute to abnormal wiring of the brain, it also contributes to the behavioral dysfunction in the adult brain. Moreover, mTOR manipulation could putatively serve as a therapeutic for improved behavioral outcomes in ASD [534]. However, while rapamycin has shown to improve behavioral outcomes associated with ASD in animals, a recent double-blind randomized placebo-controlled phase-II study indicated that rapamycin failed to improve neurocognitive or behavioral outcomes in children with TSC [538]. However, newer clinical trials in syndromic ASDs such as Rett, Fragile-X and Phelan McDermid Syndrome are testing the efficacy of IGF-1 (an mTOR pathway activator) on behavioral and medical outcomes [539]. Early reports have suggested that in girls with Rett syndrome, IGF-1 administration reduced anxiety and depression and reduced social avoidance [540]. In double blind [lacebo controlled Phase 2 trials, IGF-1 improved social impairment and restrictive behaviors in 9 patients with Phelan McDermid Syndrome, without any serious adverse events [541]. Likewise, in Fragile-X, clinical studies on IGF-1 are currently in phase-2. Thus, early clinical trials using IGF-1 are suggesting that targeting mTOR may be a useful mechanism to treat many sub-types of ASD [542, 543]. However, as discussed in the genetics section, syndromic forms of ASD and idiopathic ASD do not necessarily share similar genetic factors. However, children with idiopathic ASD have also shown brain pathological defects and behaviors that are similar to animal models and humans that have dysregulated mTOR. Indeed, genetic studies looking for convergence of ASD associated genes have

implicated that the mTOR pathway is a point of convergence for ASD pathology. Thus, it is possible that mTOR may play a role in the pathogenesis of idiopathic ASD. One recent study looked at mTOR levels in the post-mortem brain of adolescent patients with idiopathic ASD and found hyperactivation of the mTOR pathway [544]. However, the sample size was a meager 5 brains though all 5 brains exhibited hyperactive mTOR. Curiously, another study of postmortem brains from 11 individuals with idiopathic ASD (ages 5-56) found lower activity levels in the mTOR pathway [545]. Thus, both hyperactivity and hypoactivity of the mTOR pathway seem to be correlated with autism spectrum disorders, developmental brain pathology, and abnormal behavioral phenotypes in both idiopathic ASD and multiple subtypes of syndromic ASD [546]. Interestingly, mTOR dysregulations are also a feature in some environmental models of ASD. For example, rats exposed to valproic acid, which has been suggested to increase autism risk in both animals and humans, had lower levels of mTOR signaling in their brain. Models of maternal immune activation, which have also been associated with increased autism risk, have also shown increased expression of genes associated with the mTOR pathway[547]. Thus, mTOR dysfunction seems to be closely linked with neurodevelopmental disorders and autism.

In conclusion, numerous subtypes of ASD with varied etiological origins show dysregulation in the mTOR pathway. This suggests, that the mTOR pathway may be a unique point of convergence for numerous neurodevelopmental disorders. Alteration of the mTOR pathway in mouse models embryonically, neonatally, and in adulthood lead to autism-like behaviors or the presence of comorbid conditions commonly associated with ASD. Specifically, embryonic alteration of mTOR pathway activity in rodents produces brain pathology that is strikingly similar seen in individuals with ASD. Furthermore, we

see that mTOR pathway has vast roles in the regulation of embryonic brain development though these studies are very limited. Thus, studying mTOR pathway in humans with ASD could potentially help us understand the molecular and biochemical pathology of the disorder. Moreover, these studies could also help uncover novel therapeutics that could be used to treat ASD. Indeed, in numerous syndromic ASDs, IGF-1, a known activator of the mTOR pathway has already shown great therapeutic potential.

The Adenylyl Cyclase Pathway:

Many G-protein coupled receptors, when activated by ligand, activate an intracellular molecule known as Gs which then binds to an enzyme known as adenylyl cyclase (AC). AC then catalyzes the conversion of ATP into cyclic adenosine monophosphate (cAMP), an important intracellular 2nd messenger with numerous roles [548, 549]. An increase of cAMP activates an enzyme called protein kinase A (PKA) which goes on to phosphorylate many downstream effectors to ultimately phosphorylate CREB which promotes the transcription of genes in the nucleus (Figure 3). PKA also interacts with other proteins to modulate many other cell functions. Developmentally relevant EFs such as PACAP and 5-HT, as discussed before, signal through G-protein coupled PKA pathway. Thus, proper function of PKA would be essential for 5-HT and PACAP mediated regulation of development and could potentially be involved in disease states where PACAP and 5-HT responses are aberrant. In addition to mediating signaling of these EFs, in the brain, the PKA pathway has been shown to play important roles hippocampal dependent memory and synaptic plasticity [550, 551]. The PKA pathway also regulates neuronal cytoskeletal dynamics and mitochondrial function and dynamics [552]. As discussed before, proper cytoskeletal function is essential for numerous processes in the

developing brain. Yet, despite the importance of the PKA in mediating EF responses, regulating synapse formation, and cytoskeletal dynamics, there are very few studies that have looked at the roles of this pathway in neurodevelopment and neurodevelopmental disorders. Dysregulated PKA pathway has been noted in neurodegenerative disorders, addiction, and anxiety suggesting that altered PKA could alter brain circuits and behavior.
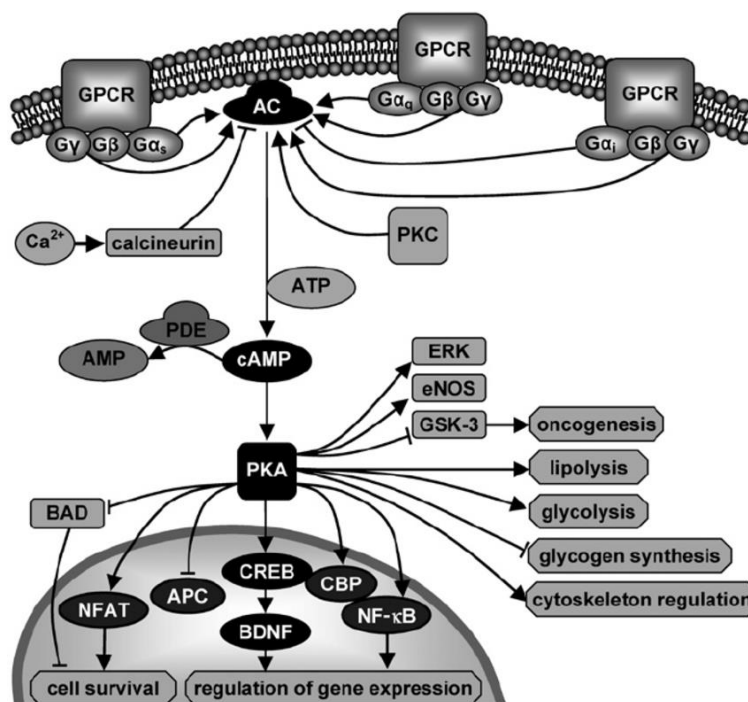
Figure 3: The members and functions of the PKA pathway [553]

While direct studies of PKA's role in development are limited, PKA is a kinase that is important for phosphorylating many important regulators of neurodevelopment. For example, Toriyami et al 2012 showed that PKA phosphorylates doublecortin in order to promote migration of E16.5 mouse neural precursor cells. PKA also phosphorylates SAD-A/B kinase which phosphorylates MAPs that are required for the developmental specification of axons in E15.5 mouse cortical neurons [290]. Likewise, PKA can also phosphorylate DISC-1 which leads cortical precursor cells to switch from a proliferative state to a migratory state [554]. Interestingly, DISC-1 is an important molecule that has been

implicated as a susceptibility factor for a wide range of neuropsychiatric illnesses such as schizophrenia, mood disorders, and autism [555-558]. Thus, alterations in PKA activity could lead to altered phosphorylation of developmental modulators leading to aberrant development. cAMP, which activates PKA, has also been shown to regulate neurodevelopmental processes. For example, depletion of cAMP in E15 motor neurons reduced the percentages of cells with neurites and neurite length in a dose dependent manner [559]. Likewise, in human neuroblastoma lines, cAMP activation of PKA leads to the extension and elongation of neurites which was blocked by a PKA antagonist [560]. cAMP is also involved in guidance of axonal growth cones in developing neurons [561-563]. As mentioned before, PACAP can act as an anti-mitogenic and pro-differentiation signal in the developing cortex. Studies in our lab have found that PACAP exerts its effects on the development by increasing cAMP levels and causing CREB phosphorylation [281]. Thus, PKA activity is important for the regulation of important developmental molecules, mediates the activity of developmentally relevant EFs, and is involved in the regulation of neurites and growth cones in the developing brain The PKA pathway has also been implicated in the pathogenesis of aberrant neurodevelopment seen in rat offspring born to mothers with hypothyroidism. In the first 12 weeks of fetal development in the human, maternal thyroid hormone is necessary for the proper development of the fetus including normal brain development. Children born to mothers with hypothyroidism can often exhibit cognitive defects, intellectual disabilities, and impairments in learning and memory [564]. In rat models, reduction of maternal thyroid hormone results in animals with impaired spatial memory and learning. In these animals, reduced levels of CREB which is downstream of many pathways including PKA were observed [565, 566]. Thus, while studies

of the PKA pathway in development are limited, the few studies presented do implicate a role in PKA for regulation of normal development and brain function.

Alterations in members of the PKA pathway have been associated with neurodevelopmental disorders. For example, CREB is thought to be involved in the pathology of Rett syndrome, a syndromic form of ASD. For example, studies by Klein et al 2007 found that CREB regulates the levels of MECP2, the gene associated with Rett syndrome, indirectly through microRNA132 [567]. Bu et al 2017 found that female mice that were heterozygotes for MECP2 (+/-) had lower levels of both total CREB and phosphorylated (active) CREB in the cortex [568]. Treatment with a CREB agonist, rolipram, reduced anxiety behaviors, improved performance on cue test of fear conditioning paradigm in MECP2 +/- mice suggesting CREB is contributing to Rett pathology. Another ASD associated syndrome, Neurofibromatosis, is characterized by heterozygous loss of NF1 gene function which leads brain tumor formation and behavioral deficits. NF1 is a positive regulator of cAMP such that Nf1 deficient neurons and astrocytes have reduced cAMP levels [569-571]. In hippocampal neuronal cultures derived from E13.5 mice, loss of 1 copy of NF1 led to shorter neurite lengths and smaller growth cone areas [569]. These hippocampal neurons also exhibited reductions in intracellular cAMP and reduced expression of p-PKA substrate proteins indicating reduced cAMP/PKA pathway activity. Interestingly, in this study, depletion of cAMP in control neurons led to reduced neurite outgrowth and reduced growth cone area which mirrored the NF1 phenotype. Thus, cAMP/PKA pathway once again is an important developmental regulator of neurite outgrowth and axon dynamics. Moreover, alterations in cAMP are associated with disorders that show increased incidence of autism suggesting the involvement of cAMP in

neurodevelopmental disorders. Some researchers have postulated that altered cAMP contributes to the neurobehavioral alterations seen in Fragile-X syndrome [572, 573] . In humans with Fragile-X syndrome, decreased cAMP levels were seen in platelets [574]. Moreover, overexpression of FMR1 in mouse neural cells increases levels of cAMP showing a positive correlation between these factors. Kelley et al (2015) acquired Fragile-X and unaffected neural cells from the cortices of human fetuses and compared the levels of cAMP in these cells [575]. This study also assessed the levels of cAMP in the cortex of Fragile-X mice and in the heads of Fragile-X drosophila (572). In all three models, induced cAMP levels were reduced in both the fly and the mouse suggesting deficient stimulation of cAMP production. Some researchers have also suggested that PKA may also be involved in the pathology of other forms of ASD and in ADHD. However, there are few experimental studies establishing this connection. Rather, based on the vast functions associated with PKA which relate to the pathology of autism and ASD, integrative hypothetical review papers have been put forward suggesting a role for PKA in autism and other neuropsychiatric disorders [576]. One study by Ji et al 2011 examined the expression and activity of PKA in post-mortem brain tissue samples from the frontal, temporal, parietal, occipital cortices, and cerebellum of individuals with regressive autism and non-regressive autism [577]. PKA levels were significantly decreased in the individuals with regressive autism when compared to unaffected controls. Thus, overall there are scattered studies suggesting the importance of PKA in neurodevelopmental disorders.

In the adult brain, many studies have investigated the roles of PKA/cAMP/CREB in synaptic plasticity, activity dependent synapse formation, memory, and addiction. These studies have given us clues that PKA is involved in numerous important functions such as

the regulation of mitochondrial and cytoskeletal dynamics. Yet, despite the importance of these functions in development, studies that focus on the roles of the PKA pathway in development are sparse. The limited studies reviewed above, however, do suggest that PKA is essential for regulating later stages of neurogenesis such as migration, neurite outgrowth, and synapse formation. With roles in regulating development and important roles in brain function, it seems likely that PKA may be involved in the pathogenesis of developmental disorders. While PKA alterations have been well studied in memory and certain degenerative disorders, again studies in developmental neuropsychiatric disorders are limited. The few studies available, however, do show that various syndromic forms of ASD seem to be associated with cAMP alterations. Thus, with clear importance of PKA and the obvious dearth of studies on PKA in development, further studies looking into this pathway, particularly in human development and ASD would be valuable and informative.

The MAPK/ERK Pathway

In mammals there are more than a dozen MAPK enzymes that regulate proliferation, differentiation, motility, and survival of numerous cell types including neurons and their precursors. Much like the mTOR pathway the MAPK pathway is known to integrate signals from numerous EFs to alter cell behavior. The role of the MAPK pathway in proliferation and survival has led to extensive studies of its function in the pathogenesis of many tumors. Indeed, numerous pharmacological and biologic drugs in the cancer field antagonize the MAPK pathway to restrict tumor growth. Moreover, these drugs have also been shown to improve survival in individuals with cancer. MAPK dysregulation has also been implicated in brain disorders such as autism, schizophrenia, Alzheimer's disease, and Parkinson's disease suggesting important roles in both the

development and maintenance of the brain [578]. The canonical activation of the MAPK pathway is preceded by the binding of growth factors to receptor tyrosine kinases (TRKs). Thus, EFs reviewed in the previous section such as neurotrophins and FGF utilize this signaling pathway to exert their effects on brain function and development. As seen in Figure 4, receptor binding leads to the activation of a small GTPase protein known as RAS which then activates a kinase known as RAF. RAF phosphorylation leads to downstream phosphorylation of other kinases including MEK which then phosphorylates MAPK (ERK). ERK then goes on to activate transcription factors such as Myc. ERK is also known to phosphorylate many other downstream targets such as microtubule associated proteins [579, 580] The MAPK pathway also includes activation of JNK which has been implicated in apoptosis and maintenance of microtubule stability.
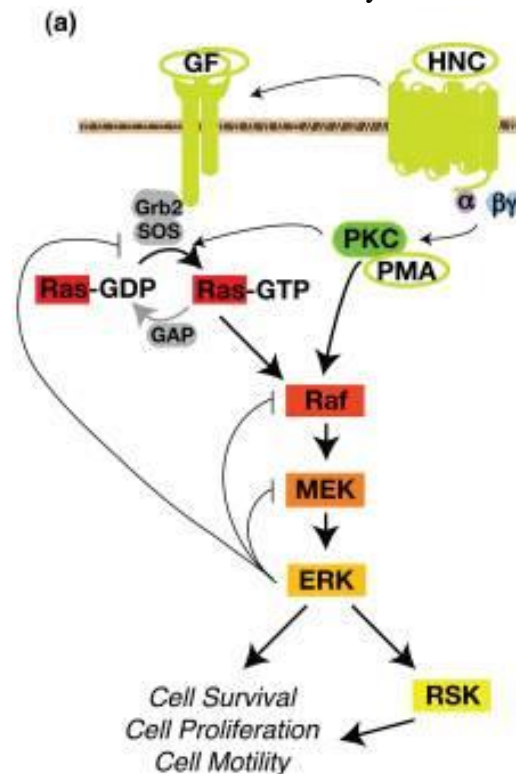
Figure 4: The MAPK/ERK pathway and the functions it regulates Tab[581]

Interestingly, mutations in the MAP-K pathway are associated with disorders known as RASopathies that have craniofacial abnormalities, heart defects, delayed development and growth, intellectual impairments, cognitive deficits, and autism. Many individuals with these disorders also show macrocephaly [582-584]. Thus, MAPK/ERK pathway seems to be associated with regulation of development and cognition in the brain. One RASopathy, NF1, was reviewed earlier in the context of mTOR dysregulation. NF1 encodes for a RAS-GAP protein that is a negative regulator of Ras, the kinase that begins the MAPK cascade. As reviewed mouse models of NF1 show abnormalities in their brain and changes in behavior that are associated with autism and other developmental disorders. These animals also had altered levels and activation of MAPK pathway members such as ERK [585]. Another disorder, Legius syndrome, involves an inactivation of the gene SPRED1 which regulates the activity of RAF. This disorder also shows mild learning impairments and ADHD suggesting alterations in brain function and development [586]. Abnormal MAPK signaling has also been observed in models of monogenic autism including Fragile-X syndrome and Tuberous sclerosis [587, 588]. Moreover, an idiopathic mouse models of autism, the BTBR mouse, shows elevated levels in p-ERK in the prefrontal cortex but not the cerebellum [589]. Altered RAS/MAPK signaling has also been identified as a common downstream mediator of diverse mutations linked to ASD and schizophrenia [108, 590, 591]. Moreover, ERK1 is present in the CNV 16p11.2 region. Deletions and duplications of this region contribute to about 1% of all cases of ASD showing again that ERK may be involved in brain development and the pathology of ASD [592-595]. Yet, despite all these studies linking ERK to neurodevelopmental disorder, there are very few studies looking at how ERK dysregulation alters neurodevelopment and

contributes to these disorders.

In developing mouse embryos, phosphorylated (active) forms of ERK1 and ERK2 were found in the neuroectoderm as early as E8.5 and maintain activity across development [596]. In the embryonic mouse cortex, ERK activity is confined to proliferative zones with low medial to high lateral expression which mirrors the gradient of neurogenesis that occurs in the brain [582]. Studies have shown that alteration of members of the MAPK pathway is important for regulating neurogenesis and gliogenesis. In the cortex of mammals including rodents and humans, neural precursor cells initially differentiate into neurons which migrate and form the 6- layer structure. In mice this neurogenic phase is thought to occur from E13.5-E17.5. At E18.5 the radial glial cells that were generating neurons, now begin to generate astrocytes. This glial generation continues to occur postnatally and includes genesis of oligodendrocytes. Multiple groups have identified MEK signaling as important for the regulation of this neurogenesis-gliogenesis change in the brain [597]. In cultured E13.5 coritcal cells, enhancing MEK signaling increased the percentage of neurons generated while inhibiting the number of glia generated [598-601] Likewise, inhibiting MEK reduced neurogenesis and caused cells to remain as undifferentiated neural precursor cells [598]. This function of MEK activation and inhibition were also replicated in vivo in E14.5 mice [599]. In vivo, MEK blockade also caused NPCs to remain in the VZ/SVZ in an undifferentiated state. Mutations in scaffolding proteins that link receptor tyrosine kinases to MAPK activation also led to similar changes in the differentiation of cortical progenitors [602-604]. In 2008, Samuels et al generated a conditional ERK2 knockout mouse model which led to the inactivation of ERK2 in cortical NPCs at E14.5, when neurogenesis is at its peak [605] This loss of ERK2 resulted in the

generation of fewer neurons and more astrocytes in the cortex. These mice also had significant reductions in cortical thickness and displayed abnormal fear conditioning behavior. Prior to activation of the CRE-lox system, BrdU incorporation and pH3 immunoreactivity are identical in WT and floxed mice. Activation of the conditional knockout leads to significantly less BrdU and pH3 immunoreactivity in the KO cortex with a specific reduction of dividing TBR2+ IPCs. In 2012 the same group deleted both ERK-1 and ERK-2 in murine models to study the effects of complete loss of ERK on cortical development [582]. While ERK-1 knockout animals are viable and healthy, early loss of ERK2 leads to embryonic lethality. Thus, Emx-1 CRE was used to knockout ERK2 in ERK1 null animals and in WT animals. The ERK double knockout (DKO) model died during the first postnatal week while ERK-2 CKO survived well past P21. At P2, DKO mice had 32% smaller brains, showed a significant decrease in cortical area, and cortical length compared to WT controls. CKO mice had 11% smaller brains along with alterations in cortical area and length at P2. Both CKO and DKO mice also showed alterations in cortical lamination, reductions in pyramidal neurons of layer II-IV, an increase in layer 5 subcortical neurons and no change in layer 6 neurons. The deficits were more pronounced in DKO mice. Moreover, neuronal morphology was altered in ERK2 CKO mice. Reduction of ERK-2 activity led to 50% reduction in mean total axonal length and a reduction in dendrite complexity. These changes observed in the brain were due to premature progenitor pool depletion and early differentiation. Studies conducted on ERK DKO mice by another group also showed altered cortical development, particularly changes in NPC proliferation and neuronal maturation in ERK DKO mice [606]. Likewise Inamura et al found that ERK2 DKO led to proliferation defects in late glial progenitor in the ventricular zone and caused

severe cortical lamination defects as late generated cortical neurons were misplaced and failed to migrate to upper cortical layers [607]. Thus, we see that proper ERK signaling is very important for the normal development of the cortex by regulation of proliferation, cell cycle, migration, differentiation, and neuronal morphology.

As mentioned, ERK dysregulation is associated with autism spectrum disorders. Recently, Pucilowska et al developed a mouse model of 16p11.2 deletion. As mentioned, the 16p11.2 locus codes for the ERK1 gene along with several other proteins. Deletions of this region contribute to 1% of all cases of ASD whilst duplications are associated with schizophrenia. In humans, deletions of the 16p11.2 locus is associated with macrocephaly while duplications are associated with microcephaly. Behavioral studies by Pucilowska et al and others showed that the 16p11.2 deletion mice exhibited anxiety like behavior, had impaired memory, had diminished ultrasonic vocalization to common social cues, and had hyperactivity and repetitive movements which are all phenotypes or comorbidities found in ASD [608-611]. In the 16p11.2 deletion mouse, deletion of 1 copy of the ERK1 gene at E14.5, resulted in a paradoxical increase in the ratio of P-ERK/ERK when compared to WT mice. This ERK "hyperactivity:" was associated with reductions in brain size, cortical area, cortical length, and perturbations in cortical cytoarchitecture which surprisingly mimics the phenotypes seen in ERK2 null mice studied by the same group and reviewed above. Surprisingly, despite having smaller brains, between E12.5 and E14.5, 16p11.2 del mice had increases in the level of PH3 a marker of mitosis in the VZ/SVZ. Yet, despite this early acceleration in proliferation, there was premature exit from the cell cycle of progenitor cells in the 16p cortex suggesting early differentiation. Moreover, reduced divisions of progenitor cells decreased the pool of cells available to become neurons

thereby decreasing brain size. Thus, just like conditional deletion of ERK2, an animal model of ASD with heterozygous ERK1 loss shows alterations in neurodevelopment which leads to abnormal brain structure and behavior. Similar alterations therefore could be occurring in humans with ASD that is associated with ERK defects.

In summary, the RAS/MAPK/ERK pathway plays vital roles in the regulation of basic cellular processes in the body and in the brain. Dysregulation in ERK leads to abnormalities in the brain due to improper proliferation, cell fate, migration, differentiation, and maturation of neural cells. Indeed, mutations in ERK often lead to syndromes with macrocephaly, autism, and cognitive impairments indicating the importance of ERK in normal brain development and function. Thus, it is important to continue study of ERK's role in neurodevelopment particularly in human diseases.

Summary of Signaling Pathways:

There are a multitude of signaling pathways active in a cell that help regulate cellular responses to environment and other signals. Due to the critical roles of these signal pathways, perturbations can lead to a host of diseases from cancer to obesity to neurodevelopmental and neurodegenerative disorders. During neurodevelopment, it is essential that cells properly sense and respond to environmental cues in order to have proper brain structure. Dysregulation in signaling pathways can alter multiple cellular processes and disrupt brain structure, function, and behavior. In ASD, disrupted signaling is often implicated in the pathogenesis. Traditionally, it was believed that each signaling pathway was an independent mutually exclusive set of processes that led to cellular action. However, more and more studies are finding that not only do different pathways cross-talk, regulate, and feedback on each other, but canonical step-wise activation postulated is most

likely not accurate. Thus, dysregulations of one node in a signaling pathway can have wide-spread changes in multiple internal cellular parameters in the cell. This could explain why there are dysregulations in multiple pathways associated with the same disorders and why there are numerous etiologies for disorders like ASD.

Metabolism, Neurodevelopment, and Autism

Thus far, we have seen that EFs and signaling pathways are integral for the regulation of neurodevelopment. Moreover, dysregulations in these molecules are also associated ASD and other neuropsychiatric disorders. In addition to regulating cell morphology, division, and maturity, signaling pathways, EFs along with environmental cues, are also important for fine tuning and changing cellular metabolism. Homeostasis of metabolic processes is integral for proper survival and function of an organism. Thus, the interplay between metabolism, internal growth factors, and cellular signaling together are important for regulation of development and normal brain functioning. In adults, the brain is a particularly energy-intensive organ- taking about 25% of total body glucose for optimal functioning. In development, the early embryo requires the production and expenditure of large amounts of cellular energy for growth, division and differentiation [612, 613]. Thus, alterations in metabolism during development or later in life can have significant impacts on brain functioning and behavior. Indeed, there are many genetic disorders of metabolism that display abnormal brain structure and neuropsychiatric symptoms. Moreover, defects in important nutrients such as folate in the maternal diet have also been associated with developmental abnormalities such as neural tube defects. Thus, the fetal metabolome, which is influenced by fetal genome and fetal environment, can impact neurodevelopment. In addition to the finding of altered neurodevelopment and behavior in genetic metabolic

disorders, new studies have uncovered dysregulated metabolism in individuals with neuropsychiatric disorders including autism, schizophrenia, depression, and bipolar disorder. Thus, metabolism and metabolic status seems to have great impact on the brain both during development and in adulthood. Therefore, studying metabolism can give us further insight into the etiologies of neurodevelopmental disorders and could even help us identify diet and vitamin-based supplements that can mitigate symptoms.

Genetic disorders that lead to alterations in the metabolism of molecules such as amino acids, nucleotides, lipids, and sugars often coincide with neuropsychiatric conditions and altered neurodevelopment [614-616]. These disorders, known as inborn errors of metabolism (IEM), are caused by a single gene defect that blocks a biochemical pathway leading either to the deficiency of an important enzyme or cofactor or the accumulation of toxic intermediate compounds. As early as the 1980s, researchers began to uncover abnormalities such as dysgenesis of the corpus callosum in numerous forms of IEMs [617]. Moreover, many IEMs are associated with neuropsychiatric features such as seizures, intellectual disabilities, and autistic phenotypes. With further research, other forms of cortical alterations have been observed in IEMs though abnormalities in white matter tracts are commonly seen suggesting that myelin or the process of myelination may be particularly vulnerable to metabolic changes. For example, Zellweger syndrome is caused by defects in peroxisomes which are important for the catabolism of fatty acids and for the reduction of reactive oxygen species. It is characterized by neuropsychiatric symptoms such as intellectual disabilities and seizures [618]. In addition to having reduced cortical myelin sheaths individuals with Zellweger's also show changes in brain size, periventricular cortical heterotopias, and abnormal layering of the cerebellum and cortex

which are suggestive of alterations in neurogenesis and migration [618-622]. Indeed, altered migration is a common phenotype seen in peroxisomal disorders showing that proper metabolism is necessary for the normal occurrence of early developmental processes such as neurogenesis and migration [623]. Metabolic pathways associated with mitochondrial energy production can also manifest with severe neurological phenotypes. In children with pyruvate dehydrogenase (PDH) deficiency for example, we again see dysgenesis of the corpus callosum, abnormal cerebellar Purkinje cells, and migration abnormalities such as cortical and subcortical heterotopias [624-627]. Other disorders with migration and/or defects in neurogenesis include genetic defects in fumarate, cholesterol, carnitine, and fatty acid metabolism [614, 615]. On the other hand, disorders of amino acid metabolism do not usually cause brain malformations embryonically. For example, phenylketonuria (PKU) is an IEM caused by a defect in the enzyme phenylalanine hydroxylase which leads to the toxic buildup of dietary phenylalanine [628]. In utero, children with PKU are not generally affected because phenylalanine metabolism can be compensated by maternal phenylalanine hydroxylase. However, PKU can have drastic effects on postnatal development leading to reduced myelination, and alterations in axon growth, dendrite formation, and synapse formation in the cortex [629]. Interestingly, in mothers that have uncontrolled PKU, the excess build-up of phenylalanine in the maternal system is teratogenic to the fetus [630-632]. Children born to these mothers often have microcephaly, low birth weight, hypoplastic corpus callosum, and the presence of heterotopias. In mothers with controlled PKU however, these effects were not seen. Thus, we see that embryonic and postnatal brains are exquisitely sensitive to metabolic changes in both the maternal environment and the fetal environment. Thus, well-regulated metabolism is essential for

proper neurodevelopment and brain structure.

How do metabolic abnormalities contribute to alterations in brain structure and function [614, 615]? One hypothesis is that altered metabolism could cause the build-up of toxic molecules in the developing brain which can lead to altered cell survival or inhibition of proteins and enzymes necessary for normal brain structure. For example, in Zellweger's syndrome, studies have suggested that a toxic metabolite produced by abnormal peroxisomes inhibits the production of a cell adhesion molecule known as L1 which is integral for normal cell maturation and migration [633]. Another hypothesis suggests that altered metabolism leads to altered energy balance in the fetal environment. Studies have shown that aerobic metabolism in the brain increases during periods of neuronal proliferation, differentiation, and migration. Thus, a mutation such as changes in pyruvate dehydrogenase which reduces aerobic respiration can reduce the "energy availability" to these developing cells thereby slowing or inhibiting important developmental processes. As many metabolic disorders alter fat metabolism another hypothesis for why metabolism influences neurodevelopment is by changing the biophysical property of cell membranes. Normal membranes are essential for maintenance of concentration gradients needed for signaling, help cluster receptors and ligands, are necessary for efficient signaling, and are critical to axonal guidance [561, 634]. Similarly, certain metabolites such as cAMP are important second messengers in signaling systems. Thus, increases or decreases in these metabolites or changes in membranes can alter signaling systems which as reviewed previously can alter neurodevelopment.

New studies are showing that dysregulated metabolism is seen in neuropsychiatric disorders [635]. For example, states of metabolic disruption such as diabetes, insulin

resistance, and obesity often occur with disorders such as bipolar disorder, schizophrenia, depression, and Alzheimer's disease. A recent meta-analysis in ASD found that the prevalence of obesity was significantly higher (odds ratio: 1.85, p<0.0001) in individuals with ASD [636]. Nationally, about 18.5% of children are considered to be obese. Studies of weight in children with ASD generally find prevalence of obesity is between 17-40% which suggests a potentially higher prevalence than the general population [637]. However, many of these studies analyzed data acquired from pediatrician's office, national registries, or school reports instead of conducting parallel studies on large sample populations of typically developing children to autistic children to directly compare prevalence. In addition to co-occurrence of ASD with metabolic disorders, dysfunction of energy metabolism, particularly mitochondrial dysfunctions, have been implicated in ASD. One meta-analysis of 18 publications (112 with ASD and mitochondrial disorders) found that the prevalence of mitochondrial dysfunction was 50-fold higher in children with ASD (5% prevalence) when compared to children without ASD (0.01% prevalence). This study used numerous metabolic biomarkers of such as lactate, pyruvate, carnitine, and ubiquinone levels along with known alterations in mtDNA as markers for "mitochondrial dysfunction" [638]. However, it is important to note that these biomarkers are not always specific to mitochondrial dysfunction and could be due to alterations in biochemical enzymes involved in metabolic pathways. Thus, could be a potential "inflation" of the prevalence of mitochondrial dysfunction in ASD. Moreover, 79% of the children with ASD and "altered mitochondrial function" did not possess a known genetic alteration associated with mitochondrial dysfunction. Autism has also been associated with abnormalities in fatty acid metabolism including long chain fatty acids, cholesterol, and poly-unsaturated fatty

acids (PUFA) [639-641]. For example, alterations in the balance of PUFAs such as EPA and DHA have also been observed in ASD while supplementation with these FAs have shown to be beneficial in some studies [642-644]. However, many of these studies showing FA supplementation benefits have small sample sizes (n=5-20), do not control for diet or other medication, often rely on parental reports, and are not based on randomized placebo-controlled trials [645]! Moreover, the few randomized control trials have largely indicated no efficacy [646]. Newer technology has allowed for the unbiased analysis of metabolites in individuals with the use of metabolomics. These studies have observed dysregulations in amino acid metabolism, antioxidant status, nicotinic acid, purine and pyrimidine metabolism, and mitochondrial metabolism in children with ASD [647, 648]. However, these metabolome studies are often conducted on blood or urine samples which may not necessarily reflect brain metabolic status. In children with the IEMs reviewed including those with abnormalities in amino acid, certain purine and pyrimidine, lipid, and vitamin metabolic disorders all have shown symptoms of autism. With some metabolism related disorders alteration in diet or supplementation can lead to beneficial outcomes. For example, in PKU, removal of all phenylalanine from diet can either completely prevent neurological alterations or at least mitigate them [615, 629]. Likewise, in some cases of epilepsy, a ketogenic diet can reduce the number of seizures experienced [649, 650]. Even in some subtypes of ASD, the ketogenic diet has shown to be useful [651-653], though these studies are conducted by one group and has yet to be replicated or assessed by other labs. Overall, metabolic studies in ASD are currently limited by small sample sizes and lack of rigor. However, recent metabolomic analyses and some of the more rigorous studies suggest that metabolism could be altered in ASD. If metabolism in is indeed altered, then

in some cases, supplementation could be used as a therapeutic to help with either comorbid conditions or behavioral problems.

In summary, the metabolic profile of an individual can have great impacts on brain and body. During development, abnormal metabolism can change the way the brain is formed and lead to neuropsychiatric disorders. Likewise, in many neuropsychiatric disorders, metabolic dysregulations have been uncovered. The study of metabolites, in neuropsychiatric disorders, however, has been very limited. Moreover, metabolic analyses are often conducted on blood or urine, which does not always reflect the metabolic state of the brain. Further studies could help us uncover new etiological factors and even new treatments for disorders like ASD.

Regulation of Neurodevelopment: Summary

Thus far, I have individually reviewed some important regulators of neurodevelopment which include EFs, signaling pathways, and metabolism. It is important to note, of course, that none of these systems are acting in isolation. For example, metabolism and energy balance have important roles in the alteration and regulation of cellular signaling pathways. Many cells have a mechanism the sense the AMP/ATP ratio to monitor cellular energy balance. An increase in the AMP to ATP ratio activates a kinase known as AMPK which phosphorylates and activates TSC2 [499]. This prevents activity of mTORC1 and prevents the anabolic processes that this pathway regulates. Likewise, release and response to an extracellular factor can be dependent upon signaling pathway activities in a cell. Thus, while I took a reductionist approach to explain individual regulators of neurodevelopment and disease, it is important to remember that neurodevelopment is a highly complicated process where numerous factors are constantly

interacting to lead to appropriate developmental outcomes. Of course, with so much interplay, sometimes slight differences can change the whole cellular system leading to massive consequences on development. However, in other cases, this cross-talk between numerous systems prevents dire consequences from being apparent due to compensation.

The Rodent Model: Benefits and Limitations:

Thus far, a majority of the studies reviewed on neurodevelopment, its regulation, and disease have been conducted in mice models [654, 655]. As mice are mammals, share 85% of their genes, and have common brain circuits with humans, they are a good model to study basic neurodevelopment and disease. Moreover, as mouse models can be readily genetically modified, including conditional alterations of genes in specific compartments or developmental times, they have greatly enhanced our knowledge about brain development and function. Indeed, the knowledge gained from these models has informed us about everything from signaling pathways to brain development and disease as reviewed above. Moreover, mice have given us the essential platform on which to conduct cause and effect studies in a complex *in vivo* system. However, it is still salient to point out that mice are not humans nor are they close ancestors. Therefore, mouse physiology, brain anatomy, brain development, and behavior are quite different from humans. Indeed, this difference is readily apparent when we look to numerous clinical trials of drugs which show great potential in mouse models but ultimately fail in humans. In fact, by some estimates more than 50% of drugs that show promise in mice have failed in humans [656-659]

In 2014, numerous papers were published in Science and Nature based off of work from the NIH ENCODE project that aimed to compare mouse and human genomes. Overall, mice and humans shared about 70% similarities in protein-coding DNA. However,

there were many variations in DNA and gene expression patterns that were not shared between these species. For example, the regulatory elements and activity of many genes in the immune system, metabolic processes, and stress response vary between humans and mice [660-663]. Of course, these systems are also important for communicating with and regulating brain and behavior, thereby making these species rather different in multiple physiological respects. Even an inspection of the adult mouse and human brain readily shows differences that can be observed superficially. In addition to vast differences in brain to body ratio in humans and mice, we see that mouse brains lack the folds and gyri that are readily apparent in normal human brains. These folds are indicative of greater neocortical expansion in the humans that is not present in rodents [205, 664, 665]. Of course, with neuropsychiatric disorders, defects in the cortex are commonly implicated and thus mice with their smaller and less expanded cortex may not be the best of models. Studies of gene regulation across mice and humans in the brain have also shown vast differences. Zheng et al 2012, looked at 700 genes in human vs mouse cortex by in situ hybridization and found that 25% of these genes had differences in either area of expression or cellular patterning [666]. Glial gene expression and expression of genes associated with neuropsychiatric disorders were also found to be widely different between humans and mice [667], suggesting that the mouse may not be the best representative for study of human neuropsychiatric disease. In terms of development, the gestational period of a mouse is approximately 20 days while human gestation takes 40 weeks! As reviewed, the time stamp for brain developmental processes such as neurogenesis are vastly different in humans and mouse. While neurons are generated in 1 week in an embryonic mouse brain, it takes up to 18 weeks in humans. Moreover, in humans newly derived neurons have to travel farther

distances and cross more densely packed layers of cells to reach their ultimate destination. Humans also have greatly expanded progenitor regions such as the VZ/SVZ containing cell subtypes that are hardly ever seen in the mouse brain. In addition, the outer-SVZ, which barely exists in mice, is a major source for the neurons that populate the expanded cerebral cortex [222, 223, 668-672]. Thus, for neurodevelopment and neuropsychiatric disorders, mice models may fall short in their ability to truly capture and reflect the human condition.

One of the most useful aspects of mouse models is the ability to illustrate the function of important genes on neurodevelopment and behavior. However, alteration of some genes which have devastating consequences in humans have no effect in mice. For example, Lesch-Nyhan syndrome (LNS) is an X-linked recessive disorder characterized by uric acid build up, abnormal catecholamine metabolism, and self-injurious behavior due to hypoxanthine-guanine-phosphoribosyl-transfersase (HGPRT) deficiency. Due to catecholamine similarities to schizophrenia and self-injurious behaviors seen in ASD, scientists attempted to model LNS in mice by knocking out HGPRT. Surprisingly, animals did not display the LNS phenotypes due to differences in metabolic pathways emphasizing the differences between human and mouse systems [673]. Likewise, in humans there are highly penetrant mutations in the gene that encodes neuroligin-3 (NL3) associated with ASDs. However, knockout of NL3 in mice led to no autism like behavior and no alterations in neuronal synaptic transmission [674]. In addition to differential function of disease-associated genes, study of mouse behavior and comparison to human behavior in itself may be flawed. Disorders like autism, which are characterized by defects in behaviors that are uniquely human, mouse models cannot truly capture the human condition. In mouse studies, language is often represented by changes in ultrasonic vocalization, social

impairment by object over conspecific preference, and repetitive behaviors by excessive grooming. While these behaviors may superficially resemble autism symptomology, it is unclear whether the same brain regions control these behaviors in humans and mice. More importantly, it is unclear how analogous any mouse behavior is to human behavior.

Lastly, as I touched upon earlier, most models of ASD are created by the alteration of a single highly penetrant gene. However, about 80% of cases of autism are idiopathic meaning they are genetically undefined. Moreover, researchers have postulated that accumulations of numerous low-penetrant or small effect common variant genes may contribute to many cases of ASD. Either way, idiopathic and polygenic ASDs cannot really be modeled in mice. Thus, due to the multiple limitations posed by mouse models, to truly understand brain development and diseases such as ASD, we need a model system that will allow us to study live human neurons and their development particularly in the context of complex idiopathic and polygenic contributing factors. Importantly, human induced pluripotent stem cell technology (iPSC) provides us with such a model system.

Human Pluripotent Stem Cells and IPSC Technology

As discussed in the introduction to neurodevelopment section, a developing embryo first starts out as a fertilized egg undergoing rapid proliferation. Ultimately, this simple 2-cell fertilized egg develops into a blastocyst. The blastocyst consists of a layer of trophoblast cells that become the placenta and an inner cell mass of pluripotent stem cells which ultimately becomes the embryo. These pluripotent stem cells are characterized by a unique ability to ultimately form almost any cell in the body with the exception of placental tissues. Moreover, these early blastocyst cells can also divide and self-renew. In 1981 Gail Martins and Martin Evans independently made the first embryonic stem (ES) cell cultures

by removing and plating the pluripotent mass of cells from a mouse blastocyst [675, 676]. These cells were able to replicate for extended periods of time in culture, self-renew, and form the cells of every tissue in the adult body including germ cells. About 20 years later, in 1998, using similar methods, Thomson et al derived the first human ES cells from surplus fertilized eggs donated by couples who were undergoing fertility treatment [677]. Much like mouse ES cells, human ES cells (hESCs) were stable in culture for extended periods of time, could self-renew, and could form almost any cell type in the body. The derivation of these ES cells provided researchers with the opportunity to study the live embryonic development of human tissue. Until the derivation of these ES cells, human developmental studies were restricted to analyzing tissue samples derived from donated abortion materials. Of course, the ethical concerns and donor sparsity very much limited these studies. Study of live cellular development in humans were restricted to cancer cell lines like SH-SY5Y. While cancer lines did have some shared similarities to their tissue of origin they also had unstable karyotypes and other genetic alterations that made them less than ideal models. For neuroscientists, ES technology provided an avenue to study live human neurons in culture- a nearly impossible feat before the generation of ES lines.

The first published paper that generated neural cells from hESCs was published by Zhang et al in 2001- 3 years after the generation of human ESCs [678]. This protocol along with optimizations by other groups did allow for studies on human neurodevelopment in vitro. Later, hESCs that had genetic mutations associated with disease were acquired from pre-implantation genetic diagnosis. Using these hESCs, researchers could, for the first time, study abnormalities in the development and function of human neurons derived from disorders like Fragile-X syndrome, Down Syndrome, Huntington's Disease, and Lesch

Nyhan Syndrome [679-688]. Yet, only a few diseases were checked for in these pre-implantation screens and modeling of other genetic disorders were rather difficult in ESCs. Before the invention of genetic editing techniques like CRISPR, genetic editing of human cells was a very low efficiency and costly procedure. While CRISPR did greatly improve the ability to generate mutations in human cells, its first use to edit human cells wasn't until 2014, more than 15 years after the first hESC was derived[689]. Finally, hESC, much like mouse models, could not be used to model idiopathic disorders. This in conjunction with ethical concerns regarding the handling and destruction of human embryos limited hESC studies despite their powerful ability to model human development [690, 691].

In 2006, Takahashi and Yamanaka revolutionized the pluripotent stem cell field by showing that ESC-like cells could be derived from adult mice fibroblasts by transfecting them with retroviral vectors that contained just 4 pluripotency inducing factors: Oct3/4, Sox-2, c-MYC, and Klf4 [692-694]. These ESC-like cells, known as induced pluripotent stem cells (iPSCs), could form all 3 germ layers of a developing embryo and could contribute to different tissues in mice when injected into the blastocyst of a developing embryo. Moreover, they expressed similar markers and had similar morphology to ES cells. This showed that mature, somatic adult cells could be reprogrammed into cells that were "embryonic" and pluripotent. By 2007, the Yamanaka lab and other groups showed that similar techniques could be used to reprogram human fibroblasts into ESC-like cells too [695, 696]. Further advances in iPSC technology now allows for the derivation of these cells from numerous tissue types including white blood cells, keratinocytes, melanocytes, liver, stomach, and neural cells. Moreover, iPSCs have now been derived from numerous species including humans, mice, rats, and other primates. Initially, generation of iPSCs was

conducted by the transfection of constitutively active retroviral vectors that stably integrated into the host genome to introduce the 4 pluripotency factors. This method often resulted in partially reprogrammed iPSCs, left residual activity or reactivation of viral transgenes which could intervene with developmental potential of iPSCs, and elicit reactivation of some of the transcription factor such as c-MYC, which caused the formation of tumors when injected in vivo. More recent advances have allowed delivery of the pluripotency factors using non-integrating viruses like Sendai Virus or through methods that do not use viral or plasmid vectors at all [697]. For example, iPSCs have been derived from both mouse and human fibroblasts by delivery the pluripotency inducing factors as recombinant proteins [698, 699]. However, both the non-integrating and non-viral methods have very low efficiencies of iPSC generation when compared to the retroviral integrating methods (0.1-1% for retroviral, 0.001% for nonintegrating, and 0.0001-0.001% for recombinant protein) [697]. New studies are looking into chemicals such as valproic acid which increase the efficiency of iPSC generation [700]. As the field continues to evolve, further optimization of iPSCs will allow researchers to have easy access to pluripotent cells that they can use to conduct in vitro developmental studies on almost any species.

The advent of iPSCs truly revolutionized the study of disease and development due to the many advantages they have over ESCs. Unlike ESCs, iPSC source material is not limited as they can be derived any time from somatic mature cells. Moreover, iPSCs do not carry the ethical concerns associated with destruction or use of embryos that ESCs do. The most exciting aspect of iPSCs is that they retain the genetic signature of the individual from whom they were derived. This means, for the first time, idiopathic and polygenic disorders can be can be studied by deriving cells directly from humans who have the disease! This

was particularly valuable in the field of neuropsychiatry, where diseased neurons from individuals with genetically complex brain disorders could not be studied directly. Excitingly, iPSCs allow us to study the function and structure of live neurons and even the development of these neurons in the context of disease relevant genetic signatures. Moreover, iPSCs also have opened the gate for personalized medicine approaches in neuroscience. Specifically, with complex heterogenous diseases, we can spend time understanding individualized personalized biology and etiology that may contribute to one person's disorder and then tailor therapeutics to help that person. iPSCs also allow for high throughput screening for disease genes, common molecular abnormalities, and useful therapeutics. This high-throughput approach could allow for classification of neuropsychiatric disorder by molecular pathology rather than behavioral symptoms, allow for early diagnosis and screening for devastating disorder, and unearth new therapeutics that could help individuals with disease function better. Thus, particularly in the context of complex idiopathic disorders, iPSCs are a revolutionary technology. However, it is important to ensure that iPSCs are a good model for human neurodevelopment by comparing them to ESCs. Thus, in the next section, I will discuss studies that have compared hESC and iPSCs, hopefully revealing that iPSCs are indeed a valid model to use to study human development. Then, I will describe different differentiation techniques and model types that have been employed to study neurodevelopment and neurodevelopmental disorders. Lastly, I will discuss relevant studies that have employed these models to assess normal neurodevelopment and neurodevelopmental disorders.

hiPSCs vs hESCs: How similar are they?

While iPSCs have marker expression and morphology that is similar to ESCs,

shortly after their inception, researchers began to ask how similar these two cell types were. Of course, if iPSCs are drastically different from the ES cell they are thought to mimic, utilizing iPSCs to study human development and disease may not be ideal. Moreover, as iPSCs are generated from a procedure that involves changing cell identity and altering DNA function concerns regarding the fidelity of iPSCs emerged [701-704]. During iPSC generation, most of the somatic cells that are plated for reprogramming die and only a few cells emerge as viable iPSC colonies. This indicates that iPSCs are a "rare event" and thus could indicate that the cells that ultimately became reprogrammed were unusual or carried some mutation that allowed for reprogramming. There were also concerns that mutations could accumulate or occur due the reprogramming process that iPSCs undergo. These mutations could alter the biology of iPSCs, lead to deleterious effects on chromosome stability, impact genomic integrity of the iPSCs, and in studies of disease, could lead to phenotypes that arise from the mutation rather than due to disease state. Secondly, as iPSCs are derived from mature cells, there were concerns that the epigenetic imprint of the original cell would not be completely erased or that only partial reprogramming of the cell could be achieved. In 2009, Chin et al found that the transcription profiles of high quality hiPSCs and hESCs were highly similar [705]. Yet, at early passages (lower than P10) about 22% of genes were significantly differentially expressed between hiPSCs and hESC lines. Of these genes 79% were expressed at a lower level in iPSCs than ESCs. These genes had roles in processes such as energy production, RNA processing, DNA repair, and mitosis. On the other hand, genes related to differentiation were more abundantly expressed in hiPSCs than hESCs. This suggested that iPSCs perhaps were in a more "differentiated" and less "proliferative" state than hESCs. Interestingly, comparing hiPSCs to hESCs and

hESCs to the original fibroblast tissue indicated that there was an incomplete silencing of the genes from the original fibroblast and an inadequate upregulation of "ESC" genes in iPSC cells. Upon extended passaging (greater than P40) however, hiPSCs do become more similar to hESCs. Yet, even at late passages, differences between hESCs and hiPSCs persisted which were considered to reflect an "imperfect resetting of somatic cell expression to an ESC-like state," a finding that other studies have also confirmed. Chin et al also took iPSC lines derived by other labs and compared their expression profiles. Strikingly, there were 15 genes that were consistently differential between the iPSCs from all 4 different labs and the Chin lab when compared to hESCs. hESCs can also efficiently differentiate to form multiple cell types of the body including neurons. Likewise, iPSCs have shown to be able to form numerous cell types of the body. However, to be equivalent to an ESC cell, iPSCs should be able to form differentiated cells at the same efficiency an ESC can [706, 707]. The few studies that have applied side-by-side protocols to induce differentiated cells from iPSCs and ESCs have found that in general hESCs more efficiently and reproducibly generate differentiated cells than iPSCs. A study by Hu WEick et al (2010) conducted side-by-side neural inductions on 12 human iPSC lines established through lentiviral, retroviral, or episomal methods in comparison the 5 hESCs lines taken from 5 different labs [708]. After initial neural induction, hESC lines all yielded a similar high proportion of PAX-6 expressing neural progenitor cells (NPCs) (90-97%). In contrast, iPSCs exhibited a lower and more variable neural differentiation ability when compared to iPSCs (15-79% PAX-6 positive cells). Even among iPSC lines derived from the same exact fibroblasts there were vast variations in neural induction (15% PAX-6 vs 50% PAX-6). While the retrovirally induced lines showed the least efficient neural differentiation,

integrating method alone did not account for the large variability and reduced efficiency of neural differentiation. However, despite the difference in efficiency, hESCs and hiPSCs had the same time-stamp for differentiation [702, 708]. For example, both hESCs and hiPSCs took about 15 days in culture to form PAX6+ NPCs, about 1 month in culture to make immature neurons, about 2 months to generate astrocytes, and 2-3 months to generate oligodendrocytes. Moreover, iPSC-derived NPCs were able to respond to the same set of EFs as hESCs to differentiate into regional progenitors and make functional neurons. Thus, both hiPSCs and hESCs are able to differentiate into mature somatic cells of multiple tissue types, however, iPSCs do so with lower efficiency and higher variability than hESCs. Studies have also compared the epigenetic profile of hiPSCs and hESCs. Again, the DNA methylation patterns between hiPSCs and hESCs were highly similar when compared with lines such as fibroblasts. However, studies have detected there is a hypomethylation of CPGs in hiPSCs and that the patterns were suggestive of incomplete epigenetic reprogramming of the differentiated cell-of-origin genome [709-714]. Lastly, studies have also compared the mutational load differences between hESCs and hiPSCs [715-718]. Accumulations of CNVs, aneuploid cells, and alterations in cell cycle genes were largely similar between iPSCs and hESCs with iPSCs bearing no significant increased risk of acquiring mutations. In summary, overall hiPSCs and hESCs share similar transcriptomic, epigenetic, and developmental profiles. However, it seems like iPSCs still retain some of the transcriptomic and epigenetic imprints of the cells from which they are derived. This could potentially mean, iPSCs have less "multipotentiality" than ESCs. Indeed, iPSCs are also less effective at generating differentiated cells. However, despite this, iPSCs are almost like a "less efficient" ESC and thus are a good modeling system to study human

development and disease. Further studies of course are needed to optimize iPSC generation protocols and culturing techniques to reduce the disparity between hESCs and hiPSCs.

The modeling of Neurodevelopment: The Generation of Neural Precursor Cells, Neurons, and Organoids from hiPSCs

The first protocol for the derivation of neural cells from human pluripotent stem cells was published by Zhang et al in 2001. In this study, Zhang et al derived neural progenitor cells (NPCs) from human ES cells. In general, there is no clear consensus on the term "neural progenitor cell" used in the human stem cell culture field [678]. It is often interchangeably used with terminology such as neural stem cell or neural precursor cell and seems to refer to a population of neural epithelial cells that are proliferative, self-renewing, and have the multipotency to make neurons and glia. The NPCs generated by Zhang et al formed neural-tube like structures, expressed NPC markers, and were able to differentiate into neurons, astrocytes and oligodendrocytes. Moreover, when these NPCs were injected in the lateral ventricle of newborn mice, they were able to successfully migrate and integrate into the mice brain forming both neurons and astrocytes! Thus, hESC-derived NPCs were able to recapitulate aspects of neurodevelopment both *in vitro* and *in vivo*. Thus, in 2001, the first opportunity to generate live neurons to study development and disease became possible. Over the years, numerous other protocols for NPCs generation were developed [719]. More recently, 3-D culture of these NPCs as "mini-brains" has also become possible. Some groups have also begun to optimize protocols for the generation of astrocytes and oligodendroctyes, however, this will not be the focus of my studies. The various NPC models that are employed to study neurodevelopment will be described and discussed below.

To generate NPCs, Zhang et al first grew iPSCs as detached colonies grown in suspension known as embryoid bodies (EBs). EBs are 3D aggregates of pluripotent stem cells which allow preservation of the cell-to-cell contact seen in a developing embryo. To reliably generate neural-tube like structures known as neural rosettes, EBs were cultured with FGF2 in media containing serum. Later, other laboratories were able to remove the serum addition into culture media and tested the effects of adding different inductive factors to these embryoid bodies to generate NPCs. Watanabe et al 2005 found that treatment with WNT and Nodal antagonists during the first 5 days of EB culture (serum free) led to selective differentiation of ESCs into neural cells (~90%) [720]. They also found that Wnt3a and Shh treatment altered the fate and determined the subtype of telencephalic progenitor cells that were yielded in culture. Further improvement of differentiation was achieved by plating EBs on coated dishes instead of leaving them in suspension in both human and mice ESCs [721]. This allowed for better control of EB size and led to more efficient NPC generation. The plated method, known as the serum-free EBq (SFEBq), approach has been utilized for developing methods to make NPCs from a variety of brain regions including retina, cerebellum, forebrain and hippocampus [722-725]. This SFEBq method has also been tested in hiPSCs and has successfully induced neural fate in these pluripotent cells too. By 2009, Chambers et al published a paper showing that neural rosettes could be produced directly from PSCs by applying dual-SMAD inhibition to the culture [726]. This "monolayer method" allowed the bypassing of the intermediate EB stage thereby saving both time and technical expertise needed to culture and select EBs. This method was further modified by additions of retinoids which further improved the efficiency of forebrain specification of the monolayer method [727, 728]. More recently, EBs

were embedded into a 3-D extracellular matrix which led to the formation of "mini-brains" known as organoids that exhibited a variety of brain regional identities and self-organized into layers and structures that were similar to the brain [729]. Some organoids had progenitor zones including the SVZ/VZ and even OSVZs which is a progenitor region specific to higher primates. Some organoids even had primitive cortical layering, showed hippocampal-like structures, and had marker expression similar to the developing brain [724]. Thus, these methods presented the first opportunity to study human neurodevelopment in an aggregate model similar to the developing brain. While the organoids are an invaluable method to study development in a 3-D model with regionalization and cell-to-cell contact, organoids systems often take months to culture, require expensive tools and extensive experience to make, and are often low-throughput and high cost.

By and large, the neural induction protocols reviewed were largely pioneered in ESCs and then adopted by the iPSC field. Thus, it is possible that techniques may need to be optimized for use in iPSCs. While there are numerous neural induction protocols, many are improvements or optimizations of previous protocols. Overall, there are two main methods of generating NPCs. In the first method, a 3D embryoid body is utilized as an intermediate to generate NPCs from iPSCs whereas in the 2nd method, iPSCs are cultured in a monolayer and directly induced into NPCs using dual-SMAD inhibitors. As 2D culturing abolishes the cell-to-cell contact that is essential in *in vivo* development, there were concerns that the NPCs generated using monolayer methods were different and inferior to those generated by EB method. Indeed, even now, most iPSC studies of neurodevelopment and disease have mostly utilized EB methods to generate neurons and study disease. In 2017 Chandrasekaran et al compared the NPCs generated by these two

methods [730]. While early formation of rosettes and acquisition of neuroepithelial fate was similar in monolayer and EB NPCs, a higher proportion of EB-derived NPCs were Nestin/PAX-6 positive than monolayer derived NPCs (~94 % vs 83%). Moreover, lower levels of PAX-6 protein were expressed in the monolayer NPCs than the EB-derived NPCs indicating that forebrain neural fate is more efficiently generated by EB method. However, 2-D cultured had more SOX-1 positive cells (NPC marker) and less SOX-9 + neural crest cells than EB-derive NPCs. In terms of differentiation potential, NPCs from both methods were able to efficiently generate cortical neurons that had similar electro-physiological properties. At later time points in culture, NPCs derived from both methods also were able to successfully generate glia at similar rates. However, 5 days after terminal differentiation, neurons derived from monolayer method NPCs had shorter neurites suggesting slower maturation of these lines. Other studies have also found minor disparities between monolayer and EB derived neurons [731]. Interestingly, a study by Chen et al 2013 looked to see whether NPCs that were derived from iPSCs reprogrammed from different somatic cells had different characteristics [732]. Comparison of NPCs induced from iPSCs derived from dental pulp cells vs fibroblasts found that for the most part transcriptomes were similar between these cells. However, dental pulp NPCs had less expression of hindbrain developmental genes and higher expression of forebrain developmental genes when compared to fibroblast NPCs. This suggests that NPCs derived from iPSCs generated from different tissue sources may have slightly different regional cell fates. However, as a whole NPCs generated from different iPSCs or generated by 3D vs 2D methods expressed forebrain NPC markers, were able to differentiate into neurons and glia, and remarkably, when injected into the ventricles of neonatal mice could migrate and integrate into the

cortex as neurons and astrocytes [678, 733, 734]. This and the fact that NPCs follow the general developmental time-window of neuron and glia differentiation seen in the fetal brain suggest that they are a good model for studying early brain development.

While NPCs are an excellent way to model and study neurodevelopment, most studies of ASD and other developmental disorders, utilized NPCs as an intermediate to generate postmitotic neurons for further studies. Generation of these neurons was largely done by allowing NPCs to stay in culture for prolonged time or by use of inductive factors. However, more recently, a new technique known as the "induced neuron" has emerged that allows for generation of neurons directly from either iPSCs or even somatic tissue such as fibroblasts by expression of neuronal factor Neurogenin-2 [735-737]. This allows researchers to bypass the NPC phase to generate neurons and save many weeks of time. While these neurons generate synapses, have electrical activity, and express neuronal markers, it is unclear how similar or different they are from neurons generated through NPC differentiation. Of course, while marker expression and function of these neural cells suggest that they are similar to human NPCs and neurons, it is important to compare these iPSC derived NPCs and neurons to the analogous cells in the human brain to ensure they are an appropriate model to study brain development and disease. In 2012 Mariani et al cultured EB cells using the SFEBq method for up to 50 days to produce neural rosette-like structures that contained multiple layers of cells including NPCs, radial glia, and early neurons [738]. The gene expression profile of these neural structures was compared to tissue acquired from post-mortem brains at 15 different developmental stages. The 50-day multilayer aggregates showed the highest correlation with human cerebral cortex at 4-10 post-conception weeks with an enrichment for genes expressed in the frontal regions at 8-

10 weeks. Thus, the developmental time-point of the cell correlated most closely to the early developing cortex where events such as proliferation, migration, and differentiation are occurring. In 2015, Brennand et al used EB methods to generate NPCs which they later dissociated and studied in a monolayer (unlike Mariani et al who kept the NPCs as a 3-D structure) [739]. Brennand et al compared NPCs and neurons derived from their iPSCs to the microarray gene expression profiles of the developing human brain found on Allen BrainSpan. In general, hiPSC derived cells shared the most similarity with 1st trimester human fetal brain tissue. Specifically, hiPSC NPCs were most similar to fetal brain tissue 8-16 weeks post conception while neurons that were in culture for 6 weeks resembled the fetal brain between 10-24 weeks. Likewise, Stein et al in 2014, compared primary fetal NPCs (derived from live fetal tissue), hIPSC derived NPCs, hESC derived NPCs, and SY5Y cells to transcriptomic data acquired from developing fetal brains [740]. The primary fetal NPCs matched strongly with cortical developmental periods from early to mid-fetal time points (8-24 weeks). Just as with primary fetal NPCs, all 3 other neural cell types also matched most closely with germinal zones (VZ/SVZ) of the cortex in mid-fetal development. However, SY5Y cells showed the least degree of matching while hESC-derived cells matched relatively well to in vivo cortical development. There was some variability in the hiPSC derived cells depending upon lab and culture but overall these cells were again matching fetal brain. Even after months in culture, neither hESC derived or hiPSC derived neural cells did not become similar to adult or even postnatal brains. The "oldest" neurons generated matched closest with week 24-27 fetal brain (mid 2nd trimester). These studies along with studies along with others suggest that neurons generated from iPSCs are immature and much more similar to 24-week-old fetal neurons than adult

neurons [741]. NPCs derived from PSCs represent an earlier developmental window that more closely matches the mid-to-late first trimester. Thus, iPSC derived cells may be better suited to study early neurodevelopment and disease susceptibility than to study functional synaptic alterations that may be present in mature neurons.

iPSC derived neural cells provide us with the perfect opportunity to study the early neurodevelopment in humans. For developmental disorders like ASD, this provides us with great insight into what processes may be going awry early in development to lead to disease. As mentioned, in mouse models, these studies of early neurodevelopment are very few, and iPSCs could be used to fill the knowledge gap that exists regarding the aberrations that occur in early development in neuropsychiatric disorders. Surprisingly, many hiPSC studies of NDDs have focused on in-depth analysis of post-mitotic neurons. Indeed, while these studies have found aberrations in these neurons, NPCs and their functions have been largely overlooked. However, since 2010, numerous studies have been published on developmental pathology in everything from monogenic diseases to idiopathic disorders like schizophrenia, ASD, and bipolar disorders. Excitingly, these studies have found fascinating differences in disease neural cells that were not always seen in mouse models. Moreover, some of these studies were even able to test potential therapeutics in iPSC models. Overall, unlike mouse models, iPSCs have provided us with the ability to study idiopathic disorders and human neurodevelopment in a simplified culture model system.

iPSC studies of ASD and Other NDDs:

Some of the earliest studies of hiPSC models of neuropsychiatric diseases were conducted on the highly penetrant monogenic developmental disorders such as Rett Syndrome (RTT), Fragile-X syndrome and Timothy Syndrome [742-744]. These studies

largely derived NPCs and neurons using the EB method. In 2010 Marchetto et al derived iPSCs from the fibroblasts of 4 female patients with Rett syndrome and 5 control individuals [742]. While NPCs were generated in this study, the only experiment this group conducted to study the cells was to do FACs analysis of the percentage of cells in G1, S, and G2/M at a single passage. No differences were found in the percentage of cells in each phase between WT and RTT NPCs. No cellular assays or assessment of NPC morphology was conducted. The neurons derived from Rett patients had reduced glutamatergic synapses, had smaller soma sizes, had lower number of dendritic spines, and had changes in the frequency and amplitude of mIPSCs and mESPCs. The RTT neuronal phenotype could be rescued with IGF-1 treatment and with increasing MECP2 expression. Thus, even though Rett Syndrome doesn't manifest until 6-18 months of age, fetal-like iPSC derived neurons from Rett patients showed abnormalities. Shortly thereafter, a study on Timothy Syndrome was published by Pasca et al in 2011 [743]. Timothy Syndrome (TS) is a syndromic ASD caused by a mutation in an L-type calcium (Ca2+) channel. Surprisingly, in addition to the expected defective Ca2+ signaling, iPSC derived neurons from TS patients displayed changes in catecholamine biosynthetic enzyme, tyrosine hydroxylase (TH), which subsequently led to increased norepinephrine (NE) and dopamine (DA). Excitingly, excess NE and DA production was reversed by treatment with an atypical L-type Ca2+ channel blocker, roscovitine. However, when TS channels were expressed in transgenic mice, the Ca2+ signaling defects were observed but the TH dependent changes were not found, illustrating the value of using a human model to study neuropsychiatric disorders. Further studies in 2013 on TS iPSCs from the same group uncovered dendritic spine abnormalities and abnormalities in cytoskeletal protein RhoA [745]. In the original

2011 study, NPCs were derived from TS patients. The transcriptome and gene expression of these NPCs were compared to iPSCs and neurons which showed that NPC gene expression was more similar to neuronal than iPSC. Moreover, NPCs from a single TS patient was also assessed for proliferation and migration defects and none were found. However, the 2014 transcriptomic analysis of TS NPCs and neurons suggested that genes involved in proliferation were upregulated in TS NPCs while differentiation and morphogenesis genes were downregulated [746], suggesting that more careful analysis of NPCs could uncover earlier developmental defects. Neurobiological analysis of neurons derived from Fragile-X syndrome (FXS) is fairly sparse. Transcriptomic analysis of NPCs and neurons derived from FXS patients suggests defects in the ability of NPCs to differentiate and in neuronal maturation [575, 688, 747] . However, again the one study that has looked at neurobiology of FXS did not assess NPCs at all but found that there were neurite outgrowth differences in post-mitotic FXS neurons (Doers et al). More recently, a study of iPSC derived neural cells from a patient with tuberous sclerosis did study NPCs to find proliferative defects [748]. Alterations in glial and neuronal development were also seen. Thus, in summary, iPSC studies of monogenic disorders were able to uncover interesting changes in post-mitotic, differentiated neurons derived from individuals with disease. This indicated that as early as fetal development individuals with these diseases have abnormal neurons. Moreover, in some cases, studies in a human model were able to uncover defects that rodent models were not able to show, indicating the value of iPSCs. However, a majority of these studies have barely characterized NPCs derived from these patients thereby missing the study of an earlier developmental window.

In addition to monogenic disorders, other genetically defined forms of autism

including disorders caused by copy number variations (CNVs) in genes such as the 16p11.2 deletion and duplication syndrome and Phelan McDermid Syndrome (22q13.3 deletion Syndrome), have been examined. There are also defined forms of autism that arise in families due to the presence of a rare genetic variant in genes such as neuroligins. In 2013, Shcheglovitov et al studied iPSC-derived neurons from 2 patients with Phelan McDermid syndrome [749]. Phelan McDermid syndrome is usually caused by heterozygous deletions in the 22q13.3 region which contains the SHANK3 gene that encodes a scaffolding protein in the postsynaptic density. Individuals with this deletion have a higher risk for ASD. The iPSCs were generated from fibroblasts and neural induction was done through monolayer methods. Again, this study focused on post-mitotic neurons and found reduced amplitude and frequency of spontaneous synaptic events, had decreased number of synapses, and reduced expression of glutamate receptors. Restoration of SHANK-3 levels and treatment with IGF-1 both rescued the excitatory synaptic transmission defect in these neurons. In 2016, the Sudhof lab also studied neurons derived from iPSCs of individuals with Phelan McDermid Syndrome and found reductions in neurite length, reduced number of primary processes, reduced neurite branching, altered dendritic arborization, altered, intrinsic electrical properties, and alterations in synaptic transmission [750]. More recently, the same group that published the 2013 studies on 22q13.3 chose to study neurodevelopmental phenotypes in immature neurons rather than in mature-synapse forming and electrophysiologically active neurons to better understand the role of SHANK3 in early development [751]. Again, despite having access to NPCs, which are also an excellent model for early neurodevelopment, the author again focused on post-mitotic neurons. This not only took longer culture times but also led to missing developmental phenotypes like

proliferation. However, the immature 22q13.3 neurons had smaller cell bodies, more extensively branched neurites, and reduced motility (migration) when compared to controls. Thus, Shank-3 has a critical role in early neurodevelopment including regulation of early neurite outgrowth, cell size, and migration. In 2017 Deshpande et al published the first study of 16p11.2 deletion and duplication patient derived neurons [752]. NPCs were derived in this study and proliferation was studied by Edu incorporation assay. Surprisingly, despite macro and microcephalic phenotypes observed in the deletion and duplication respectively, there were no changes in cell proliferation in either set of NPCs. Neurons from the 16p11.2 deletion cells showed increased soma size, increased dendrite length, increase mEPSC amplitude, and reduced synaptic density. On the other hand, while duplication neurons also showed increased mEPSC amplitude and reduced synaptic density, soma size and dendrite length were lower in these neurons. Thus, increased and decreased dosage of the genes in the 16p11.2 locus lead to common functional and synaptic defects but differential morphometric defects in neurons. Lastly, there are few iPSC studies on rare variant genes associated with ASD, however there are groups currently working on these models- though data has not yet been published. One study looked at one individual with heterozygous deletions in CNTNAP2 [753]. Compared to NPCs from unaffected parents along with 3 unrelated control, NPCs derived from the individuals with CNTNAP2 deletions showed significantly reduced radial migration. Thus, by using NPCs, this group detected an aberration that could not be found by using mature neurons. In summary, much like the monogenic diseases, CNV disorders associated with ASD and schizophrenia can also be modeled using iPSCs. These studies have revealed abnormalities in post-mitotic electrophysiologically active neurons similar to the monogenic disorders suggesting

perhaps that excitatory synaptic transmission changes may be a common pheneotype in neurodevelopmental disorders. Moreover, by studying deletions and duplications together, studies have uncovered the effects of altered gene dose on human neural development.

iPSC studies of Idiopathic ASD and other NDDs:

Of course, one of the most exciting things about iPSCs is that they retain the genetic signature of the individual from whom they are derived. Thus, iPSCs allow us to study idiopathic and polygenic disorders for the first time. However, initially, the heterogeneity of these diseases largely limited study of idiopathic disorders using iPSCs. In 2011, one of the first idiopathic iPSC papers was published by Brennand et al on 4 patients with schizophrenia (SCZ) and no known genetic mutations [754]. Fibroblasts were acquired from these patients and reprogrammed into iPSCs via lentivirus. iPSCs were subsequently induced into NPCs or neurons using EB methods. This study found that SCZ neurons had reduced connectivity, which could be rescued by treatment with the antipsychotic loxapine. Moreover, SCZ neurons had a decrease in number of neurites, and slightly decreased PSD95 synaptic density. Interestingly, gene-expression analysis of NPCs and neurons showed perturbations in glutamate, cAMP, and WNT signaling pathways. Thus, Brennand et al's study is also one of the first studies of iPSC derived neural cells that implicated signaling abnormalities in regulation of neurodevelopment. Thus, Brennand et al showed that despite having no known genetic similarities, idiopathic SCZ patients in this study had similar defects, some of which (but not all) were observed in post-mortem studies and animal models. After the publication of this study, Brennand et al published another paper, this time focusing on the NPCs from the same set of patients in their 2012 study [739]. The NPCs derived from control and SCZ patients had no difference in doubling time, and in

the percentage of cells in G1, S, or G2/M phase, suggesting no differences in proliferation. Network analysis of gene expression of control and SCZ NPCs showed perturbations in genes associated with neuronal maturation and cellular adhesion. Moreover, proteomic analysis revealed alterations in oxidative stress proteins, cell adhesion proteins, and cytoskeletal remodeling proteins (like cofilins) Interestingly, the NPC gene signature overlapped significantly with the gene signature of iPSC-derived 6-week old neurons from these patients. This suggests that the molecular events contributing to SCZ were established even before differentiation into post-mitotic neurons, providing further evidence of the value of studying NPCs. Using 3 independent assays, including neurosphere migration, microfluidic migration, and laminin spot-chaining, aberrant (reduced) migration was also observed in SCZ NPCs. The aberrant migration could not be rescued with co-culturing with control NPCs or murine tissue indicating a cell autonomous defect. Characterization of the migrating cells revealed a majority of them were Tuj1 positive immature neurons though Nestin positive cells were also capable of migration. Thus, in 4 patients with idiopathic SCZ, both NPCs and neurons showed abnormalities indicative of altered neurodevelopment. This showed that idiopathic disorders, despite there heterogeneity, may have some phenotypes in common. Moreover, studies by Brennand et al showed the value of studying NPCs neurobiology in additional to neuronal abnormalities.

In 2015, the first iPSC study of non-syndromic ASD was published by Griesi-Oliveira et al [755]. In this study iPSCs were derived from a single individual with a de novo mutation that lead to disruption of the cation channel TRPC6. Before this paper, TRPC6 had not been implicated in the pathogenesis of ASD. Neurons and NPCs were derived by EB method and NPCs were studied for proliferation differences using FACs cell sorting.

Again, there were no differences in the percentage of cells in G1, S or G2/M in TRPC6 mutant cells vs control cells. Calcium signaling revealed reduced Ca2+ influx into TRPC6 mutant NPCs. Neurons derived from this individual had shorter neurites that were less arborized and had less dendritic spines. These defects were fixed by treatment with a TRPC6 channel agonist or by genetically increasing TRPC6 levels via viral induction. In 2015, one of the first organoid based studies of idiopathic ASD was published by Mariani et al [756]. Mariani et al focused on individuals with macrocephaly and ASD in order to pick an endophenotype that could help reduce heterogeneity within the cohort. iPSCs were derived from fibroblasts of members of 4 families that each included an ASD proband with macrocephaly, and 1 to 3 unaffected first-degree family members. Organoids were generated from 2-3 iPSC lines per person using a modified version of the free-floating SFEBq method. The organoids generated had autonomously organized layers of radial glia cells, intermediate progenitors, and neurons. After 11 days of terminal differentiation (TD11) the organoids were composed of polarized proliferating progenitors expressing radial glial markers NESTIN, SOX2, BRN2, and PAX6. The radial glia were mitotic on the luminal side and produced immature neurons expressing TUJ1 and DCX. AT TD31, mature NeuN+ neurons accumulated on the basal side of the radial glia (similar to layering found in the developing cerebral cortex). Comparison of organoid transcriptomes to the Allen BrainSpan human developmental data showed that TD11 organoids closely resembled the human brain during early fetal development (9 weeks post-conception) while TD31 organoids had significant similarities to early 2nd trimester human fetal brain samples (13-16 wks post conception). The cells in these organoids were more similar to human dorsal telencephalon including the cerebral cortex and hippocampus. The ASD organoids

had a significant upregulation in transcription factors associated with the acquisition of neural cell fate and precursor cell proliferation. There were also upregulation in members of neural cell adhesion family and genes involved in cytoskeletal regulation of many cellular functions including neurite outgrowth, axon guidance, cell proliferation, and migration. At TD11, ASD-derived organoids showed a transient increase in size, indicating potentially increased proliferative rates in early development. This size difference normalized by TD31. Moreover, BrdU incorporation experiments showed a significant decrease in cell-cycle length in ASD derived NPCs. Unlike many studies that found reduced synapse formation in ASD, Mariani et al found increases in Synapsin puncta in ASD derived neurons. Moreover, unlike other studies, glutamate synapses were unchanged yet, there was an increase in GABAergic synapses. Thus, it seems that ASD cells that have NPCs with proliferative defects seem to show different neuronal phenotypes than neurons derived from individuals whose NPCs have no proliferation defect. This could suggest that there are different subtypes of ASDs and NDDs that cluster based on NPC proliferation. In addition to these two studies, two other studies were published on idiopathic ASDs in 2016 and 2017 [757, 758]. One study by Marchetto et al used a cohort of 9 individuals with macrocephaly and idiopathic ASD to find alteration in NPCs proliferation and NPCs WNT signaling. Neurons from these patients also displayed reduced synaptogenesis and functional defects in neuronal network [757]. Interestingly, neuronal network defects were rescued by IGF-1. In 2017 Liu et al studied 4 male patients with idiopathic ASD excluding patients with severe intellectual disabilities, seizure disorders, or known syndromes or malformations. iPSCs were derived from fibroblasts and differentiated into NPCs using EB methods [758]. The NPCs were differentiated neurons and studies were conducted on

neurons 80 days post-differentiation. Compared to neurons from unaffected siblings, neurons from ASD patients had slightly increased synapsin, PSD-95, and VLGUT puncta. Moreover, ASD neurons also displayed altered excitability, aberrant Na+ and K+ channel currents, and alterations in genes associated with synaptic transmission. Liu at al did not study NPCs from these patients. Thus far, the studies conducted in patients with idiopathic ASD have shown relatively similar phenotypes for patients within each study. Moreover, comparing patients from all the studies, we see that in general, ASD and other NDDs seem to be characterized by alterations in neurites, dendritic spines, synapse formation, and neuronal excitability. However, there are differences between patients in different studies on the direction of these alterations.

Summary of iPSCs and iPSC studies:

iPSCs are truly a revolutionary technology. Not only do iPSCs allow us to study neurons and their development, these studies can be conducted in the context of the complex genetics that are often associated with neuropsychiatric disorders. In ASD and other NDDs, use of iPSC technology has revealed numerous defects in post-mitotic neurons derived from patients with monogenic, CNV, and idiopathic versions of ASD. Overall, these studies have uncovered some common alterations in neurites, dendritic spines, synapse formation, and electrical activity in ASD-neurons. Some of these alterations have been previously described in mouse or post-mortem studies, however, a few discoveries have been unique to the live human neurons that we have been able to study for the first time. Yet, while iPSCs have been such as useful technology to study early neurons, they are not the perfect model [759]. As iPSCs are a relatively new technology, there are still technical challenges in working with these cells. For one, researchers have

noted that there can be vast differences in phenotypes between iPSCs that are derived from the same original cells. Moreover, even iPSC colonies (clones) that emerge from the same reprogramming experiment can have different behavior. Because of this, substantial experimental variation between iPSC lines derived from the same person can arise. Further variability is introduced during induction into cells such as NPCs. Moreover, both the iPSC generation process and the NPC generation process are relatively low efficiency and sometimes can lead to generation of contaminant cells that can alter results. Thus, often multiple iPSC derivations or clones must be studied and multiple neural derivations from these clones must be used in experiments to ensure that phenotypes are an expression of patient biology rather than an artifact of culture. This is both expensive and time consuming. iPSCs are also prone to acquiring mutations in culture, and thus, this needs to be screened for carefully too. Secondly, as reviewed, there are concerns that iPSCs retain "epigenetic imprints" of the cells they were originally derived from. Thus, it is possible that the characteristics of the original tissue may appear in iPSC-derived cells like neurons. Thirdly, many iPSC studies have used post-mitotic neurons to study disease phenotypes. These studies sometimes have drawn conclusions that the neuronal aberrations observed in the dish may be present in the individual from who they were derived. However, iPSC-derived neurons are more similar to fetal brain cells than to adult or post-natal brain cells. Thus, these studies are largely a reflection of what may occur in the developing fetal brain. This concern is particularly relevant in the study of degenerative disorders where aged neurons would be a more appropriate model. There are also questions on selection of best patient and control groups. Early studies on iPSCs did not always consider possible confounds such as sex, age, and genetic background differences between controls and

patients. Some researchers suggest a mix of both unrelated controls along with unaffected family members represent the best controls for studies. Lastly, due to the work that it takes to make iPSCs, the number of clones and neural inductions needed to get consistent data, most iPSC studies are very underpowered and thus techniques to be become faster and perhaps automated are needed for more effective use of this model system. (Falk 2016). There are some cases where iPSC models are not as effective as ESCs for modeling certain diseases. For example, in the cases of genetic aberrations that lead to early lethality, iPSC derived from individuals that survive to term would not be representative as they are reflective of an exception. Likewise, in the case of disorders like Fragile-X syndrome, ESCs with this mutation often expressed FMR1 mRNA until they are differentiated into a neural fate. This is suggestive of a developmental fate based transcriptional silencing of FMR1. However, iPSCs derived from individual with Fragile-X do not express FMR1 protein. Thus, iPSCs may not be the best model to study developmental transitions from pluripotent to differentiated cells [701]. Lastly, iPSCs derived neural models are still *in vitro* systems, thus the result derived from iPSC cannot fully reflect the complicated events that occur in an in vivo system. The iPSC field as a whole however has only continued to improve since its inception. Newer technology, optimization of protocols, automation, and careful experimental design are largely helping overcome the technical caveats associated with iPSCs. Moreover 3-D modeling systems are allowing iPSCs to become more similar to the in vivo tissue they model. Thus, despite some issues, iPSCs are still the best model system we have to study the human neurodevelopmental phenotypes of complex heterogeneous diseases.

Chapter 2: Rationale, Hypothesis, and Goals

Autism is a disorder of brain development. Despite decades of study, elucidation of ASD etiology has been hampered by inability to study human neurons, the disorder's heterogeneity, and the relevance of animal model systems. A look at the brains of individuals with ASD in either post-mortem or MRI studies show defects that are suggestive of dysregulation of early embryonic and post-natal development. Newer genetic studies have also suggested that ASD risk genes largely converge upon the cortex of developing humans at between weeks 8 and 24 in utero. Yet, despite decades of mouse studies, an overwhelming majority of studies in autism models have focused on postnatal development or adult synaptic transmission defects in autism. Thus, studies looking at early developmental processes such as proliferation, migration, and early differentiation, which are essential to build the brain, are largely limited. The few studies that did focus on early development, found that alterations in brain structure and function associated with NDDs began as early as the initial formation and patterning of the neural tube. By the early to mid-2000s, the derivation of hESCs and later iPSCs bestowed us with the ability to study live human neural cells in culture for the first time. Specifically, in the case of iPSCs, cells could be derived from individuals with complex heterogeneous disorders, allowing insight into these disorders for the first time. Studies indicate that iPSC derived neural cells largely resemble cortical cells of embryonic humans from weeks 8 to 24. Thus, these cells are an excellent model to study early human neurodevelopment, particularly in the context of disease. Yet, despite all the developmental potential of iPSCs, these iPSC studies have largely focused on post-mitotic neurons. While these neurons are fetal in nature they are indeed post-mitotic and thus cannot be used to study developmental processes that occur

before terminal differentiation. The few studies that have looked at NPCs in ASD have focused largely on proliferation. However, almost no published studies to date have looked at the early differentiation phenotypes and migration phenotypes of NPCs in ASD.

At the inception of my project, there were only about 5-10 studies in total that utilized iPSCs to study neurodevelopment. None of these studies were focusing on idiopathic autism or CNV based ASDs. Furthermore, there were no studies that compared different sub-types of ASD to one another. Moreover, as mentioned, most of these studies had focused on post-mitotic neurons and largely neglected to deeply characterize early neural phenotypes in NDDs. The major exception was the Brennand et al study in 2015 that focused on SCZ NPCs [739]. Thus, the initial goals of my project were to characterize basic developmental processes in idiopathic autism NPCs compared to sibling controls. At the time, our study design was one of the only ones using siblings as controls to reduce genetic heterogeneity. Based on post-mortem, imaging, and the few mouse studies of early neurodevelopment in ASD, I hypothesized that ASD NPCs could have defects in processes such as proliferation, migration, or early neurite outgrowth. I chose to focus on migration and early neurite outgrowth which are studied even less than proliferation in cortical development. As the iPSC field was in its infancy and NPC biology was rarely studied, my first goal was to establish and optimize culture conditions to study neurite outgrowth and migration. Then I utilized these methods to study 3 randomly selected sibling-pairs from our cohort of 8 sibling pairs to understand how common defects in NPC migration and neurites were amongst our different patients. It was unclear whether all patients studied would have similar defects or common neurobiology due to the heterogeneity of ASD.

Now, in 2018, there are many more studies of ASD and other NDDs using iPSC

technology. There are even 4 studies that have focused on looking at neural cells derived from individuals with idiopathic ASD [755-758]. Interestingly, most of these studies of idiopathic ASD have selected for patients who have macrocephaly as a mechanism to reduce inter-patient heterogeneity. However, only about 20% of the ASD population had macrocephaly, and thus, more studies need to be done on other "subtypes" of ASD to get a broader view of phenotypes associated with idiopathic ASD. Our cohort was selected based on language endophenotype rather than macrocephaly. That is, all the patients in our cohort also have a primary relative who has a language disorder known as specific language impairment. Moreover, to reduce heterogeneity we have selected patients who are most severely affected. Even with these new studies, there remains a gap in the understanding of NPC neurobiology in ASD. While 3 out of 4 of these studies have checked for proliferation defects in NPCs, the bulk of these papers have focused on neurons that have been post-mitotic for weeks and are capable of more mature functions like synapse formation and firing of action potentials. In addition, very few of these studies have looked at the effects of important developmental regulators such as neurotrophins or FGF on cell biology. The one exception is the use of IGF-1 to rescue phenotypes in certain models. In mouse models, altered responses to EFs have been seen in ASD and has been linked to abnormal neurobiology. Thus, based on this, I hypothesized that the idiopathic ASD-NPCs from our cohort would have abnormal responses to important EFs. Thus, in addition to studying basic neurobiology in control conditions, I studied both neurite outgrowth and migration under the stimulation of numerous EFs. These studies not only help us understand the roles of these EFs in early human development but also, the use of EFs could help uncover defects that may not be present in control conditions and are only

present when the cell is challenged. Thus, many ASD-iPSC studies could be missing subtle differences in cell biology between ASD and unaffected cells due to most studies being done in control conditions.

iPSC based studies of ASD have also largely neglected to study underpinning subcellular and biochemical mechanisms that may contribute to the neurobiological defects seen in ASD neurons and NPCs. In general, these studies have done transcriptome analyses to show alterations in genes associated with cell adhesion, signal transduction, or oxidative stress but these "omic" analyses were rarely followed up with western blots, other protein-based studies, or functional studies. In addition, the metabolism of neural cells in neuropsychiatric diseases has yet to be studied in ASD. In mouse models and in blood samples from humans with NDDs, defects in signaling pathways and metabolism have been commonly observed in ASD. In fact, studies that reviewed and integrated numerous studies from mouse and humans have suggested that signaling pathways are a major point of convergence in ASD. Thus, I hypothesized that NPCs derived from our idiopathic ASD cohort would have defects in important signal transduction pathways. Moreover, I postulated that these signaling abnormalities would be contributing to the developmental dysregulation observed in ASD lines. Furthermore, I posited that targeting dysregulated signaling could potentially ameliorate the neurodevelopmental defects seen in our cells. Thus, I analyzed 3 pathways commonly associated with ASD (PKA, mTOR, MAPK) and analyzed them via western blots. Moreover, I used utilized agonists and antagonists to manipulate these pathways to observe the effects signaling had on migration and neurite outgrowth. Lastly, I applied metabolomic analyses to assess one sibling pair from our family to see if dysregulations in metabolism were also observed in idiopathic ASD.

While the initial goals of my project were to characterize idiopathic ASD patients, we were lucky enough to acquire somatic cells from 3 patients with a genetically defined form of ASD, the CNV 16p11.2 deletion patients. This put our lab in the unique position of being able to study and compare two distinct types of ASD and understand whether NPCs developmental phenotypes, EF responses, and signaling aberrations were common or different amongst these ASDs. Moreover, with the 16p11.2 patients, we also acquired unaffected unrelated controls, thereby providing 2 control groups for both cohorts of our patients. Other than a very recent paper by Deshpande et al 2017, which compared neurons derived from 16p11.2 deletion and duplication patients, no studies published have compared between different types of ASD [752]. Due to the heterogeneity of ASD, I anticipated that the 16p11.2 cells would most probably show different behavior than the ASD cells however, I had no certain hypotheses about the cells.

Ultimately, my studies aim to uncover neurobiological and biochemical defects in ASD that are present before the terminal differentiation into neurons. This gives insight in the cellular behavior of ASD neural cells in a developmental window that has rarely been studied in both mice and humans. The methods utilized in my study are described in the next chapter. Moreover, in the results section, I have also described the many steps taken to establish and optimize NPC culture and experimental methods.

Chapter 3: Materials and Methods

Cohort Characteristics

Idiopathic Autism (1-ASD) Cohort

Blood samples were collected from eight individuals with idiopathic autism and their unaffected sex-matched siblings as controls. These individuals were selected from a larger cohort of 85 New Jersey families recruited by collaborator Dr. Linda Brzustowicz. Each family has five members where there is one individual with autism (proband), an unaffected sibling (Sib), and a 3rd individual with specific language impairment (SLI). Each family member was extensively phenotyped with a battery of behavioral tests by the [760, 761] lab. To be included as an autism proband in the larger cohort, the individual could NOT have any known genetic mutation contributing to autism (Fragile-X, Rett, TSC). Furthermore, the autism proband was required to meet the criteria for Autistic Disorder on two of the three following measures: 1) Autism Diagnostic Interview-Revised (ADI-R), 2) Autism Diagnostic Observation Scale (ADOS), and Diagnostic and Statistical Manal IV (DSM-V). The SLI individual was identified using the following inclusionary/exclusionary criteria: 1) A core standard score of ≤ 85 on an age appropriate version of the Comprehensive Test of Language Fundamentals OR a subtest score of at least 1 standard deviation below peers on 60% of language measures along with a significant history of language and reading difficulties. 2) A non-verbal IQ score ≥ 80 on the Wechsler Abbreviated Scale for Intelligence. 3) Hearing within normal limits. 4) No motor impairments or oral structural deviations affecting speech or non-speech movement of articulators as assessed by a speech-language pathologist. 5) No history of autism or apparent neurological disorders such as mental retardation, seizures, or brain injury as

determined by parental report. ADI-R and ADOS were also administered to formally rule out ASD. Finally, all family members were given age-appropriate measures of language and reading including: The Clinical Evaluation of Language Fundamentals (CELF-4), a Comprehensive Assessment of Spoken Language (CASL), The Comprehensive Test of Phonological Processesing (CTOPP5), Gray Oral Reading Test (GORT-IV), The Woodcock Reading Mastery Test-Revised, and the Wide-Range Achievement Test-3 (WRAT-3). All family members were also assessed with the DSM-IV and ADOS to ensure that unaffected siblings did not meet criteria for Autism. To reduce heterogeneity, the 8 families with the most severely affected individuals (ADOS scores 1 or 2) with sex-matched SLI, sibling, and ASD individual were selected. Lymphocytes were isolated from collected blood samples and cryopreserved.

16p11.2 Deletion Autism & NIMH Control Lines

Fibroblasts and lymphocytes were acquired from two males and one female patient with the 16p11.2 deletion and autism. These individuals were derived from the Simon's VIP cohort. Data derived from 16p11.2 individuals will be compared to two control groups: Siblings from the Idiopathic cohort and control individuals acquired from the NIMH Regenerative Medicine Common Fund. Control individuals from NIMH were confirmed to have no known disease associated genetic mutations at birth.

Generation & Validation of iPSCs

iPSCs were made by Dr. Percy Yeung in the Lu Lab. iPSCs were generated by infecting cryopreserved lymphocytes with a non-integrating Sendai virus containing the four Yamanaka Factors: SOX2, OCT, KLF and a temperature sensitive C-MYC. First, lymphocytes were plated onto dishes coated with an anti-CD3 antibody and IL-2 to allow

for selective proliferation of T-lymphocytes. After five days, T-cells were infected with the Sendai Virus containing the Yamanaka factors for 24 hrs. The infected T-cells were then re-plated onto mouse fibroblast feeder cells, and media is removed and replenished daily. After 20-25 days clusters of iPSC colonies known as clones were formed. Five to ten clones were picked manually per patient. Morphology and TRA-1-60 live staining were used for selection of clones. selected clones were then expanded and cryopreserved.

iPSCs for the 16p11.2 cohort were generated by RUCDR from lymphocyte, erythroblasts, and fibroblasts provided by the Simons VIP collection, using Sendai Virus. NIMH control iPSCs were generated from CD34+ cord blood by Episomal Plasmid method. Cryopreserved iPSCs for the 16p11.2 and NIMH control individuals were stored, expanded, and then further cryopreserved by Millonig lab for future use.

Once iPSCs were generated or acquired, they were characterized for pluripotency using immunocytochemistry and QRTPCR for the following markers: NANOG, OCT4, TRA-1-60, SSEA4, CD24, and E-Cadherin. iPSCs were assayed for chromosomal abnormalities via G-band karyotype assay or via Array Comparative Genomic Hybridization (Company: Cell Line Genetics). The comparative array allows for a higher resolution karyotype analysis that can detect structural and numerical chromosomal alterations that would not be visible by typical G-banding and microscope analysis.

Preparing Plates and Media for iPSC Maintenance:

Coating Matrigel Plates: As described in Williams and Prem et al 2018, iPSCs and NPCs were cultured on Matrigel (Corning) coated 6-well plates (Corning). Matrigel received in 100 mL vials were thawed overnight at 4°C [762]. Then aliquots necessary to make 6 mL of working solution were made and stored in the -80°C freezer. Matrigel varies from batch to

batch and thus, the volume of Matrigel required to make 6 mL of working solution was calculated based on dilution factor found on the Certificate of Analysis sheet. To coat plates, aliquots of Matrigel were thawed for a minimum 10 minutes at 4°C and then dissolved into 6 mL of cold DMEM/F12 (ThermoFisher Scientific). 1 mL of the Matrigel/DMEM/F12 solution was then placed into each well of a 6-well plate. Plates were then incubated at room temperature (RT) for 1 hr. Then, plates were used to culture cells or stored at 4°C for up to two weeks.

iPSC Media: iPSCs were cultured in mTESR medium (STEMCELL Technologies, Catalogue # 85850). mTESR medium is sold as a kit containing 5X mTESR supplement and mTESR Basal Medium. To make mTESR, 10 mL of 5X mTESR supplement was added to 40 mL of mTESR Basal medium. Then, 1:500 (100 µL/50 mL) of 500X Primocin (Invivogen) antibiotic was added to media. Media was then stored for up to 2 weeks at 4°C.

Maintenance of iPSCs:

Thawing iPSCs for Culture: Cryopreserved vials of iPSCs were obtained from the Millonig lab and placed in liquid nitrogen to ensure preservation. Generally, 1-2 million iPSCs were frozen in each tube. To culture iPSCs, vials were thawed by swirling tubes in 37°C water until a small crystal of ice remained. iPSCs were removed from the Cryotube and centrifuged at 100g for 5 minutes to pellet cells. The cell pellet was then resuspended in 1mL of pre-warmed mTESR media with 5µM ROCK inhibitor (Y-compound, STEM CELL Technologies:72304). Since iPSCs should be plated in clumps, resuspension was done gently with a P1000 or a serological pipette no more than 5 times. Prepared Matrigel plates were acquired and excess Matrigel was aspirated. 2 mL of mTESR media with 5µM Y-compound was then added to each well. Then, iPSC clumps were plated onto Matrigel

coated wells with mTESR such that the density was between 10 to 20% confluent. Over or under-plating can lead to changes in the confluency rate of cells which can lead to higher chances of karyotype abnormalities or improper differentiation. Media was changed daily.

Passaging & Expanding iPSCs: When cells reached between 70-90% confluency with densely packed colonies, approximately 3 to 7 days after initial plating, they were passaged. iPSCs should not be allowed to reach 100% confluency as this can alter cell behavior and induce karyotype abnormalities. To passage iPSCs, media was aspirated and 1 mL of 0.05 mM EDTA in 1X Calcium and Magnesium Free PBX (CMF) was added to the iPSCs and incubated at 37°C for up to 30 minutes until cells detached from well. Cells were then collected, pelleted, resuspended, and plated in clumps as described above. Generally, multiple wells of iPSCs were made to ensure enough cells for cryopreservation and neural induction. However, to ensure proper quality and stability, neural induction and cryopreservation of iPSCs was only conducted at least one passage after initial thawing.

Cryopreservation of iPSCs: To ensure enough iPSCs for future use, iPSCs must be expanded and cryopreserved. To cryopreserve iPSCs, cells were detached, pelleted and resuspended in 1 mL of mTESR media per well of iPSCs collected. For example, if 2 wells of iPSCs were collected, the pellet would be resuspended in 2 mL of mTESR. iPSCs were kept as clumps for cryopreservation. One well of iPSCs can produce 3 Cryotubes of about 1.5 million cells. Approximately 300 μL of iPSC + mTESR solution was added to each Cryotube (ThermoFisher Scientific; 1.8 mL size, 5000-1020). Then equal volumes of 20% DMSO in mTESR was added drop-wise to the Cryotubes. Cryotubes were then placed in a Freezing Container (ThermoFisher Scientific, 5100-0001) and then put into the -80°C overnight. Then, tubes were moved to the liquid nitrogen tank for permanent storage.

Coating Plates and Preparing Media for NPC Generation & Maintenance

Preparing plates: iPSCs were induced into NPCs on Matrigel coated 12-well plates. Briefly, 500 μL of Matrigel was added to each well of a 12 well plate and incubated for 1 hour at RT before use. NPCs were maintained on 6-well plates coated with Matrigel as described.

Neural Induction Media: To make NPCs, iPSCs were cultured in media obtained from a commercially available neural induction kit from GIBCO ThermoFisher. The kit consists of Neurobasal (NB) media and a 50x Neural Induction Supplement (NIS), which was used to make a 1X Neural Induction Medium (NIM). NIS was also used to make 100% Expansion Medium in which the NPCs are maintained after induction. To make 50 mLs of neural induction medium, 1mL of NIS was added to 49 mL NB. Then, 100 μL of 500X Primocin was added to the media.

100% Expansion Media (100 Exp): After induction, NPCs were maintained in 100% expansion medium. This medium was made by diluting 50X NIS into equal volumes of NB and DMEM/F12. For example, to make 50 mL of 100 Exp, 1mL of NIS was added to 24.5 mL of DMEM/F12 and 24.5 mL of NB. Then, 100 μL of 500X Primocin was added.

Generation, Validation, and Maintenance of NPCs

Generation: To generate NPCs, iPSCs were induced using a modified version of the ThermoFisher GIBCO Neural Induction Protocol. First, confluent iPSCs were dissociated from plates using 1X Accutase (room temperature (RT) Gibco, ThermoFisher Scientific). Unlike for passaging, for Neural Induction, iPSCs must be dissociated into single cells to allow for cell quantification. After detachment, iPSCs were collected, pelleted and resuspended into NIM with 5 μM of Y-compound. Cells were quantified and plated at multiple densities to ensure successful induction of at least one group of iPSCs. The

following densities of iPSCs were plated into Matrigel coated 12-well plates containing 1mL of NIM (with 5μM Y-compound): 80K, 120K, 200K, 300K. Two wells of each density were made. One set of wells at each density were induced for 7 days while the other was induced for 8 days. After the 1st day, media was aspirated and replaced with 1 mL of fresh NIM without Y-compound. Afterwards, media was changed every 2 days until cells reached confluency. Once cells reach confluency, media was changed daily. At day 7 or day 8, induction was complete. Wells with good neural morphology, less than 20% contaminating flat cells, little or no giant multi-nucleated or pale nucleated cells, and less than 50% cell death were selected for passaging.

Lifting, Dissociating, and Pelleting NPCs for Maintenance or Experiments: To passage or experiment with NPCs, medium was removed from wells and then 250 μL (12 well plate) or 500 μL (6 well plate) of Accutase at room temperature (RT) was added per well of confluent NPCs. Cells were then incubated for 10 minutes at 37°C. Afterwards, cells were collected and centrifuged at 300xg for 5 mins to form a pellet. The supernatant was removed and cells were resuspended in 1-5 mL of media (type of medium varies from procedure to procedure). The volume of medium added varied from line to line but the cell density was kept between 1 to 5 million cells per mL of media. Cells were then quantified on a hemocytometer after a 1:10 dilution in PBS (50 μL cells into 450 μL of 1X PBS).

Maintenance of NPCs: After the induction period (7 or 8 days), NPCs were passaged as described above. Cells in neural induction condition were sometimes more firmly attached to Matrigel and thus a cell scraper (Fisher Scientific) was sometimes necessary to remove cells from wells. Once cells were pelleted, they were resuspended in NB + 5 μM Y-compound and plated into Matrigel coated 12-well plates with 1 mL of 100 Exp + 5 μM y-

compound. Cells were plated between a density of 400k and 600k. Media was changed every 2 days. After initial passaging from induction conditions NPCs were considered to be P0. After 3-12 days, when cells reach confluency, NPCs were passaged (as described above) and now plated into Matrigel coated 6-well plates at a density between 1 and 1.5 million. Multiple wells of cells were made to ensure enough for experiments and cryopreservation. Cryopreservation was conducted at passages 2, 3, & 4. Experiments, validation of markers, and differentiation were not done until P3 to ensure stable cells. At every passage, NPCs were assessed for culture homogeneity, neural morphology, and cell death. Cells with poor morphology, excessive heterogeneity (>50%), excessive cell death, or growth rates slower than 15 days for initial confluency were discarded. See Figure 5 for examples of normal and abnormal cells. Some contaminating cells can be cleaned with differential Accutase techniques to selectively expand NPCs (Figure 5). Some cell types cannot be removed by differential Accutase and these mixed cultures must be discarded if contaminating cells exceed 20% (Figure 5). To clean cells, Accutase was added to heterogeneous wells. Cells were observed under the microscope and when flat cells begin to lift while NPCs still remain attached, Accutase is removed and wells were washed with 1X PBS to detach contaminating cells. The remaining NPCs were passaged as described.

Cryopreservation of NPCs: NPCs were cryopreserved as described in above in the iPSC cryopreservation section. Briefly, NPCs were resuspended in 100% Exp. Then, a minimum of 1.7 million NPCs, at a volume less than 700 μL, were placed into Cryotubes. Equal volumes of 20% DMSO in 100 Exp was added in drop-wise to the NPCs. Cells were then frozen and stored as described in the iPSC cryopreservation section.
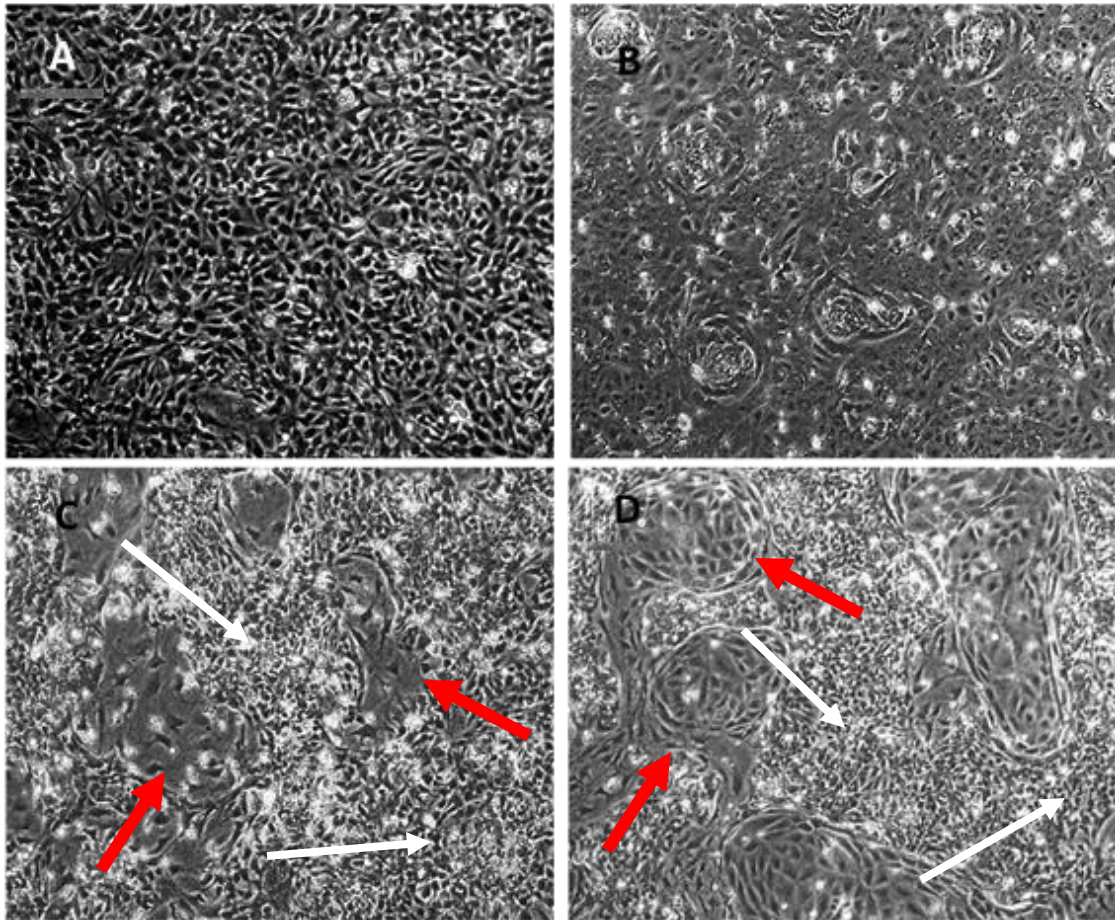
Figure 5: Representative images of high and low-quality NPC cultures. A) shows high quality NPCs as evidenced by homogeneous cells with dark nuclei and little cytoplasm. Cells are closely packed, yet individual cell bodies can be distinctly visualized. B) Cultures of NPCs that have lost normal morphology and have become flat, poorly defined cells. These cells cannot be used for experiments. Note, the dark nuclei are no longer present and distinct cell borders cannot be seen. C) Heterogenous cultures with white arrows point to normal NPCs while red arrows point to "flat" contaminating cells. These cells can be cleaned with differential Accutase treatment. D) Heterogeneous culture with white arrows pointing to normal NPCs and red arrows pointing to another contaminating cell type. These cells cannot be removed with differential Accutase and thus, cultures where these cells are more than 20% of the population must be discarded.

Validation of NPCs: In addition to visually assessing morphology, health, and homogeneity of cultures, immunocytochemistry and differentiation of NPCs into 3 lineages was conducted to confirm that generated cells were indeed NPCs. The Millonig lab also conducts Luminex Panels on NPC lines to ensure presence of markers.

Immunocytochemistry (ICC): All NPCs lines were validated by ICC. At P3, NPCs were

plated into Matrigel-coated 24 well plates (Corning) with 100% Exp at a 100k density per well to assess for markers. After 48 hours, marker plates were fixed in ice-cold 4% paraformaldehyde (PFA) for 15 minutes. Then, wells were washed 3 times with PBS. Cells were then blocked in 10% Normal Goat Serum with PBS containing 0.3% Triton-X (PBS-T) for 1 hour at RT. Then, cells were incubated in primary antibody which was diluted in the 0.3% PBS-T overnight at 4°C. To confirm NPC identity, cells were incubated with the following primary antibodies: Nestin (1:200 Santa Cruz), SOX2 (1:1000 Abcam), and PAX6 (1:500 BioLegend). To assess immature neurons and astrocytes, B-3 Tubulin (1:100, Santa Cruz) and GFAP (1:4000, Sigma) were used respectively. Finally, to ensure that NPCs were completely induced and that no iPSCs remained in culture, iPSC marker, OCT3/4 (1:250 SantaCruz) was used. After incubation in appropriate primary antibody overnight, wells were washed 3 times with PBS and then cells were incubated in the appropriate secondary antibody for 1 hr at RT. All antibodies for marker staining were made in mouse or rabbit and thus, the secondary antibodies used were red (594 Alexa Fluor, ThermoFisher Scientific) or green (488 Alexa Fluor, ThermoFisher Scientific) goat-anti-mouse antibodies or goat-anti-rabbit antibodies. All secondary antibodies were used at a 1:1000 dilution into PBS. After incubation in secondary, cells were washed 3 times in PBS and then incubated for 5 minutes with DAPI (1:1000, Invitrogen) to visualize nuclei. Cells were then washed again with PBS and stored in 1X PBS + 0.05% Sodium Azide for preservation. Stained cells were visualized at 10 and 20X using fluorescent microscopy (Zeiss). 1-2 images were taken per marker. Images were analyzed for presence or absence of marker (at minimum 2 fold background). To quantify percentages of markers per line, low density culture (50k cells in a 35mm dish) were stained with the aforementioned

antibodies. Then, 10-12 systematically random images were acquired on fluorescent microscope (Zeiss) and then total cell number and cells with marker (minimum 2-fold background) were counted. Percentage of marker positive cells were calculated.

Differentiation of NPCs: NPCs were differentiated into neurons, astrocytes and oligodendrocytes as described in the ThermoFisher GIBCO NPCS differentiation protocol. NPCs from 1 clone per patient were differentiated to ensure multi-potency. After allotted differentiation periods, cells were stained using immunocytochemistry (as described above) with the following markers.

- Neurons: Tau (1:500, SantaCruz), MAP2 (1:500), TUJ1 (1:100, SantaCruz)

- Astrocytes: GFAP (1:4000, Sigma), S100B (Sigma)

- Oligodendrocytes: 04 (R&D Systems), Gal-C (SantaCruz)

Cells were imaged on a fluorescent microscope. The presence of at least one marker (min 2-fold background) from each category in a minimum of at least 1 cell was necessary to consider the cells as NPCs.

Media Preparation for Experimental Conditions

All experiments were conducted in 30 % expansion media (30 Exp) which was made by diluting 100 Exp by 70% in a 1:1 ratio of DMEM/F12 and NB media. Primocin antibiotic was also added. For example, to make 20 mL of 30% Exp, 6 mL of 100% Exp was diluted with 7 mL of DMEM/F12 and 7 mL of NB and then 40 μL of Primocin was added.

Neurite Outgrowth Assay:

Plating Conditions: As described in Williams & Prem et al 2018, neurite experiments were conducted on 35 mm dishes (Corning) coated with 0.1 mg/mL poly-d-lysine (PDL)

(Sigma-Aldrich) and 5 µg/mL fibronectin (FN) (Sigma-Aldrich) [762]. PDL was dissolved in dH20 to make a 1 mg/mL stock solution and filter sterilized. To coat plates, a working solution of 0.1 mg/mL of PDL was made by dissolving the stock PDL (1:10) into $dH_2 0$ and then applied to dishes for 20 minutes. Then, dishes were washed 3x with $dH_2 0$ for 5 min each. Fibronectin was added directly to 30 Exp. Appropriate vehicles and Extracellular Factors (EF) were then added to the 30 Exp+ FN and then 1 mL of the media solution was added to each dish. Numerous EFs were tested and used in the neurite experiments. In some cases, multiple doses of EFs were tested and serial dilutions were conducted. The following factors and concentrations of EFs were used in the neurite experiments:

- Nerve growth factor (NGF, Peprotech): 3, 10, 30, 100 ng/mL

- Serotonin (5-HT, Sigma-Aldrich): 30, 100, 300 µg/mL

- Pituitary adenylate cyclase activating polypeptide (PACAP, American Peptide /BACHEM): 1, 3, 10, 30, 100 nM

- Oxytocin (American Peptide): 3, 10, 30, 100 nM

- Brain Derived Neurotrophic Factor (BDNF, Peprotech): 3, 10, 30, 100 ng/mL

- Neurotrophin-3 (NT3, Peprotech): 3, 10, 30, 100 ng/mL

- Fibroblast Growth Factor (FGF, Peprotech): 3, 10, 30, 100 ng/mL

Plating NPCs: Once media and dishes were prepared, NPCs were dissociated, pelleted, and resuspended as described. 50,000 cells were then plated into each 35 mm dish and dishes were rocked in all directions to ensure proper distribution of cells. NPCs were then incubated at 37°C for 48 hours.

Replicates: For each experiment, 2-3 dishes were set up per condition. For example, an experiment testing the effect of two doses of PACAP on neurite outgrowth would have 3

conditions: Control condition with no PACAP added, 3nM PACAP condition and 10 nM PACAP condition. For each condition 2-3 dishes with 50k cells/dish would be set up totaling 6-9 dishes in this experiment.

Fixing NPCs: After 48 hrs NPCs were fixed for 15 mins in 4% PFA. To prevent breakage of neurites, removal of medium and PBS were done using a plastic Pasteur pipette instead of vacuum suction. After PFA is washed off, cells were placed in 1X PBS + 0.05% sodium azide and moved to 4°C for preservation. At this point, analyses were conducted.

Analysis: In each dish, the proportion of cells bearing neurites was counted blind. Neurites were defined as processes that extend from the cell body that are equal to or greater than 2 cell body diameters in length. For cells with more than one process, the longest process was used for the criteria. Cells were counted directly on a phase contrast microscope at 32x. Two to four 1 cm rows were counted per dish. At least 250 cells were counted per dish and the proportion of cells with neurites was calculated in each dish. When counting, cells with the following characteristics were excluded (Figure 6): large flat cells with pale nuclei and cytoplasm: nucleus ratio greater than two (blue arrow), multinucleated cells (green arrow), or phase-bright dead cells with disrupted membranes (white arrow). Cells in clumps with greater than 5 cells were also excluded as clumping can change neurite behavior. Dishes with excess cell death (<50 cells/row), excess flat cell contamination, (>15%), or excessive clumping were not analyzed.
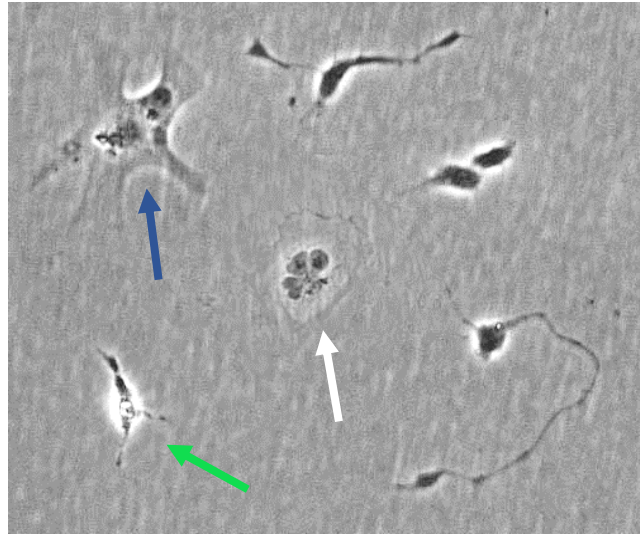
Figure 6: Representative image illustrating cell types that are excluded from neurite assay counts. Arrows point to the cells that are excluded from our neurite counts. The blue arrow points to a cell with higher cytoplasmic surface area, white arrow points to a multinucleated cell and the green arrow points to a phase-bright dead cell.

Migration Assay

Neurosphere formation: As described in Williams & Prem et al 2018, neurospheres were formed by dissociating confluent NPCs and plating 1 million cells into uncoated 35 mm dishes with 100 Exp [762]. At least 2 to 3 dishes of NPCs were made to ensure enough neurospheres for the assay. The NPCs were then incubated at 37°C for 24-96 hrs (varies from line to line) to allow the aggregation of NPCs into neurospheres of approximately 100 μm diameter. Sphere size was assessed daily using a live-ruler on a phase-contrast microscope. When a majority of spheres reached an approximate diameter of 100 μm (± 20 μm), migration assay was performed. Smaller spheres will completely disperse and break apart during the migration assay while larger spheres cannot be imaged at 10X.

Media & Plates: To allow for proper migration, Neurospheres were plated into 30 Exp + Matrigel solution. To coat plates, a Matrigel aliquot was dissolved into 6 mL of cold 30 Exp. Appropriate vehicles and EFs were added to the 30Exp/Matrigel mixture as desired.

Then, 1mL of the 30Exp/Matrix $\pm$ EF mixture was added into 1 mL of a 6-well plate. Two to 3 wells were made per experimental condition. Plates were then incubated for 30 minutes at 37°C. Unlike expansion conditions, Matrigel was NOT aspirated from wells before plating the spheres. If spheres were plated onto aspirated wells, excess and rapid migration would result. The following EFs and concentrations were used for the Migration Assay:

- PACAP: 1,3, 10, 30, 100 nM

- 5-HT: 10, 30, 100, 300 ug/mL

Plating Neurospheres: While plates were incubating, neurospheres were collected from the 35 mm dishes and placed into a 15 mL conical tube. The tube was then centrifuged at 100 g to pellet spheres. Pelleted spheres were then very gently resuspended using a P1000 in 1-3 mL of pre-warmed 30 Exp. Volume of medium added was determined by the number of neurosphere formation dishes set up. For example, if two dishes of neurospheres were collected, 2 mL of medium was added to resuspend spheres. 200 µL of the sphere suspension was then placed into the 30 Exp Matrigel plates. Spheres were allowed to migrate for 48 hours and were then fixed for 30 minutes in 4% PFA. Spheres were washed with PBS and stored in PBS + 0.05% Sodium Azide.

Analysis of Migration: Neurospheres were imaged at 10X on a phase contrast microscope. At least 30 neurospheres were imaged per condition. Only spheres with a contiguous migrating carpet, an intact inner cell mass, and lacked sphere to sphere contact were imaged. Average migration was measured using ImageJ (or Fiji) as described in Willams & Prem et al 2018 [762]. To measure migration, the outer contour of a neurosphere was traced using the freehand line tool and then area of the trace was measured. Then, the inner cell mass area was also measured. Migration was quantified by subtracting the inner cell

mass area from the total neurosphere area. At least 20 neurospheres were analyzed per condition. Exclusion criteria are detailed in JOVE methods paper [762].

Signaling Analyses

In order to assess underlying mechanisms that contribute to developmental defects in ASD NPCs, signaling pathway proteins were assessed via western blot, immunocytochemistry, and phospho-proteomics.

Experimental Paradigm and Protein Collection for Western Blot Analyses:

To collect protein for western blot, confluent NPCs were dissociated, pelleted, and plated onto PDL+ FN coated plates with 1 mL 30 Exp. NPCs were plated at a density of 1 million into 35 mm dishes.and then incubated at 37°C for 48 hours. At 48 hours, cells were treated with vehicle or EF for 10, 15 or 30 minutes. Then, dishes were washed with ice-cold PBS 2 times. Cells were then lysed in 50 µL of ice-cold lysis buffer/ dish. Lysis buffer was made by adding the following components: 50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.5% IGEPAL (), 1% Triton X-100 ()10 ug/mL aprotinin, 10 ug/mL Leupeptin, 20 ug/mL soybean trypsin inhibitor, 50 mM NaF, 0.5 mM micro-cystin-LR, 0.5 mM dithiothretiol, 2 mM phenylmethylsulfonylfluoride, and 1% CHAPS. After incubating in lysis buffer for 1-2 minutes, the sample was collected by scraping the dish with a cell scraper. At this point, protein was stored in the -80°C until further processing of samples could be conducted.

To purify and measure protein, lysate samples were thawed for 1 hr on ice. Lysate was then sonicated 2X for 1 min on ice and then samples were centrifuged at 4°C for 10 min. The supernatant was removed and saved while the pellet was discarded. Protein concentrations were determined by BCA assay (Pierce) in comparison with a bovine serum

albumin (BSA, Sigma) standard curve (range 0-12 ug/mL). Samples and standards were measured on a Spectrophotometer (Beckman) as previously reported [197, 281, 763-765]. Standard curve of absorption vs concentration were generated and sample concentrations were calculated. Protein was aliquoted and stored at -80°C.

Western Blot Analyses:

Aliquoted samples were thawed and 20 μg of protein was mixed with appropriate volumes of 4x NuPAGE LDS Sample buffer (ThermoFisher Scientific) and 10x NuPAGE Sample Reducing Agent (ThermoFisher Scientific). Samples were boiled, cooled, and loaded into wells on a 12% SDS-PAGE poly-acrylamide gel (NuPage, ThermoFisher Scientific). The samples were then run at 100 V for 1.5 hours in a running apparatus with 1X NuPAGE Mops SDS Running Buffer (ThermoFisher Scientific) running buffer to achieve separation of proteins by weight. Protein was then transferred to a PVDF membrane using a wet transfer apparatus with 1X NuPAGE Transfer buffer (ThermoFisher Scientific). Transfer can be done for 2 hours at 60V at RT or overnight at 20V at 4°C. The membranes were then washed with Tris-Buffered Saline (TBS) with Tween20 (0.1%) (TBS-T) and blocked with 5% powdered milk in 0.1 %TBS-T. Then, the membranes were probed for proteins with the following antibodies used at a 1:2000 concentration and incubated overnight at 4°C.: ERK (9102, CellSignaling), P-ERK (9101, CellSignaling), CREB (9104 CellSignaling), P-CREB (9198, Cell Signaling), AKT (9272 CellSignaling), P-AKT (9271 CellSignaling), S6 (2137 CellSignaling), P-S6 (2211, CellSignaling), PKC (2056, CellSignaling), P-PKC (9371, CellSignaling), as previously reported [197, 281, 763-765]. Total and Phoshpo-antibodies were run on different gels/ different halves of one gel to avoid stripping. GADPH was used at as a loading control and was probed with antibody

(Meridian Life Sciences, H86045M) at a dilution of 1:10,000 for 1hr. After incubation in 1°, membrane was washed and appropriate HRP conjugated 2° antibody was applied at a 1:1000 concentration for all antibodies except GAPDH. For GAPDH 2° antibody was used at a concentration of 1:5000 Then, ECL (ThermoFisher Scientific), a chemilumescent agent, was applied to membranes for 1 min to allow for visualization of protein bands of interest on medical grade X-ray film. Films were scanned into JPEGs and then quantified on ImageJ. Band intensities for phospho- and total protein were normalized to signals for GAPDH. Then, the GAPDH normalized Phospho-antibody intensity was divided by the GAPDH normalized total antibody to get a relative protein intensity.

Immunocytochemistry: ICC was conducted as described previously [197, 281]. 2-3 dishes of NPCs per condition plated at 50k density were stained for protein expression with the following signaling antibodies: P-S6, P-Cofilin (SantaCruz). Cells were also counter-stained with DAPI. Cells were imaged on a fluorescent microscope and 10 systematically random images were acquired at 10X. Percentage of cells with positive stain (min 2-fold intensity level of background) was calculated and averaged across dishes.

Phospho-proteome: In order conduct unbiased analyses on signaling systems in NPCs, phospho-proteomics analyses via mass spectrometry was conducted by the Lobel lab. As described above, protein was collected from NPCs plated on 0.1 mg/mL PDL+ 5μg/mL FB at a density of 1 million in 35 mm dishes for 48 hours. 1mg of protein was collected per clone per condition. On average, 50-150 μg of protein was acquired from one dish. Multiple dishes of protein were collected across 3 passages for each cell lines to ensure adequate sample. All samples were compiled and then sonicated and measured. When at least 1 mg of protein was acquired, collection experiments were stopped.

Isolation of RNA, Generation of cDNA, & QRTPCR

RNA Isolation & Generation of cDNA:

1 million NPCs were plated onto PDL (0.1 mg/mL) and FN (5ug/mL) coated 35 mm dishes with 30 Exp. Dishes were then incubated for 48 hrs at 37°C. After incubation, media was aspirated and cells were washed twice with DEPC treated PBS on ice. RNA lysate was isolated by adding 500 µL of Trizol reagent (Invitrogen) and scraping dishes with a cell scraper. RNA was then extracted from the lysate by phenol-chloroform methods and isopropanol precipitation as previously described [276, 281]. DNA was removed from samples by treating with an RNAse free DNAse (Qiagen). Concentration of RNA was determined using Spectrophotometer. cDNA was then generated from RNA of at least 100 µg/mL concentration using dNTPs (Invitrogen), random primers (Promega) and Superscript II reverse transcriptase (Invitrogen).

qRT-PCR Analysis:

Samples were run in a 96 multiwell reaction plate (Applied Biosystems). Gene expression was quantified using a 25 µL sample mix of the following reagents: 1 ng cDNA, 1X Sybr Green Master Mix (ThermoFisher Scientific), Ultra Pure dH_2O (Invitrogen) and a 20 µM concentration of appropriate primer pairs (Integrated DNA Technologies). GAPDH and Actin were used as loading controls. Each sample was loaded in at least duplicates. For each primer a standard curve was conducted. Samples were run using the ABI Prism 7300 Real-Time PCR system (Applied Biosystems). All reactions were run under the following conditions: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15s and 60°C for 1 min. Then, a dissociation stage was conducted to ensure primers

were removed from template by heating sample to 95°C for 15s and then cooling to 60°C for 1 min, followed again by heating to 95°C for 15s.

The following primer sequences were used for qTR-PCR analyses: **GAPDH Forward (F)**: 5'-AATCCCATCACCATCTTCCA-3' & **GAPDH Reverse (R)**: 5'- TGGACTC-CACGACGTACTCA-3'.**β-Actin (F)**: 5'-TCCCTGGAGAAGAGCTACGA-3' & **β-Actin (R):** 5'- AGCACTGTG TTGGCGTACAG-3'. **NTrk1 (F)**: 5'-GTCAGCCACG-GTGATGAAATC-3' & **NTrk1 (R):**5'-CAGCACGTCACGTTCTTCCT-3'. **NTrk2**F:5'-TCGTGGCATTTCCGAGATTG G-3' & **Ntrk2 (R):** 5'-TCGTGGCATTTCCGAGA TTGG-3'. **NTrk3 (F):** 5'-GCCAGTATCAACATCACGGAC-3' & **NTrk3 (R)**:5'-AG-CCGGTTACTTGACAGGTTT-3'. **PAC1R (F):** 5'GTCGGAACCCTTCCCTCATTA-3' & **PAC1R (R):** 5'-GG CCTTCACTGACAGGTAGTA-3'. **HTR1A (F):** 5'-TGTATCA-GGTGCTCAACAAG TG-3' & **HTR1A (R):** 5'-AGGAAGCCAATAAGCCAAGTG-3' **HTR2A (F):** 5'-CCGCTTCAACTCCAGAACTAA-3' & **HTR2A (R):**5'AAAGAGCC GATCAGGACAAA-3'. **HTR7 (F)**: 5'CTCGATCATGACCCTGTGC-3' & **HTR7 (R):** 5'-GAGTGGAGGTAAGGTGATGGA-3'. **FGFR2 (F):** 5'-GGTGGCTGAAAAACGGG AAG-3' & **FGFR2 (R)**: 5'-AGATGGGACCACACTTTCCATA-3'. **BDNF (F)**: 5'-TAACGGCGGCAGACAAAAGA-3'& **BDNF(R)** 5'TGCACTTGGTCTCGTAGAA GTAT-3'. **NGF (F):**5'-GGC AGACCCGCAACATTACT-3' & **NGF (R):** 5'-CACCACCGACCTCGAAGTC-'3

Metabolomics:

Metabolomic analyses were conducted in order to understand metabolic difference in autism NPCs. Samples were sent to Metabolon for analysis. 15 samples with a minimum of 1.5 million NPCs were collected per individual. For these analyses, samples were

collected from 1 clone at 2 passages. To acquire 1.5 million cells per sample, two 35 mm
dishes were set up with 1 million cells plated onto 0.1mg PDL and 5 ug/mL FN in 30 Exp.
7 samples were collected at a time and thus 14 dishes were set up at once per patient. 48
hours later, cells were lifted with 1X Accutase, washed, collected into 15mL conical tubes,
and pelleted. Pellets were then resuspended in 30 Exp and cell numbers were quantified
and documented. Cell suspension was then transferred to a cryotube and then centrifuged
to pellet. Pellets were briefly rinsed with PBS and all excess liquid was removed to ensure
dry pellet. Cryotubes were then drop fixed in liquid nitrogen and then transferred to the -
80°C freezer. Once all samples were collected, they were sent to Metabolon in dry ice and
shipped overnight. Analyses were done at Metabolon via LC/MS and metabolites were
normalized to both cell number and protein amount. Heatmaps were generated and
metabolite ratios were also shown for each sample. Functional pathways were generated
for measured metabolites and data was analyzed for largest fold-changes in metabolites.

Rescue Studies:

To show that identified signaling and metabolic pathways were contributing to an
autism phenotype, gain and loss of function studies were conducted with drug agonists and
antagonists when possible. These drugs were applied to the neurite assays and neurosphere
assays. Western studies were also conducted under drug treatment. The following drugs
and concentrations were used on the appropriate assays:

- Dibutyryl cyclic-adenosine monophosphate (db-cAMP, Sigma): 0.1, 0.3, 1, 3, 10, mM

- SC-79 (Selleckchem): 0.1, 0.3, 1, 2, 3, 10, 20 μg/mL

- MK-2206 (Selleckchem): 0.3, 1, 3, 10, 30, 100 nM

- Hydroxyurea (Sigma): 1, 3, 10, 30, 100, 300 μM & 1, 10 mM

- Forodesine (MedchemExpress): 30, 100, 300, & 1, 3 µM

In some cases, combination studies were conducted to assay whether combining an EF and a drug led to additive or synergistic effects. For these studies, "sub-threshold" doses of the drug were combined with optimal doses of the EF. Sub-threshold dose was defined as the dose of drug which was 10-fold lower than the lowest dose that elicited a neurite or migration response. Optimal dose of an EF was defined as the dose that elicited the highest neurite outgrowth or migration were seen as per dose response studies.

For western studies measuring immediate signaling changes, cells were exposed to drugs after 15 or 30 minutes depending on literature. For combination studies, cells were cultured for a total of 48 hrs as per usual. 6 or 24 hrs before collecting protein, subthreshold doses of a drug were added to culture. Then, EFs were added 10 or 15 minutes before collecting protein as described in the Western Blot section.

Technical and Biological Replicates

In total, my analyses consisted of 6 autism patients and five unaffected individuals. For each individual we studied, 2-5 clones were derived (with the exception of the NIMH control lines and 16pdel F which only have 1 clone). From these 2-5 clones, 1-3 different neural inductions were derived. Separate neural inductions include iPSCs induced on different dates, iPSC induced for different induction lengths (D7 vs D8), or inductions conducted with different iPSC densities. Thus, in total, per person, between 3-10 distinct NPC lines were studied. For the neurite assay, 2-3 experiments were conducted from each NPC line. For each EF tested, at least 2 experiments were done per clone. In each experiment at least 2 dishes were set up per condition. For the neurosphere assay, at least 2 experiments were done per clone on 2 clones. For western blot analyses, protein was

collected from at least 2 separate NPC lines per 2 clones. A 3rd clone was used if the data from the 2 clones was contradictory. Replicates for the metabolome and phospho-proteome were discussed in their respective sections. Finally, for neurite and neurosphere rescue studies, drugs were applied to at least 2 clones and experiments were done at least twice. In the case of the combination drug + EF studies, 1 experiment was done on 2 clones each. This rigorous approach ensures that the data we collect are not just artifacts of iPSC reprogramming or NPC induction and that our data is highly reproducible.

Compilation of Data and Statistical Analyses

Data acquired from the idiopathic autism individuals were compared to data acquired from unaffected Sibs. Data was compiled and organized in several ways. Comparisons were made within Family (Sib-1 vs ASD-1), across families (Sib-1 vs ASD-2), or as an average of all Sib (3 patients, all clones) compared to the average data from all idiopathic ASD (3 patients, all clones). For every neurite experiment, 2-3 dishes were set up per condition. For every migration condition, 20-30 spheres were set up per condition. Dishes were kept as separate N values and were not collapsed into a single mean per experiment as suggested by biostatistician Dr. Wise Young. For example, when compiling data for Sib-1 in control condition, all neurite dishes from every clone were averaged together to get a mean neurite value for this patient. Data was also analyzed by clone. In this case, dishes from each clone were averaged and compared to dishes from other clones derived from the same patient. For spheres, each neurosphere was considered a separate statistical point. Migration values from each sphere derived from multiple clones were averaged together to acquire an average migration value for each patient. Spheres were also analyzed by clone. For the 16p11.2, data was compared to composite averages of the Sibs

(dishes from all 3 Sib patients and all clones) or averages of the NIMH controls (Dishes from both patients, all neural inductions). Assuming statistical normality, in comparisons that include only two groups, unpaired t-test was be used to test differences in affected vs unaffected samples. Simple unpaired or paired t-tests will be run in Microsoft Excel. If normality assumption is not satisfied, the corresponding non-parametric Wilcoxon sign test will be used via GraphPad Prism 7 software. The presence of multiple ASD subtypes and the use of EFs leads to instances where multiple group comparison is necessary. For such cases, in normal data, analysis of variance or ANOVA (one or two way) test was used to detect statistical significance. To reduce type one error, P-values for the ANOVAs will be calculated using Tukey correction in GraphPad Prisim 7. If normality assumption does not hold, non-parametric Kruskal-Wallis test will be applied for multiple comparisons.

Chapter 4- Experimental Results: Development and Optimization of Methods for Studying Neurite outgrowth and Cell Migration

Generation and Validation of NPCs:

Classically, NPCs are generated from either embryonic stem cells or iPSCs via embryoid body formation [766-771]. Embryoid bodies (EBs) are aggregates of pluripotent stem cells that are capable of forming all three primary embryonic germ layers. From these EBs, induction of NPCs can be conducted through either spontaneous differentiation or directed differentiation protocols. However, these protocols often required multiple media types, feeder cells, precise addition and removal of growth factors at set time points, takes more than a month of time, and require manual picking and replating of aggregates of cells. In 2009, Chambers et al published a protocol that allowed for the generation of NPCs from iPSCs cultured in a monolayer [726]. This allowed for more rapid generation of NPCs without formation of EBs or selection of neural rosettes which is time were consuming and technically challenging. Thus, for generation of our NPCs, we selected a commercially available monolayer culture kit from Thermofisher GIBCO. This kit requires only 1 week to generate early NPCs, requires no sophisticated equipment or techniques, and has simple media cocktail and time schedules to generate cells. The GIBCO protocol calls for culture of iPSCs at a density of $0.5 \times 10^5 - 1 \times 10^5$ cells/cm^2. for 7 days incubation in their Neural Induction Medium (NIM) to produce NPCs [772, 773]. However, we found that this method did not consistently generate NPCs from our iPSCs. Thus, we tested multiple iPSC plating densities and induction time points to optimize NPCs generation. Ultimately, we found that

there was not one consistent density or incubation time point that reliably generated NPCs from all iPSC lines, suggesting wide variability in the induction process. Thus, to ensure NPCs were generated from each induction we chose to plate iPSCs at 4 densities and 2 times points for every induction as detailed in the methods section.

Once NPCs were generated, they were validated by conducting immunocyto-chemistry (ICC) at high density for the following markers: SOX2, PAX6, NESTIN, TUJ1, GFAP, and OCT3/4. As seen in Figure 7 most cells ($\geq$90%) express SOX2 and NESTIN. SOX2 is a marker for multi/pluripotency found in iPSCs and NPCs while Nestin is an intermediate filament protein found in NPCs Approximately 70% of cells also expressed PAX6, a marker for forebrain NPCs. TUJ1, which is generally a marker for immature neurons were also seen in a small percentage of our cells. Interestingly, low and ubiquitous levels of the astrocyte marker GFAP were also seen in NPCs. GFAP is also present in radial glia cells which are precursor cells that ultimately give rise to cortical neurons. Finally, to confirm cells were no longer iPSCs OCT3/4 staining is conducted (Figure 7). Presence of OCT3/4 marker beyond 2-fold background intensity suggested that cells were not adequately induced, and thus these lines were discarded.

In addition to ICC for markers, NPCs were validated by confirming their multipotency. Using protocols from ThermoFisher GIBCO [774], I differentiated NPCs into neurons, astrocytes, and oligodendrocytes. Then, ICC was conducted for the following markers Neurons (Tau), Astrocytes (GFAP), and Oligodendrocytes (O4) as show in Figure 8. Differentiations were conducted on one clone per individual.
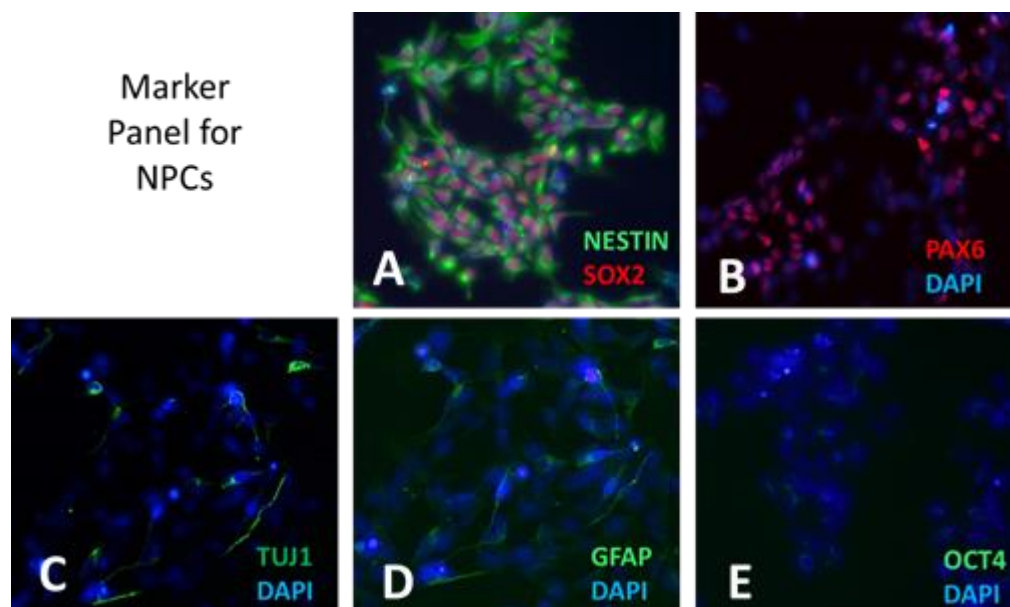
Figure 7: Representative images of high density NPCs stained for various markers. NPCs exhibit positive staining for: A) NPC marker Nestin (G), Multipotency marker SOX2 (R) B) Forebrain NPC marker PAX-6 (G). C) Some cells expressed TUJ1 (G), a marker for immature neurons. D) NPCs have low-level expression of GFAP in all cells and brighter expression of GFAP in cells that had TUJ1. E) NPCs show no expression of iPSC marker OCT-4 (G)
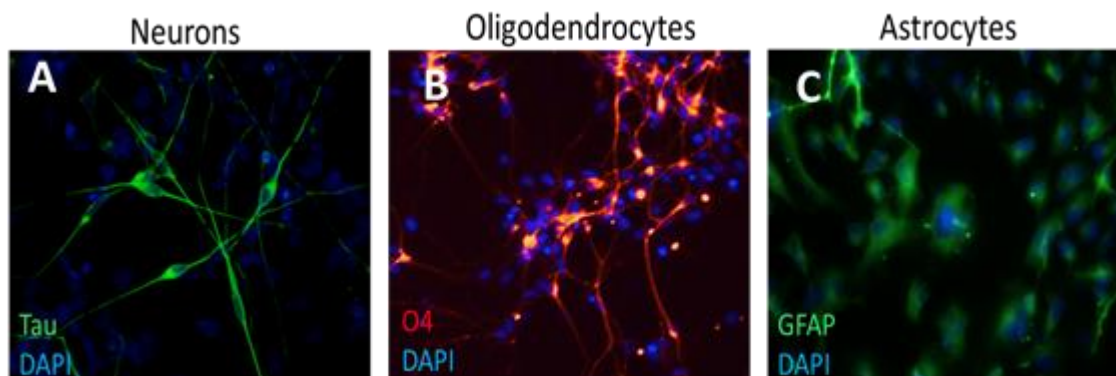
Figure 8: Representative images of all 3 cell lineages generated from our NPCs A) Neurons generated from NPCs exhibit mature neuronal marker Tau (G) and have neuronal morphology. B) Oligodendrocytes generated from NPCs exhibit oligodendrocyte marker O4(R). C) Astrocytes generated from NPCs have astrocyte-like morphology and are GFAP (G) positive.

Development and optimization of the Neurite Assay:

While iPSC technology has been employed to study both idiopathic and genetically defined autism, a majority of these studies have focused on post-mitotic differentiated neurons. Many of these studies have found defects in neurite outgrowth, including changes

in dendrite length, dendritic spine density, and dendrite branching [742, 745, 748, 749, 751, 755, 757]. However, no iPSC papers to date have utilized NPCs to study early neurite outgrowth. Thus, it is unclear whether neurite defects manifest in early developmental stages or whether these defects are only apparent after differentiation. Thus, part of my studies focused on assessing initial neurite outgrowth in our idiopathic and 16p11.2 del cohorts.

The neurite paradigm utilized for my studies was adapted from prior studies in our lab that assessed neurite outgrowth in cerebellar granule precursor cells and cortical precursor cells derived from rodent brains [197, 276, 281, 283, 763-765]. These methods are rapid, simple, and have effectively uncovered differences between ASD animal models and WT littermates. Importantly, the results obtained through these *in vitro* culture methods have translated to similar phenotypic differences *in vivo* as seen in. This indicates the validity and value of using in vitro culture to study neurodevelopmental disorders.

With rat and mouse cells, neurite quantifications were conducted by plating cells at low density onto poly-d-lysine substrate and then fixing them 24-48 hours later. The proportion of cells bearing extensions (neurites) equal to or greater than 2 cell body diameters in length was quantified and expressed as a ratio over the total cell number. However, such methods had never been applied to human NPC. Prior studies in our lab showed NPCs required different growth media and culture matrices to grow and survive. Thus, I tested multiple parameters and methods of analyses to optimize the neurite assay for use with human NPCs. Tests were conducted in control conditions and in some cases under the stimulation of developmentally relevant EFs. Testing EFs would allow us to select the optimal conditions to observe EF stimulated neurite responses.

Counting method:

Neurite quantifications could be conducted in one of two ways. Prior studies in our lab have utilized both methods without finding any statistically significant differences in results in rodent culture. Initially, for my studies, neurites were quantified by fixing cells at 48 h and then staining the dishes with fluorescent TUJ1 antibody, which marks both the cell body and the neurite. Then, 10-12 systematically random images were taken on a fluorescent microscope at 10X magnification. The total number of live cells, as marked by DAPI, were quantified along with the total number of cells bearing neurites. Neurites were defined as extensions equal to or greater than two cell bodies in length that were also TUJ1 positive. This imaging method allowed for easy visualization of neurites and measurement of neurite lengths if needed. Alternatively, cells could be analyzed with phase contrast microscopy immediately after fixing. In this method, the proportion of cells bearing neurites was quantified in three 1 cm rows. Comparison of both methods (digital image vs microscope) in parallel found no statistically significant differences in percentage of cells with neurites (Figure 9). Moreover, both methods could successfully identify increased neurite outgrowth under stimulation of EFs. Thus, both methods seemed to be valid for analysis of neurites. Ultimately, due to the rapid nature, low cost, and ease, I chose to analyze neurite directly on the phase contrast microscope.
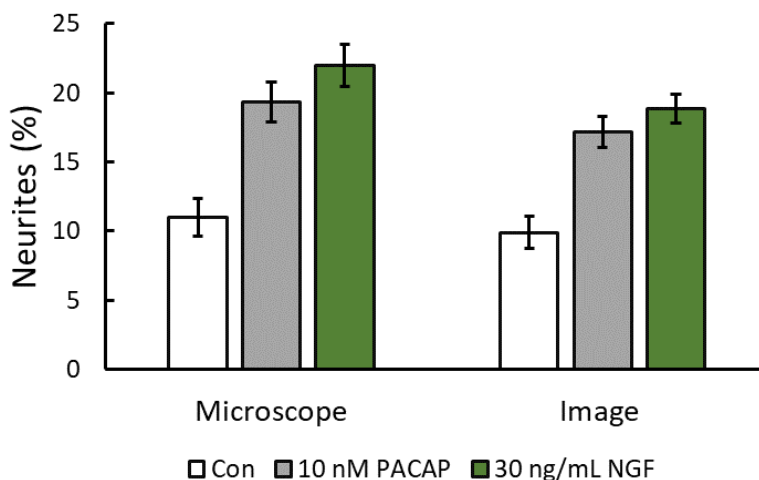
Figure 9: Comparison of neurite counts analyzed directly on a microscope or counted on phase-contrast images. Both microscope and image methods detect differences in neurites between control (White), PACAP (Grey), and NGF conditions. There are no statistical differences between neurites analyzed by microscope vs image methods in any condition. N= 2 experiments, 3 dishes/experiment, 1 patient- 1 iPSC clone

Optimization of Culture Media:

In the initial phases of studying neurite outgrowth, our lab was still optimizing media conditions for experiments with our NPCs. Traditionally with rodent culture, we had primarily utilized two types of media: Defined Medium (DM) 1:1 mix of DMEM/F12 with insulin, bFGF, BDNF and other supplements or Neurobasal/B27/bFGF medium (NBF) with Neurobasal, B27, Glutamax, bFGF and 0.1% BSA. Our human NPCs, on the other hand, are cultured in a commercially available 100% Expansion medium (100 Exp) which is made by adding GIBCO Neural Induction supplement (NIS) to equal volumes of DMEM/F12 and Neurobasal media. Initial tests in our lab found that human NPCs did not survive in DM media. However, cells survived well in NBF media though the morphology looked different than NPCs cultured in 100 Exp. Part of my experimental goals were to test the effects of EFs on neurite outgrowth. Thus, I needed a "limiting" media condition that had lower concentrations of EFs such that we could add back EFs to test their effects. Thus,

we developed 30% expansion media (30 Exp, a 70% reduction of NIS) and NB media (NBF without FGF). To find the optimal conditions for neurite outgrowth, I tested the following media: 30 Exp, NB, and NBF (Figure 10). As shown, cells had highest percent of neurites in 30 Exp and lowest in NBF. Morphology of the cells looked similar in 30 Exp and NB but were large and flat in NBF. Thus, due to typical morphology, the goal of having "limiting conditions" and a high percentage of neurites, I selected 30 % Expansion media for my experimental conditions.
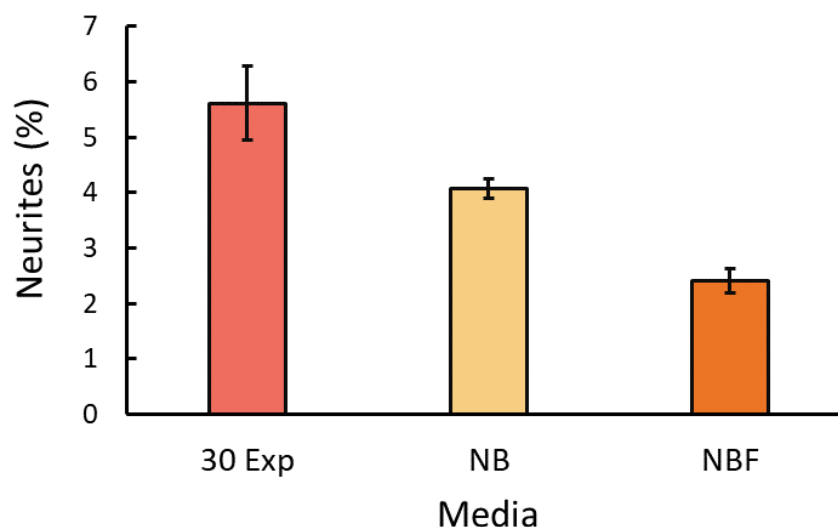
Figure 10: Comparison of percentage of neurites in 3 different media conditions. NPCs had highest neurite outgrowth in 30 % Expansion Media and lowest neurite outgrowth in NBF media. N= 5 experiments, 2-3 dishes/condition/expt, 2 patients-1 iPSC clone each.

Optimizing Plating Methods: Acute Dissociation vs Low Density

Initially, two methods of plating were utilized to study neurite outgrowth in NPCs. The acute dissociation (AD) method involved culturing 1 million NPCs on PDL+ Laminin coated 35 mm dishes. After 48 hr, these cells were dissociated with 1X Accutase, replated onto PDL+ Laminin coated dishes overnight. This method was utilized to acutely assess cells that were grown at high density. In the 2nd method, cells were plated at low density (LD) for 48 hrs and then fixed. For both methods, analyses were conducted on a phase

contrast microscope by counting three 1 cm rows for the proportion of cells with neurites. AD and LD methods were also compared under the stimulation of 10 nM PACAP and 300 ug/mL 5-HT to see if methods altered EF response. In control conditions, there was a significantly lower percentage of neurites in AD compared to LD conditions. The percent increase in neurite with PACAP and 5-HT stimulation were approximately the same in AD and LD though the absolute numbers were greater in the LD conditions (Figure 11). Thus, due to higher percentage of neurites, LD conditions were selected as the final method for my neurite studies. Moreover, unlike AD, LD culturing required less time, required no dissociating enzymes that could damage cells, and employed less coating materials.
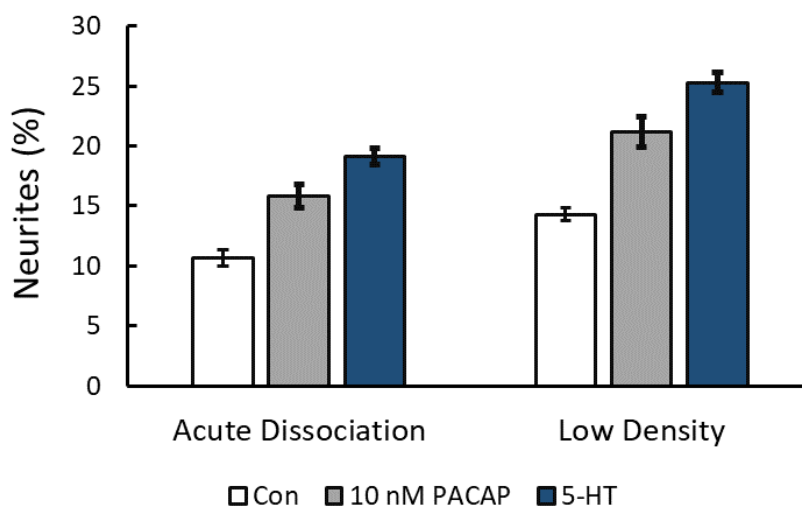
Figure 11: Comparison of two different plating methods for the neurite assay. As shown, cells express a higher percentage of neurites in all conditions in low density plating when compared to acute dissociation. N= 2 experiments, 3 dishes/expt/condition, 1 patient- 1 iPSC clone.

Optimizing Coating Substrate:

In expansion conditions, at high density, NPCs are cultured on Matrigel coated 6 well plates. However, Matrigel has an unknown composition that can vary from batch to batch. To allow for better control of coating substrate composition and concentration, we chose to select and alternate coating substrate for the neurite assay. First, cells were plated

onto 4 different coating matrices: poly-d-lysine (PDL), PDL+ Fibronectin, PDL+ Laminin, and Matrigel. Concentrations that were previously utilized in rodent cultures were selected: PDL (0.1 mg/mL), Laminin (10 ug/mL), and Fibronectin (5 ug/mL). As seen, after 48 h, neurites were sparse in both PDL and PDL+ Laminin (Figure 12). On the other hand, PDL+ Fibronectin and Matrigel had higher percentages of neurites and higher cell numbers than PDL and PDL+ Laminin. Since fibronectin is a defined substrate, this was selected for the neurite assay.
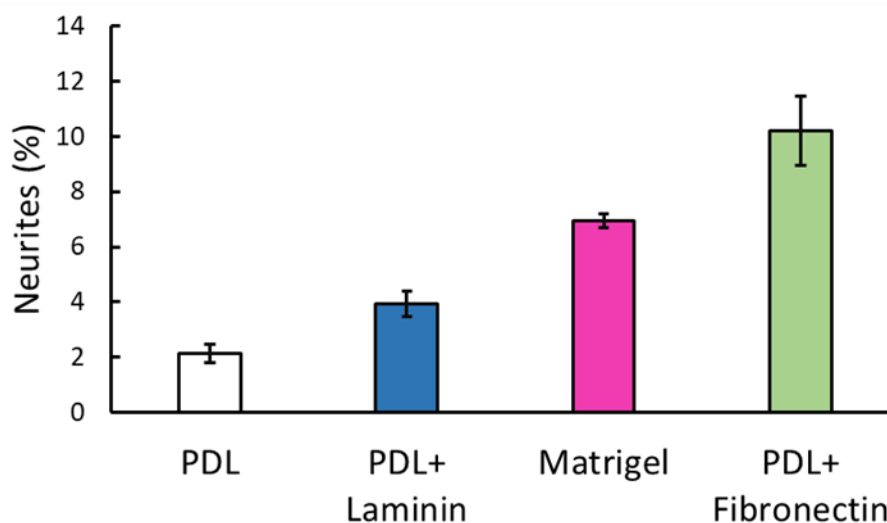
Figure 12: Coating substrate influences the percentage of neurites in NPCs. Neurites are highest in fibronectin and lowest in PDL. N= 2 experiments, 3 dishes/condition/expt, 2 patients- 1 clone each.

Once fibronectin was selected as the preferred coating substrate, studies were conducted to test the optimal concentration for neurites. The following concentrations of fibronectin were tested: 1, 5, 10, 30, 100 ug/mL. As seen in the graph (Figure 13), the percentage of neurites increased with dose until 30 ug/mL concentration, after which there was a drop. There was almost a 2-fold difference in neurites between the concentration used in rodent culture (5ug/mL) and the 30 ug/mL concentration. However, the 30 ug/mL required 6x

more fibronectin and would therefore be expensive and use a lot of material. All concentrations except for the 1 ug/mL were able to show stimulation of neurites with PACAP. Thus, to save resources, I determined that the 5ug/mL was a sufficient concentration to study neurites. However, with further experiments on different cell lines, I ultimately found that, on this concentration of fibronectin, neurites varied from 2% to 25%. Thus, in the case where a higher proportion of cells bearing neurites is desirable, a 30 ug/mL concentration of fibronectin to study neurite outgrowth may be considered.
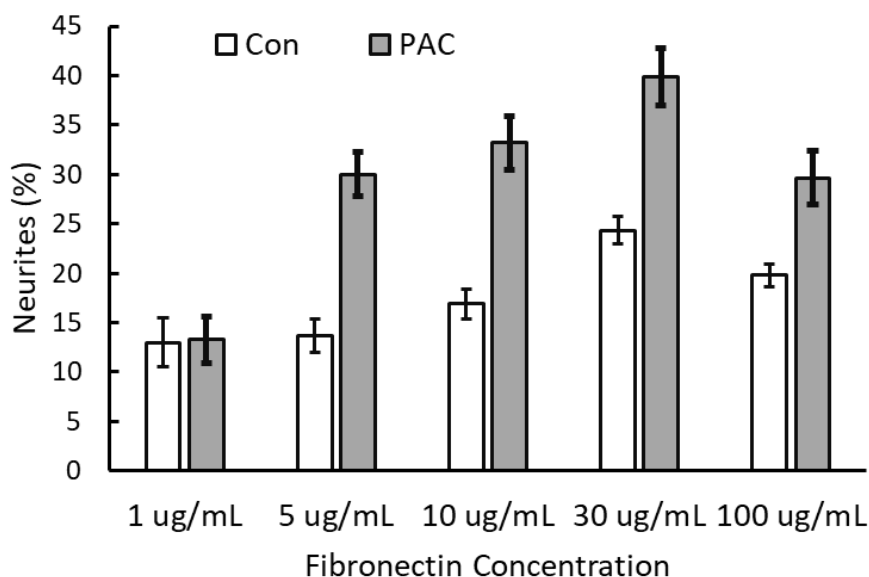
Figure 13: The concentration of fibronectin influences percentage of cells with neurites. At the lowest concentration, the effect of PACAP on neurite outgrowth is eliminated (1ug/mL). Neurites are similar on 5, 10, and 100 ug/mL fibronectin. Highest percentage of neurites is seen on 30 ug/mL. N= 2 experiments, 3 dishes/condition/expt, 1 patient-1 clone

Optimizing Plating Density

To count cells bearing neurites and total cell numbers, NPCs must be dissociated and plated as single cells. Clumps of cells and high cell densities can alter neurite behavior and make counting challenging and subjective. Thus, after selecting fibronectin as the coating, different densities of NPCs were tested to assess neurite percentage. NPCs were plated at 25K, 50K, 100K, 150K, 200K, and 250K into 35 mm dishes coated with PDL+

FN. As seen, the percentage of neurites increased with density (Figure 14). However, the cells became "clumpier" with higher densities and thus counting was more difficult. Ultimately, the 50K density was selected for my assays due to having virtually no clumps but higher neurites than 25K. However, again, in the future, using the 100 or 150K density would increase the percentage of neurites without causing excessive clumping
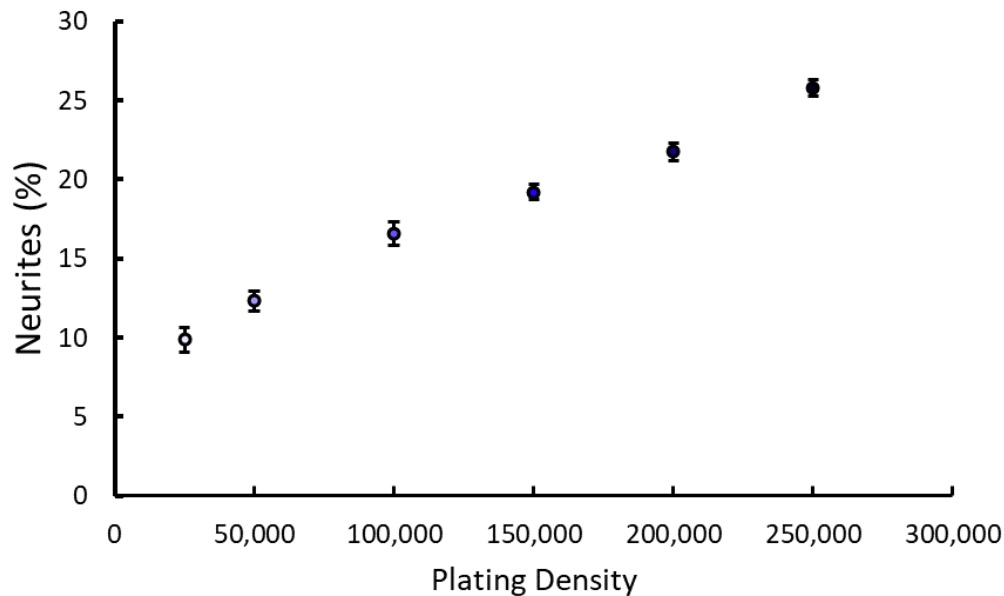
Figure 14: Initial plating density of cells influences the percentage of neurites. As seen percentage of neurite increases (almost linearly) with increase in initial plating density. N= 1 experiment, 2 dishes/condition, 1 patient-1 clone

Time in Culture:

With mouse and rat cultures, neurites could be analyzed at 24 hours. With extended time in culture, in some cases, primary cell cultures were no longer viable. NPCs however, were well suited to long term culture in high density, but their longevity in low density culture was unknown. Moreover, it was unclear whether percentage of cells with neurites would increase over time. Thus, to test the effects of time on neurite outgrowth, cells were plated at 100k density for 48, 96, and 144 hours (6 days). Tests were run in control conditions and under stimulation of 10 nM PACAP. At 24 h, visual analyses showed hardly

any neurites in control or PACAP conditions, so cells were not analyzed at this time point. By 48 h control conditions had approximately 13% neurites while PACAP stimulated conditions had 22% neurites (Figure 15). By 6 days however, neurite outgrowth in control conditions had more than tripled to 42% in control conditions and 61% in PACAP conditions. However, by 6 days, cell numbers had greatly decreased, and morphology of cells suggested differentiation into neurons and glia. Therefore, as differences in control and stimulated conditions could be seen as early as 48 hours, I selected this time point to conduct my experiments. This time point also allowed the study of cells before differentiation. However, again, while 48 hours allows for more rapid analysis and is sufficient time to observe neurite outgrowth and differences between conditions, only a small percentage of cells are expressing neurites at this point. Thus, 4 days of neurite culture may be optimal as almost 1/3 of NPCs express neurites at this point and cells are not completely differentiated as seen in Day 6.
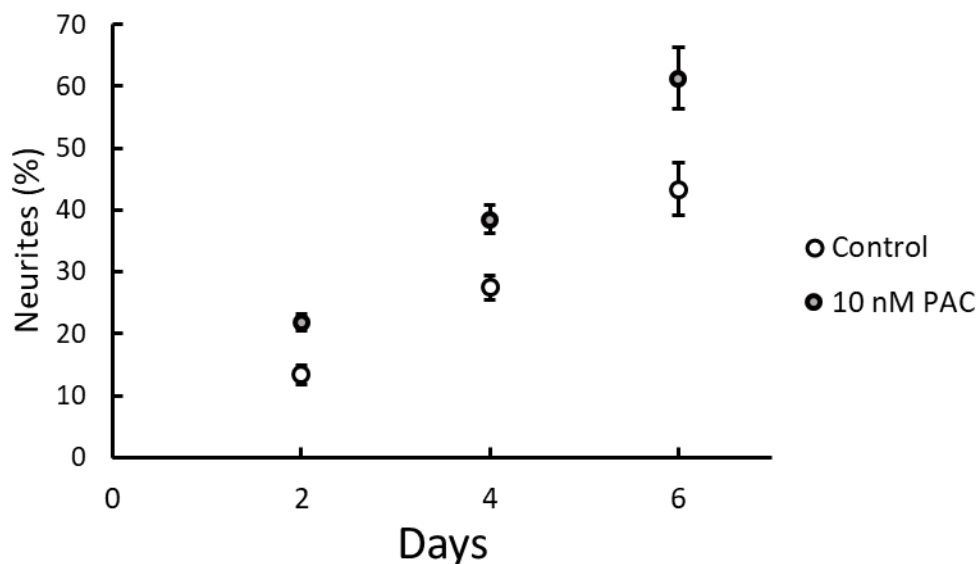
Figure 15: Percentage of cells with neurites increases with time in culture. Graph shows that there is a linear increase in the percentage of cells with neurites from D 2 to 4 to 6. PACAP continues to stimulate neurite outgrowth at all 6 days. N=1 expt, 2 dishes/D/condition, 1 patient- 1 iPSC clone.

Summary of Neurite Conditions:

After testing all the parameters mentioned above, I chose to conduct my neurite outgrowth assays using 48-hour low density culture method. Cells were plated at 50k density onto 35 mm dishes coated with 0.1 mg/mL PDL and 5ug/mL FN. Increasing FN concentration and increasing culture times can increase the percentage of cells with neurites if needed. Ultimately, the method developed can successfully detect neurite differences between lines and show differences in neurite percentages under the stimulation of EFs. These methods have been successfully and reproducibly used among numerous investigators in our lab. Indeed, Figure 16 shows neurite analyses done by 5 investigators on the same exact neurite images. All investigators were able to detect differences between control and EF conditions. Moreover, there are no statistical differences in neurite count from investigator to investigator in either control or EF conditions! This shows that our methods and results are incredibly rigorous and reproducible.
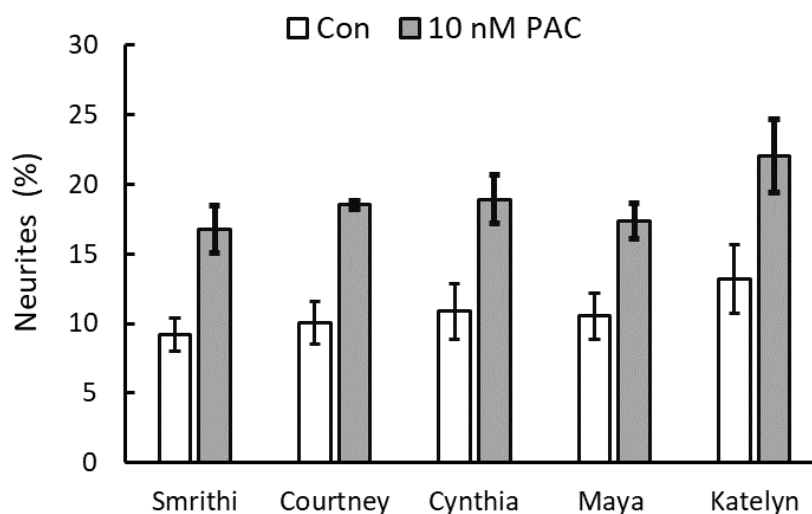
Figure 16: Comparison of data acquired by 5 investigators analyzing the same 2 dishes for neurite outgrowth. All investigators detected differences between control and PACAP dishes. There are no statistical differences in the data acquired between investigators

Marker Validation of Low Density Cultures:

Studies in mouse NPC cultures have suggested that culturing of NPCs at low density can push cells to differentiate. Thus, NPCs were stained for NESTIN, SOX2, PAX6 and TUJ1 at low density conditions that were used for neurite assay (Figure 17). The percentage of cells expressing markers were quantified over the total DAPI positive cells. Even at low density, at 48 h, most of our cells retained their "stem" or precursor cell nature. 75% of the cells were SOX2 + and 85% of cells exhibited NESTIN positive staining on average across patients (Figure 18). On average about 35% of cells also expressed PAX-6 (20-45% range). However, a noticeable number of cells also exhibit neuronal markers like TUJ1 (on average 35%, 25-55% range). By 4 days in low density culture there is a decline in NESTIN positive cells from 88% to 30% and an increase in TUJ1 positive cells from around 30% to 70%. This suggests that low density culture does push cells towards differentiation, however, a large proportion of cells remain as NPCs at day 2 and thus this system is ideal for studying early differentiation processes like initial neurite outgrowth.
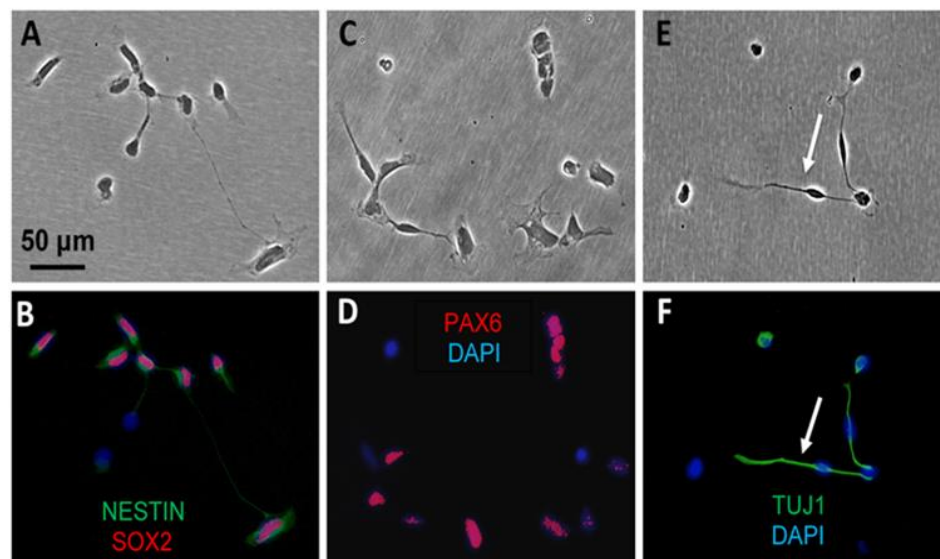
Figure 17: Expression of markers in low density NPC cultures. Panels A-C show phase-contrast images of NPCs plated at low density. These NPCs are positive for: B) NESTIN (G), SOX2 (R), D) PAX-6 (R), and F) TUJ1 (G). Figure from Williams and Prem et al 2017
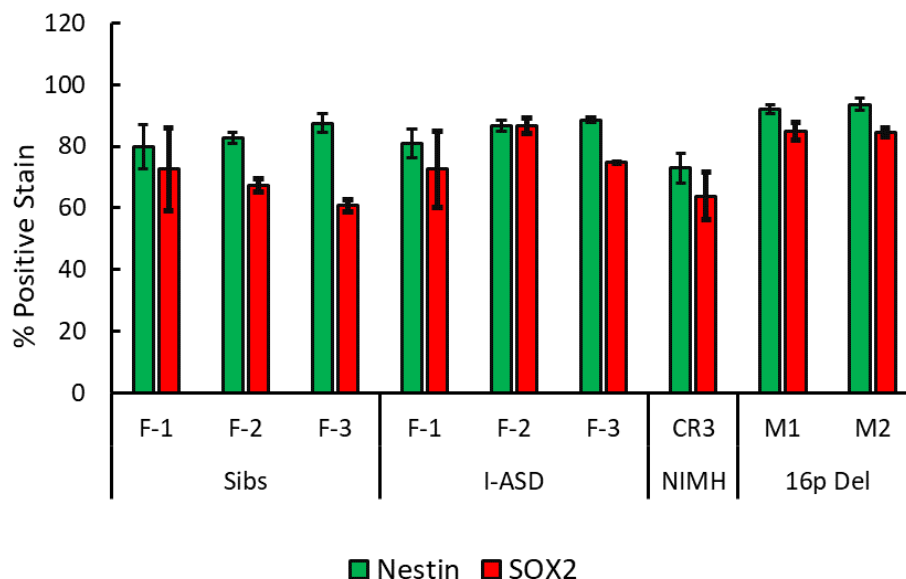
Figure 18: Quantification of Nestin and Sox2 expression in NPCs. Nestin (green) and SOX2 (red) expression in NPCs from all 3 Sibs and I-ASD (F-1, F2, F3), 1 NIMH line and both 16p11.2 deletion males (M1, M2). On average, 85% of cells were Nestin positive while 75% of cells were SOX2 positive N= 1 experiment, 3 dishes, 1 clone per line

Development and Optimization of the Neurosphere Migration Assay:

Cell migration is an important developmental process that allows for proper organization and cytoarchitecture of the brain. As reviewed, post-mortem analyses of brains from individuals with ASD have revealed/shown changes such as heterotopias and altered cortical lamination which are suggestive of disruptions in migration. Yet, despite the importance of migration in brain development and the implication that this process is disrupted in ASD, no iPSC studies of ASD have looked at NPC migration. However, the Brennand lab utilized human NPCs to uncover migration defects in schizophrenia. The Brennand lab to study migration and I adapted some of their methods to conduct my studies [775]. Much like my neurite outgrowth studies, multiple parameters were tested before conducting neurosphere migration experiments. The conditions tested are described below:

Density & Formation time:

In the Topol et al methods paper, neurospheres were formed by plating between 200,000 and 1 million NPCs into a well of a non-adherent 6-well plate [775]. Thus, for my studies I compared neurosphere formation in three different densities in 1 line. The following initial densities of NPCs were plated onto non-adherent 35 mm dishes: 250K, 500K, and 1 million. In parallel, I also cultured these densities in either 100 Exp or 30 Exp to determine if one media condition was more favorable to neurosphere formation than the other. Spheres that are too small would break apart during the migration assay and thus, based on the Topol paper, I waited for the spheres to reach a size of 100 um $\pm$ 20 um before they were collected for the assay. As seen in Table 1, spheres formed within two days when NPCs were plated at 1 million density in 100 % Expansion. The same density of NPCs plated into 30 Exp showed sphere formation to 3 days. At the 250K density, it took the cells 6 days to form spheres in 100 Exp and 8 days in 30 Exp. Thus, to efficiently generate neurospheres, I selected to culture NPCs at a density 1 million in 100 Exp.

	250K	500K	1 Million
100 Exp	6 days	3 days	2 days
30 Exp	8 day	4 days	3 days

Table 1: Time taken for Neurospheres to reach the 100 um$\pm$ 20 size needed to conduct the migration assay. Spheres form fastest when 1M NPCs are plated in a 35 mm dish in 100% Exp.

Coating & Migration Time:

Once spheres were formed to the appropriate size, they were plated and allowed to migrate. Spheres which displayed a dense inner cell mass with NPCs that migrated as a contiguous carpet were ideal for measurement. On the other hand, spheres that were dispersed or having cells that were not connected to the inner sphere could not be measured. In the Topol methods, cells were allowed to migrate for 48 hrs on Matrigel. For my assay,

I tested 24 hours, 48 hours, and 72 hours of migration on the following coatings: PDL, Matrigel, PDL-Laminin, and PDL-Fibronectin. By 72 hrs, on all coatings, spheres could not be measured as they were entirely dispersed or too large for the 10X objective. At 24 and 48 hrs, results of the migration assay varied by coating and coating concentration. On fibronectin, for example, neurospheres entirely dispersed as early as 24 hrs on all concentrations tested (1, 5, 10, 30 ug/mL). Thus, fibronectin could not be utilized for the neurosphere assay. Laminin was tested at the following concentrations: 0.3, 1, 5 and 10 ug/mL. Again, even at 24 hours, spheres were too large or broke apart in all concentrations of laminin but the 0.3 ug/mL at 24 h. In the end, at 24 h, we found that the spheres migrated the furthest in 0.3 ug/mL laminin, followed by Matrigel then PDL. This trend was largely replicated at 48 h but laminin spheres were entirely dispersed at this time point.

Ultimately, I chose a migration time of 48 h as spheres were larger, migrating carpets were better defined, and EF responses were more apparent. However, as noted above at 48 h spheres remained measurable on only PDL and Matrigel. However, PDL seemed to cause increased cell death while Matrigel was an undefined substrate. Thus, I tested a few more coating conditions at 48 h: 0.1 mg/mL PDL, 0.1 and 0.03 ug/mL Laminin, and two methods of Matrigel coating. In the first method, Matrigel was set for 30 minutes in the incubator, and then spheres were plated into the liquid matrix without removing gel (termed Matigel-Gel condition) as described in Topol et al. On the other hand, for typical cell culture (like expansion), Matrigel is allowed to set in the incubator for 30 min and then the excess liquid matrix is removed before plating cells. Thus, in the 2nd condition, I set the Matrigel for 30 minutes in the incubator, removed the excess gel, added media, and plated cells into media instead of gel. Removal of the Matrigel led to

excess migration which was measurable but almost too large for the 10x objective. Spheres plated on PDL did not survive or adhere as well and migrated too little. The 0.03 ug/mL of laminin was comparable to Matrigel-gel conditions. Yet, in a 3rd experiment, even 0.03 ug/mL laminin concentration, in a different line, led to dispersed or overly large spheres at 48 h. On Matrigel, however, this cell line's neurospheres remained intact at 48h. Ultimately due to the consistency of results and ease of coating, Matrigel (left as a gel) was selected as the coating substrate for the neurosphere migration assay

Summary of Neurosphere Migration:

Ultimately, to form spheres, 1 million NPCs were plated into an uncoated 35 mm dish in 100 Exp for 48-96 hrs until they reached a size of 100 um ±20. The spheres were then plated into Matrigel for 48 h for migration to occur. Then, spheres were fixed and average migration was analyzed on image J by measuring inner cell mass area and subtracting that value from the total sphere area. Through these conditions, migration measurements were able to be reproducibly conducted by three different investigators (Figure 19). Thus, this shows the rigor and reproducibility of the results that were obtained through these methods.
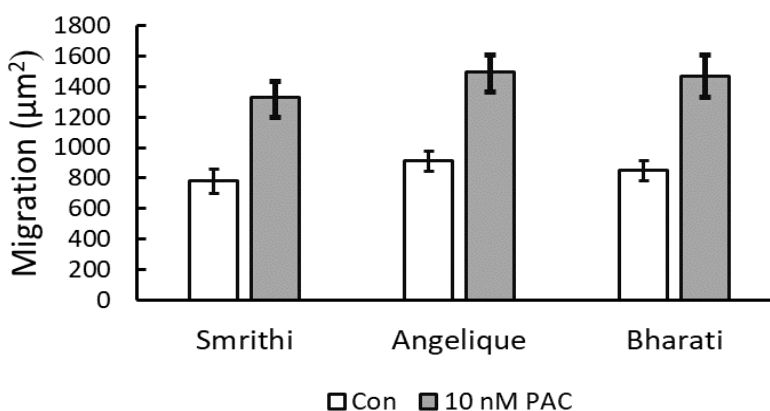
Figure 19: Data comparing migration values acquired by 3 investigators analyzing the same 20 neurosphere images. All investigators detected differences between control and PACAP images. There are no statistical differences in the data acquired between investigators.

Chapter 5- Experimental Results: Neurite Outgrowth in ASD NPCs

Family-1 (1072: Sib-1 and ASD-1)

Neurites in Control Condition

After establishing methods to assess neurites, I began to study neurite outgrowth in one randomly selected pair of brothers from our idiopathic cohort. As mentioned, from my review of human iPSC autism studies, deficits in neurite outgrowth are commonly seen in studies of post-mitotic neurons derived from iPSCs of individual with ASD. Yet, numerous studies have indicated that the mid-fetal developmental period, when NPCs are proliferating, migrating, and beginning to differentiate, is an important window in the pathogenesis of ASD. Thus, I hypothesized that neurite defects would begin to manifest in the NPC stage and would likely be observed in multiple individuals in our cohort. For Family-1, studies were conducted on NPCs derived from 5 Sib (Sib-1) iPSC and 5 I-ASD (I-ASD-1) iPSC clones (C1-C5). On average, NPCs from I-ASD-1 had approximately 57% fewer neurites than NPCs derived from Sib-1 (p= 9.63×10^{-31}) (Figure 20) However, there was some variability in percentage of neurites amongst different clones (C) (Figure 21). Yet overall, most NPCs derived from Sib clones (C1-C5) had a higher percentage of neurites than those derived from ASD clones (C1-C5).
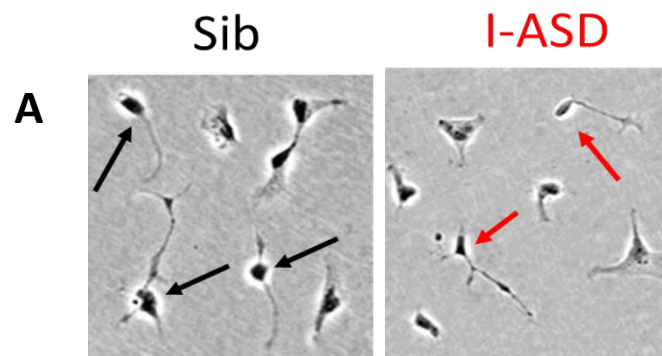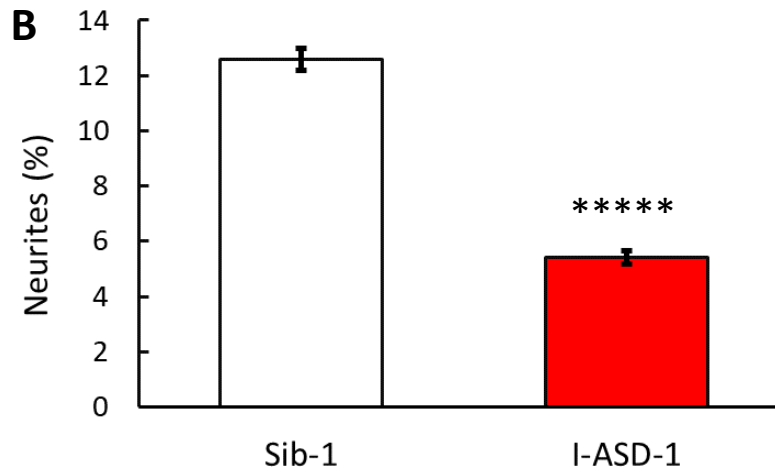
Figure 20: Reduced neurite outgrowth in ASD NPCs in Family-1. A) Representative images of neurites from Sib and ASD. The arrows point to the cells with neurites, as shown Sib-1 NPCs have more neurites than ASD-1 NPCs. B) Graph quantifying Family-1 NPC neurite outgrowth. Sib-1 N = 88 Expts, 2-3 dishes/ experiment, 5 iPSC clones, 11 neural inductions. ASD-1 N=70 expts, 2-3 dishes/expt, 5 iPSC clones, 11 neural inductions
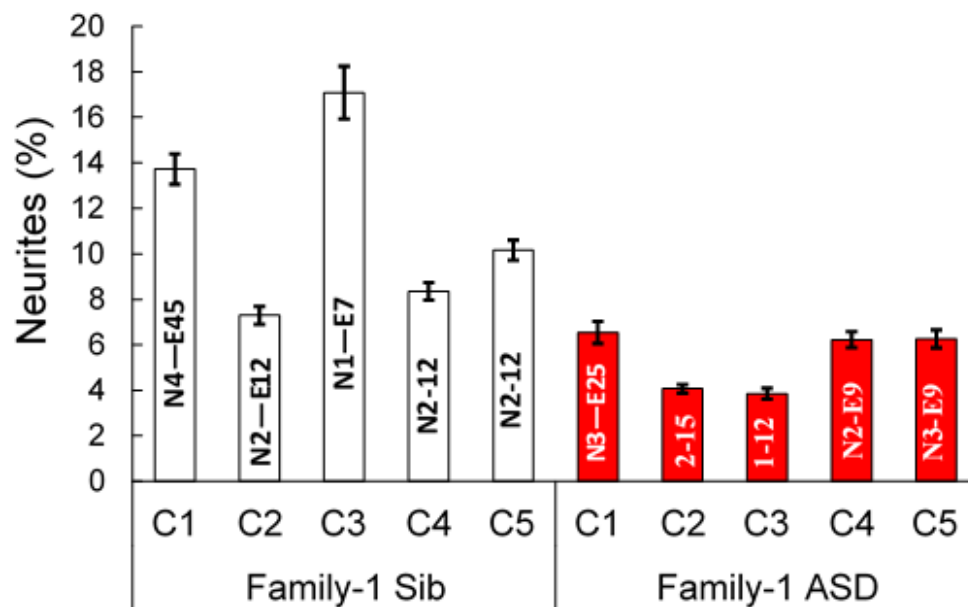
Figure 21: Percentage of neurites broken down by NPCs derived from 5 clones (C1-C5) of Sib and ASD in Family-1. In each bar, the "N "signifies the number of distinct neural inductions conducted on the clone. The "E" indicates the number of experiments conducted for each clone. There is variation in percentage of neurites from clone to clone however on average Sib NPCs have higher neurites than ASD NPCs

Neurite outgrowth under the stimulation of developmentally relevant EFs:

Most iPSC studies of neurodevelopmental disorders have only been conducted in control conditions. Yet, during brain development, there are multiple extracellular factors (growth factors, neuropeptides, and neurotransmitters) that regulate processes like proliferation, migration, and differentiation to ensure proper brain structure and function. Therefore, the effects of these important regulators remain largely undefined in human neural cells. Thus, I aimed to study neurite outgrowth under the stimulation of developmentally relevant EFs. In mouse studies conducted in our lab, we found that neuronal cells derived from ASD mice had differential responses to important EFs like IGF-1 as well as PACAP [198]. Hence, I theorized that our Sib and ASD NPCs may also respond differentially to certain EFs. My initial studies tested the effects of several EFs with known roles in development or ASD on Family-1. Based on results in this family, a narrower panel of EFs was tested in Families 2 & 3.

Neurite outgrowth under PACAP Stimulation:

Prior studies in our lab using both *in vivo* and *in vitro* rodent models have found that PACAP regulates basic developmental processes such as proliferation, migration, and neurite outgrowth [268, 277, 279, 286]. To understand the effects of PACAP on neurite outgrowth in human NPCs, I conducted dose response studies on one clone each from Sib-1 and I-ASD-1. As seen in the graph (Figure 22), when compared to Control, Sib NPCs had increases in neurites from 0.3 nM to 30 nM PACAP. There was also an inhibition of neurites to control level at 100 nM. On the other hand, ASD NPCs had no response to PACAP at any dose. Maximal neurite outgrowth in the Sib was observed at the 3 nM dose and thus, this dose was selected for further studies. At this 3 nM dose, multiple experiments

in NPCs derived from 4 different ASD and Sib clones found that, on average, Sib-1 NPCs had a 68% increase in neurites with 3 nM PACAP (p<0.0001) while ASD NPCs had no difference in neurites (p=0.99) (Figure 23). Finally, two-way ANOVA results also revealed a statistically significant difference between the action of PACAP on Sib NPCs and ASD NPCs (p=0.002). This shows that ASD NPCs in this family have an impaired PACAP response. Indeed, broken down by clone (C) we see that NPCs derived from 4 Sib clones (C1-C4) respond to PACAP while NPCs derived from the 4 ASD clones (C1-C4) all fail to respond to PACAP (Figure 24).
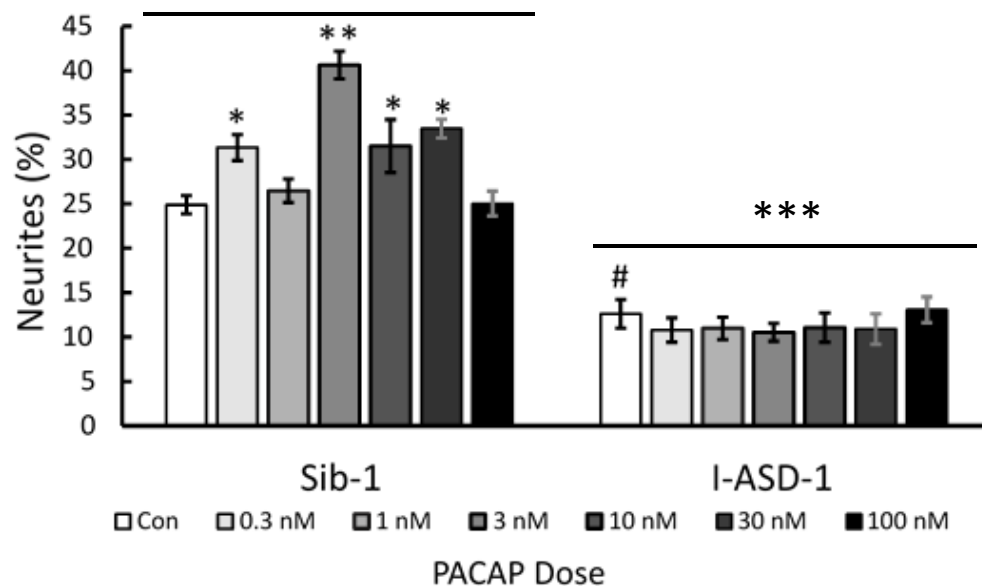
Figure 22: Dose response studies of neurite outgrowth under PACAP in Family-1. Sib NPCs showed a statistically significant increase in neurites under doses from 0.3 nM to 30 nM. ASD NPCs showed no response to any doses of PACAP. Sib N= 2 expts, 2 dishes/condition/expt, 2 clones. ASD N= 2 expts, 2 dishes/condition/expt, 2 clones. There is a statistically significant difference between each dose in Sib and in ASD (p<0.0001)
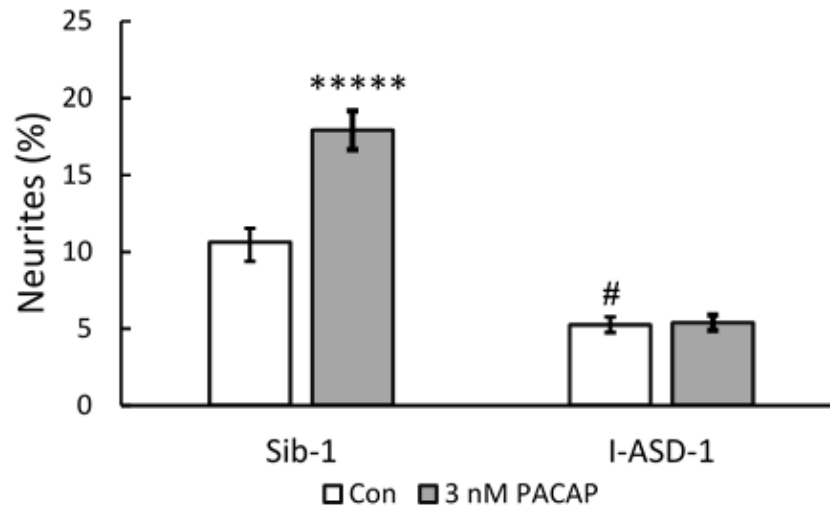
Figure 23: ASD NPCs fail to respond to PACAP. While Sib NPCs have 68% increase in neurite outgrowth under 3 nM PACAP, ASD NPCs have no change in neurite outgrowth at this dose. Sib N =21 expts, 2-3 dishes/condition/expt, 4 clones. ASD N= 18 expts, 2-3 dishes/condition/expt, 4 clones.
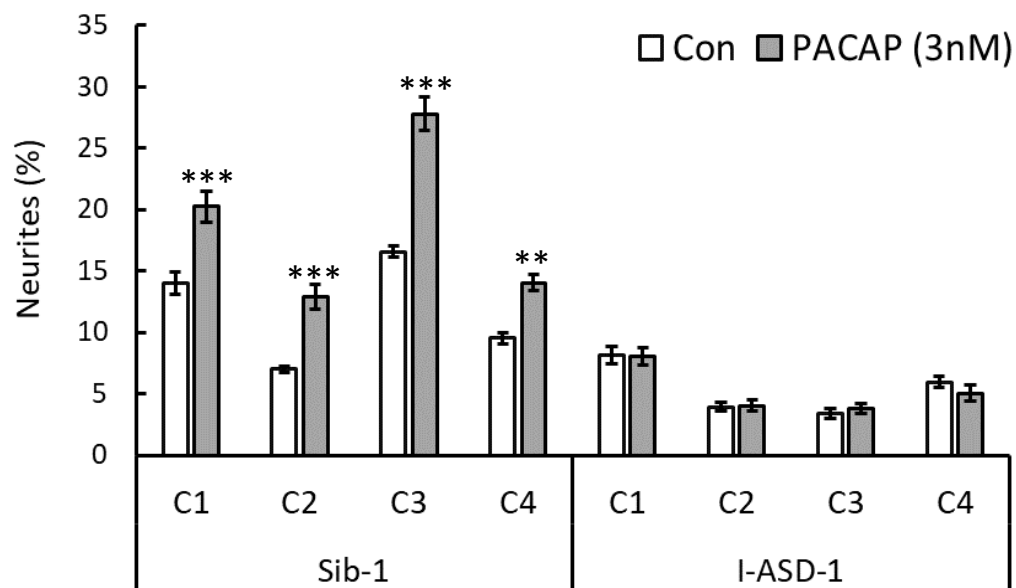
Figure 24: PACAP response broken down by clone in Family-1. NPCs derived from all 4 Sib clones respond to PACAP while no NPCs from ASD clones responded to PACAP. Sib N: 2-3 dishes/condition/expt C1: 3 neural inductions, 11 expts, C2: 1 neural induction 4 expts, C3: 1 neural induction 2 expts, C4: 2 neural inductions 4 expts. ASD N= C1: 3 Neural inductions, 7 expts, C2: 2 neural inductions: 5 expts, C3: 2 neural induction 3 expts, C4: 1 neural induction 3 expts.

Neurite outgrowth under Neurotrophin Stimulation:

The neutrophin family of growth factors include molecules such as NGF, BDNF, and NT3 which have well characterized roles in regulating the development of the mammalian nervous system. In particular, NGF and BDNF are well known for regulating neurite outgrowth. Thus, I conducted a dose response for all three neurotrophins on Sib-1 lines alone Interesting, I found that the Sib-1 did not respond to any doses of BDNF or NT3. Surprisingly, however, there were increases in neurite outgrowth at the 10 and 30 ng/mL doses for NGF (Figure 25). Thus, I chose to focus further studies on NGF responses in the Sib and ASD. I conducted a dose response for NGF in NPCs derived from 1 clone of ASD-1 and found that no dose of NGF could elicit neurite outgrowth in the ASD NPCs (Figure 25). Further studies of neurite outgrowth at the 30 ng/mL in dose in multiple clones found that Sib NPCs had an 74% increase in neurite outgrowth with NGF (p<0.0001) while ASD NPCs had no change in neurites (p=0.98) (Figure 26). Again, 2-way ANOVA results showed that NGFs actions were differential between ASD NPCs and Sib NPCs (p=0.0002)
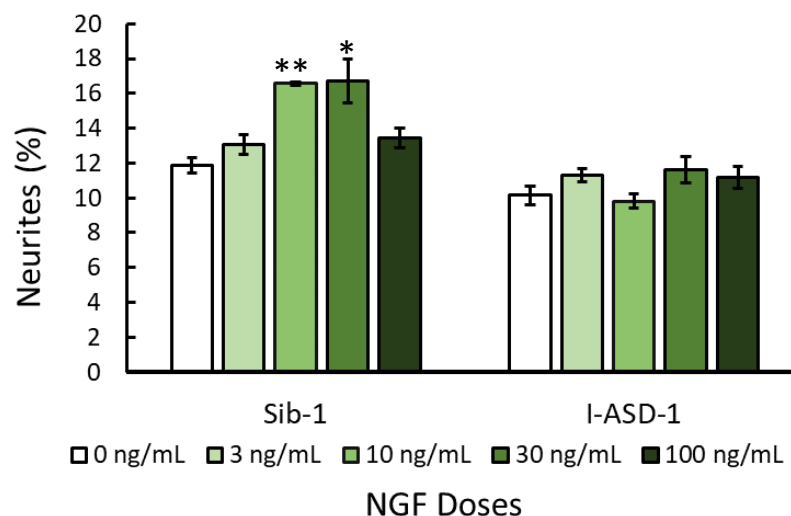
Figure 25: Dose response studies of neurite outgrowth under NGF in Family-1. Sib NPCs showed statistically significant increases in neurites under 10 ng/mL and 30 ng/mL doses of NGF. ASD NPCs showed no response to any doses of NGF. Sib N= 2 expts, 2 dishes/condition/expt, 2 clones. ASD N= 2 expts, 2 dishes/condition/expt, 2 clones

Figure 26: ASD NPCs from Family-1 fail to respond to NGF. While Sib NPCs have 74% increase in neurite outgrowth under 30 ng/mL of NGF, ASD NPCs have no change in neurite outgrowth at this dose. Sib N =14 expts, 2-3 dishes/condition/expt, 3 clones. ASD N= 16 expts, 2-3 dishes/condition/expt, 4 clones.

As BDNF was not initially tested in ASD-1 lines. I later conducted dose response studies in NPCs derived from this ASD individual (Figure 27). Unexpectedly, while the Sib NPCs remained unresponsive, ASD NPCs had increase in neurite outgrowth with BDNF at 10ng/mL (p=0.01) and 30 ng/mL (p=0.004). In short, these studies show differential responses for NGF and BDNF in Family-1 Sib vs ASD. However, we find that BDNF has the potential to increase neurites in ASD NPCs. It is important to note, that unlike PACAP, NGF does not signal through a G-protein coupled system. Moreover, the fact that cells did not respond to NGF seemed to indicate that it is unlikely that receptor dysfunction alone or alterations in the G-protein system alone were contributing to our cellular abnormalities.
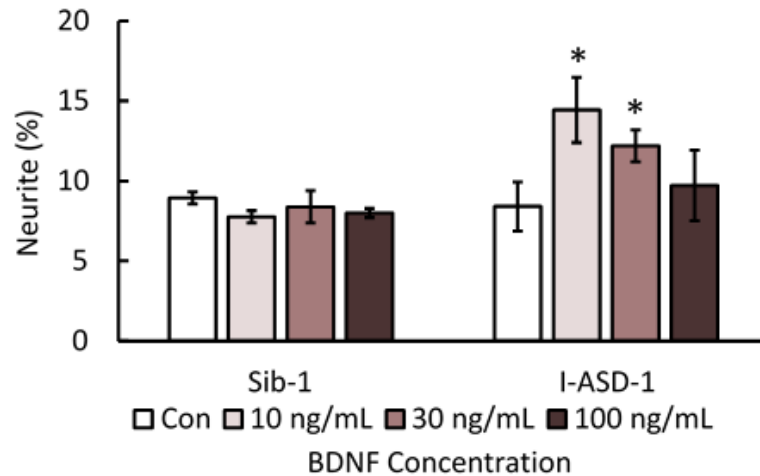
Figure 27: Dose response studies of neurite outgrowth under BDNF in Sib and ASD in family-1. Sib NPCs have no response to BDNF while ASD NPCs showed statistically significant increases in neurite at the 30 ng/ml and 100 ng/mL doses. Sib-1 N = 2 experiments, 2 dishes/condition/expt, 2 clones. ASD N= 2 experiments, 2 dishes/condition/expt, 2 clones. There is a statistical difference between ASD and Sib at the 10 and 30 ng/mL doses ($p<0.01$)

Neurite outgrowth under Serotonin Stimulation:

Serotonin (5-HT) is a neurotransmitter well known for regulating behavior. During development, 5-HT and its transporters and receptors play important roles in regulating developmental processes. Furthermore, 5-HT dysregulation is commonly found in ASD and 5-HT agonists are often used to manage and treat children with ASD. Like PACAP, 5-HT signals through G-protein systems. Thus, I anticipated that 5-HT response would also be aberrant in ASD NPCs. Dose response studies of 5-HT found that Sib NPCs begin to response to 5-HT at 30 ug/mL and have a peak response at 100 ug/mL of 5-HT. On the other hand, ASD NPCs had no response to 5HT except at 300 ug/mL dose. Further studies, done at 100 ug/mL and 300 ug/mL doses found that Sib had a 70% ($p<0.0001$) and 96% ($p<0.0001$) increase with these doses respectively (Figure 28). On the other hand, the ASD NPCs had no change in neurite outgrowth at the 100 ug/mL dose ($p=0.6704$) and an increase of 129% at the 300 ug/mL dose ($p<0.0001$) (Figure 28). Fascinatingly, according to two-way ANOVA, 100 ug/mL of 5-HT has differential effects on Sib NPCs when

compared to ASD NPCs (p<0.0001) whereas the 300 ug/mL acts the same on Sib and ASD (p=0.07). Yet, the percentage of neurites in Sib NPCs at 300 ug/mL 5-HT is still higher than the percentage of neurites in the ASD NPCs at this dose (p<0.0001). These studies suggest that NPCs derived from the ASD-1 individual have differential sensitivity to 5-HT compared to Sib. Moreover, these studies suggest that there may be aberrations in proteins or 2nd messengers involved in G-protein signaling.
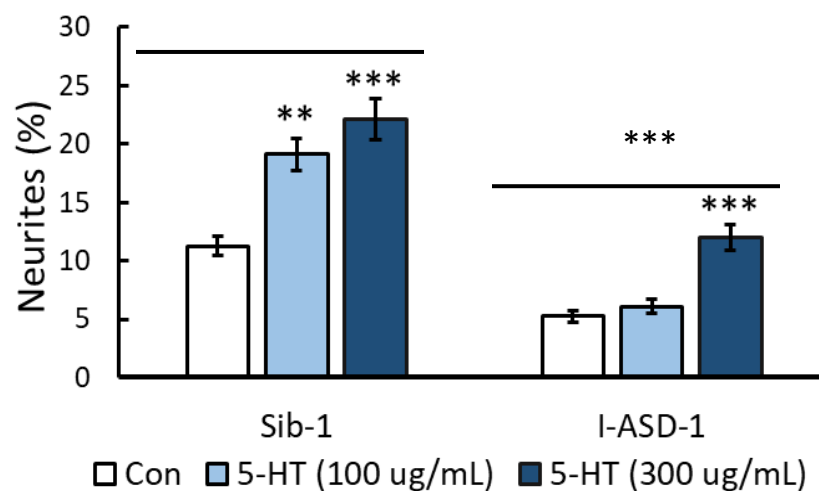
Figure 28: Dose responses studies of neurite outgrowth under 5-HT in Family-1. While Sib NPCs have a statistically significant increase in neurite outgrowth at both 100 ug/mL and 300 ug/mL dose, ASD NPCs only respond at the highest dose of 5-HT stimulation. Sib N= 12 expts, 2-3 dishes/condition/expt, 4 clones. ASD N = 13 expts, 2-3 dishes/condition/expt, 4 clones.

Neurite outgrowth under Oxytocin (OXT):

Oxytocin is a neuropeptide that has important roles in regulating social behavior and thus, it has been suggested as a therapeutic for ASD. Like PACAP and 5-HT, it works through G-protein coupled receptors. However, there are few studies on the neurodevelopmental functions of OXT. To test the role of OXT in neurites, dose response studies were conducted in our NPCs (Figure 29). Sib NPCs showed an increase in neurites at a 30 nM dose of oxytocin. In ASD, again, as with many tested EFs, there were no changes

in neurite outgrowth. Further studies with the 30 nM dose found Sib had 54% increase in neurite outgrowth with OXT (p<0.0001) while ASD had no change (p=0.098) (Figure 30). Two-way ANOVA also showed a statistically significant difference between Sib and ASD in terms of OXT interaction with ASD vs Sib (p<0.0001). Again, we see that ASD NPCs have defective responses to EFs that are important for normal development and behavior.
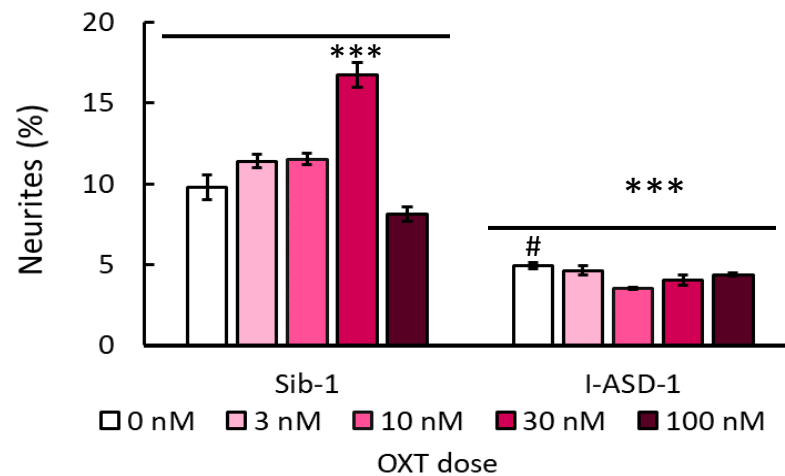
Figure 29: Dose responses studies of neurite outgrowth under OXT in Family-1. While Sib NPCs have a statistically significant increase in neurite outgrowth at 30 nM, ASD NPCs had no responses to OXT at any dose. Sib & ASD N=2 expts, 2-3 dishes/expt, 2 clones. There is a statistically significant difference between Sib and ASD neurites at every dose (p<0.0001)
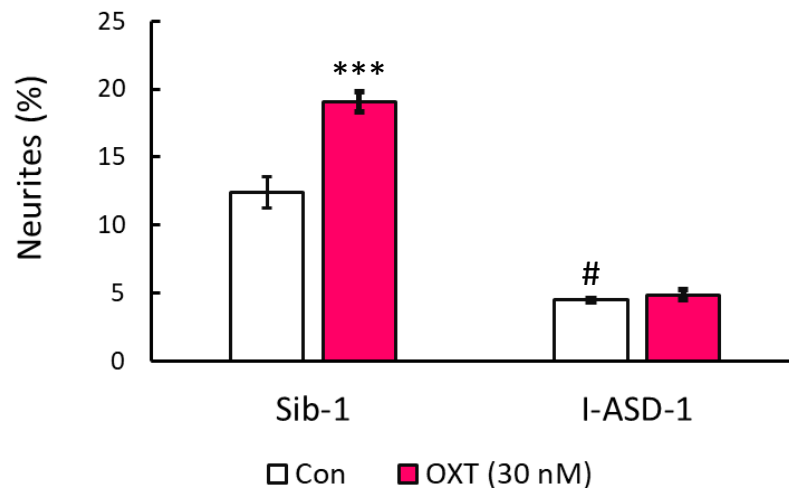
Figure 30: ASD NPCs in Family-1 fail to respond to OXT. Under 30 nM OXT, Sib NPCs have a 54% increase in neurite outgrowth while ASD NPCs have no change in neurite outgrowth at this

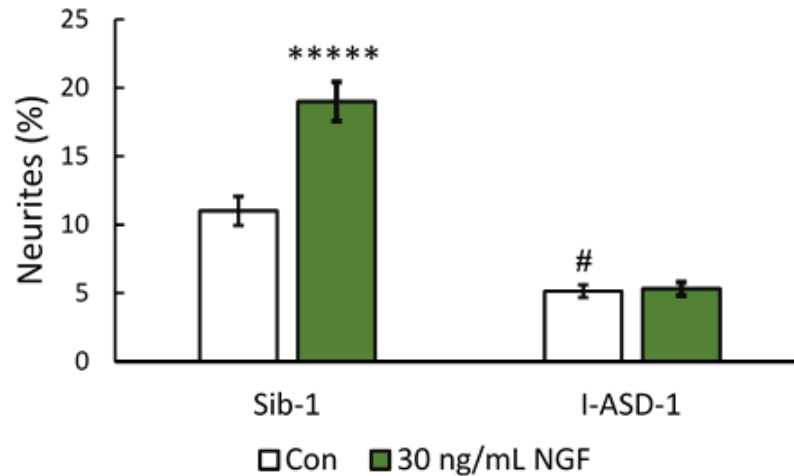dose. Sib N= 8 Expts, 2-3 dishes/condition/expt, 3 clones. ASD N = 7 expts, 2-3 dishes/condition /expt, 3 clones.

Neurite outgrowth under FGF-2

FGF is a growth factor which has well defined and important roles in neurodevelopment. Unlike PACAP, 5-HT, and OXT, FGF-2 uses a receptor tyrosine kinase system for signaling. Neurotrophins also signal using this receptor/signaling mechanism. In my studies, FGF-2 dose responses showed very interesting differential phenotypes in the ASD-1 vs Sib-1 NPCs (Figure 31). In Sib NPCs, FGF-2 causes a dose-dependent decrease in neurites while the ASD NPCs have a dose-dependent increase in neurite outgrowth with FGF-2 (Figure 31). The dose selected for further studies was 10 ng/mL. These extended studies showed that FGF reduced neurites by 30% in Sib ($p=0.01$) while it increased neurites in the ASD NPCs by 160% ($p<0.0001$) (Figure 32). Again, FGF-2 had a statistically different effect on Sib vs ASD ($p<0.00001$). Thus, it seems that FGF-2 is able to rescue neurite outgrowth defects in the ASD NPCs. However, the response to FGF-2 in the ASD NPCs is not typical.
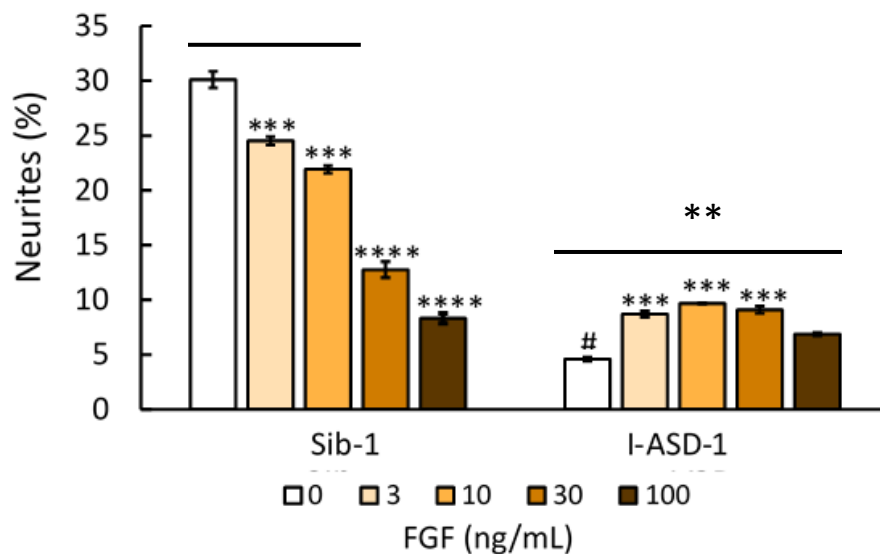
Figure 31: Dose responses studies of neurite outgrowth under FGF2 in Family-1. While Sib NPCs have a dose dependent decrease in neurite outgrowth under FGF, ASD NPCs have an

increase in neurite outgrowth at all doses of FGF tested. Sib N = 2 expts, 2 dishes/expt/condition, 2 clones. ASD N= 2 expt, 2 dishes/expt/condition, 2 clones. There is a statistically significant difference between Sib and ASD at Con, 3, and 10 ng/mL doses (p<0.001)
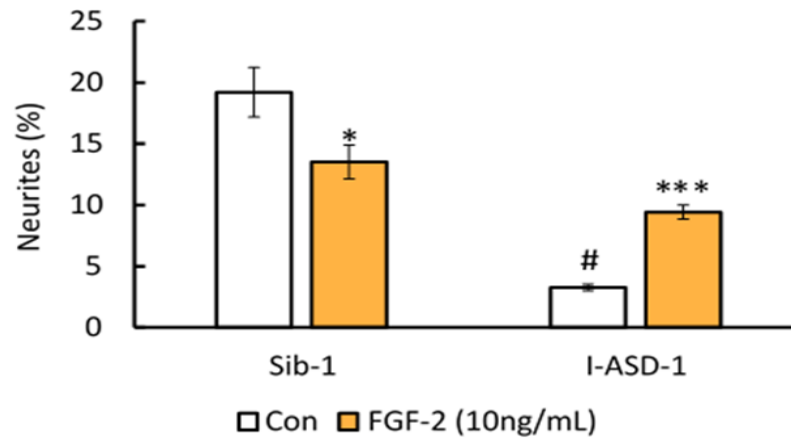
Figure 32: Family-1 Sib & ASD NPCs have differential neurite outgrowth under FGF-2. Sib NPCs have a 30% decrease in neurite outgrowth under while ASD NPCs had a 160% increase in neurite outgrowth under 10 ng/mL FGF. Sib N =11 expts, 2-3 dishes/condition/expt, 2 clones. ASD N= 10 expts, 2-3 dishes/condition/expt, 3 clones.

Neurite Length:

Thus far, all my studies have been focused on assessing the proportion of cells with neurites. However, neurite length is a common feature that is measured in studies of neurodevelopmental and neurodegenerative disorders. Initial studies in Family-1 NPCs found no difference in neurite length between Sib and ASD (Figure 33). Furthermore, stimulating the Sib cells with the EFs led to no changes in neurite length (Figure 34). Thus, neurite length analyses were not measured in other clones or in other Families.
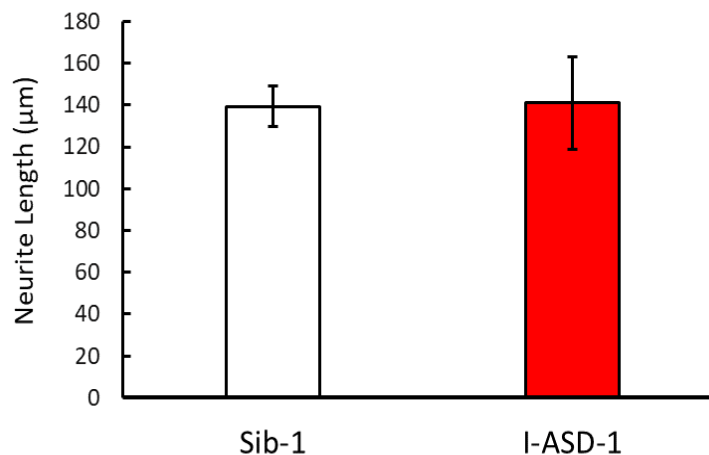
Figure 33: Average neurite lengths are not statistically different between Sib and ASD in Family-1. Sib N = 2 expts, 150 cells, 1 clone, ASD N= 2 expts, 150 cells, 1 clone.
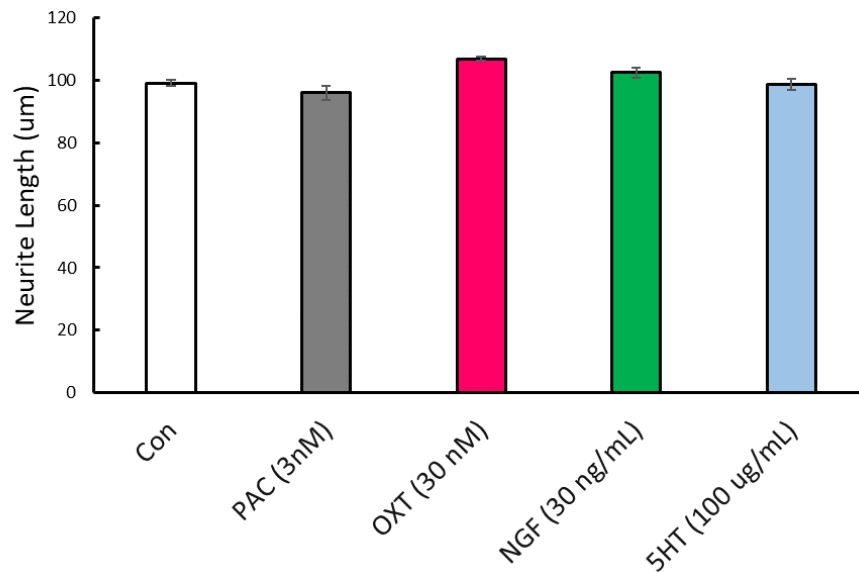
Figure 34: Graph showing neurite lengths in Sib-1 NPCs under various EFs. There are no differences in neurite length under any EF tested. Sib N = 2 expts, 100 cells/expt, 2 clones.

Family-2 (1077: Sib-2 & ASD-2) & Family-3 (1012: Sib-3 & ASD-3)

After extensively studying neurite outgrowth in control and EF conditions in Family-1, I extended my neurite studies to two more randomly selected Sib-pairs from our idiopathic cohort. Due to ASD heterogeneity, it was unclear whether the phenotypes observed in Family-1 would replicate in Family 2 & 3. In these families, I studied NPCs from fewer iPSC clones due to the relative consistency of results from Family-1. Moreover, fewer EFs were tested, particularly in Family-3, in the interest of narrowing and focusing my studies.

Neurites in Control Conditions:

Much like Family-1, my studies in Family-2 & 3 found that in control conditions, ASD NPCs on average had fewer neurites than Sib NPCs. Figure 35 shows that Family-2 Sib NPCs have an average of 8.3% neurites while the ASD-2 has 5.4% neurites, almost a 60% difference (p<0.00001). In family 3, Sib NPCs have 8.9% neurites while ASD NPCs

have 3.6% neurites with a 40% difference (p<0.00001). When we break the data down by clone in Family-2, we see that the NPCs 3 Sib clones have similar percent of neurites and the NPCs from the 3 ASD clones are all consistently less (Figure 36). The same can be seen for Family-3 (Figure 37). Thus, from studies conducted in 3 Sib-pairs, we find that impairments in neurite outgrowth are common in all idiopathic ASD NPCs studied.
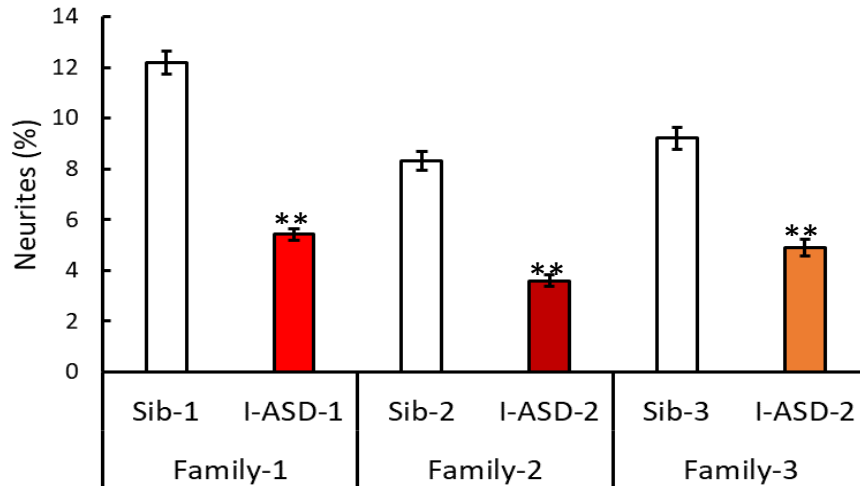
Figure 35: Neurite outgrowth in Sib and ASD in all 3 families from the Idiopathic Cohort. In all families, Sib has a significantly higher percentage of neurite than ASD. Family-1 N: Sib: 88 Expts, 2-3 dishes/ experiment, 5 clones. ASD-1 N=70 expts, 2-3 dishes/expt, 5 iPSC clones. Family 2 N: Sib-2: 34 Expts, 2-3 dishes/expt, 3 clones. ASD-2: 22 expts, 2-3 dishes/expt, 3 clones. Family-3 N: Sib-3: 15 expts, 2-3 dishes/expt, 2 clones. ASD-3: 12 expts, 2-3 dishes/expt, 2 clones .
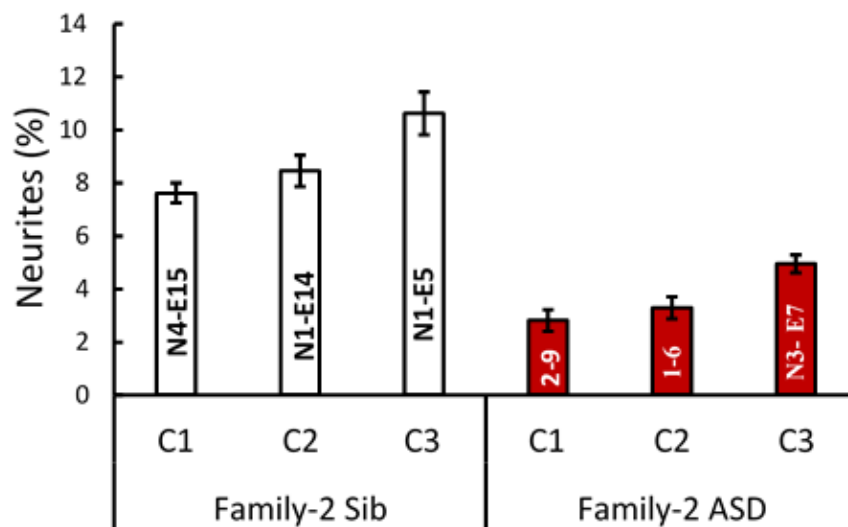
Figure 36: Breakdown of Family-2 neurites by clone NPCs derived from 3 clones (C1-C3) of Sib-2 and ASD-2. In each bar, the "N "signifies the number of distinct neural inductions conducted on the clone. The "E" indicates the number of experiments conducted for each clone. There is variation in percentage of neurites from clone to clone however on average Sib NPCs have higher neurites than ASD NPCs.
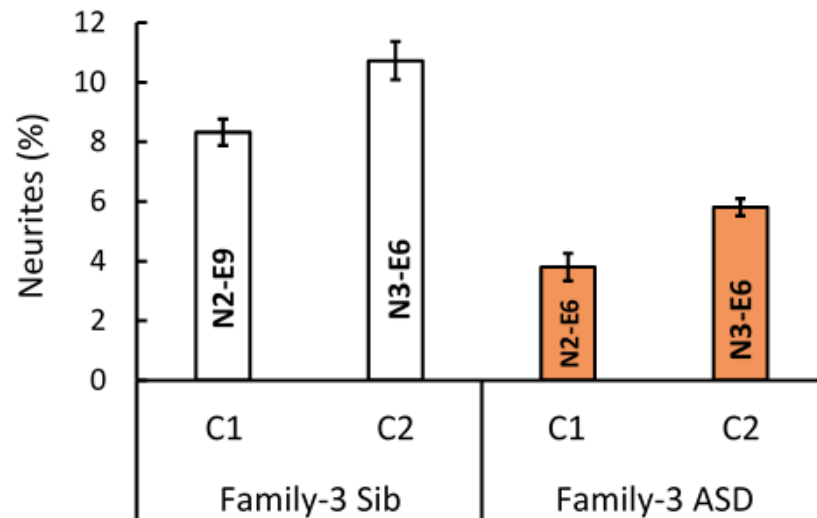
Figure 37: Breakdown of Family-3 neurites by clone. NPCs derived from 2 clones (C1,C2) of Sib-3 and ASD-3. In each bar, "N "signifies the number of distinct neural inductions conducted per clone. "E" indicates the number of experiments conducted for each clone. There is variation in % of neurites from clone to clone however on average Sib NPCs have higher neurites than ASD NPCs.

Neurites under EFs:

In Family-1, ASD NPCs failed to respond to a multitude of important EFs including PACAP, NGF, 5-HT, and OXT. ASD NPCs also had increases in neurites with FGF while Sib NPCs had reductions in neurites with FGF. These altered neurite responses could potentially be due to changes in underlying signaling pathways in ASD NPCs. Again, it was unclear if these EF response profiles were unique to Family-1 ASD NPCs or if this was a common trait in the cohort we selected. Thus, a narrower EF panel was conducted in Families 2 and 3 to understand how much our Families had in common

Neurites under PACAP Stimulation:

Dose response studies in Family-2 showed that, once again, Sib NPCs had increases

in neurite outgrowth with multiple doses of PACAP. Likewise, much like ASD-1 NPCs, ASD-2 NPCs also showed no changes in neurite outgrowth under any dose of PACAP. At the 3nM dose, where maximal responses were seen in Sib-1, we see that Sib NPCs from Family-2 had a 65 % increase in neurite outgrowth (p=0.0022) while ASD-2 NPCs had no change (p=0.844). Likewise, Family-3 Sib NPCs had a 50% increase in neurite outgrowth with 3 nM PACAP (p<0.0001) while the ASD-3 NPCs had no change (p=0.8). Thus, we see impaired response to PACAP is a common phenotype in all 3 Sib-pairs (Figure 38).
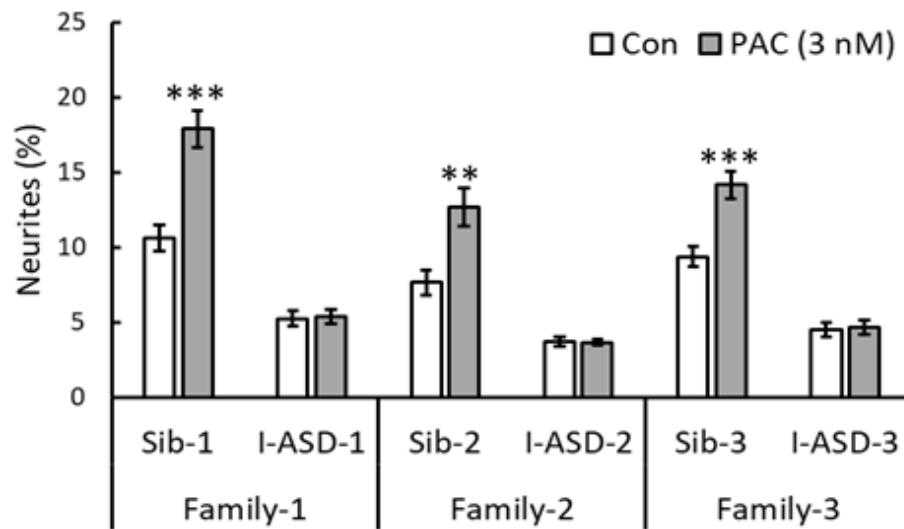
Figure 38: Neurite outgrowth in Sib and ASD in under 3 nM PAC in all 3 families from Idiopathic Cohort. In all families, Sib NPCs had an increase in neurite outgrowth under PACAP while ASD NPCs were unresponsive. Family-1 N: Sib: 21 Expts, 2-3 dishes/ experiment, 4 clones. ASD-1 N=18 expts, 2-3 dishes/expt, 4 iPSC clones. Family-2 N: Sib-2: 10 Expts, 2-3 dishes/expt, 3 clones. ASD-2: 12 expts, 2-3 dishes/expt, 3 clones. Family-3 N: Sib-3: 6 expts, 2-3 dishes/expt, 2 clones. ASD-3: 6 expts, 2-3 dishes/expt, 2 clones.

Neurites under NGF stimulation

In Family-1, ASD NPCs failed to extend neurites under NGF stimulation. Likewise, the ASD NPCs in Family-2 & 3 also failed to extend neurites under NGF. Dose response in Family-2&3 Sibs show increased neurites at 10 & 30 ng/mL while both Family-2 & 3 ASD NPCs had no neurite responses at any dose of NGF. Further studies in multiple clones

found that Sib-2 NPCs had a 50% increase in neurite outgrowth under NGF (p=0.0008) while ASD-2 had no change (p=0.52). Similarly, Sib-3 NPCs had a 60% increase in neurites under NGF (p=0.0002) while ASD-3 NPCs had no change (p=0.972). Again, these studies show common impairments in NGF response in all 3 I-ASD patients (Figure 39). BDNF was not tested in Family-2&3. However, it would be interesting to see if BDNF could rescue neurite outgrowth in these families as well.
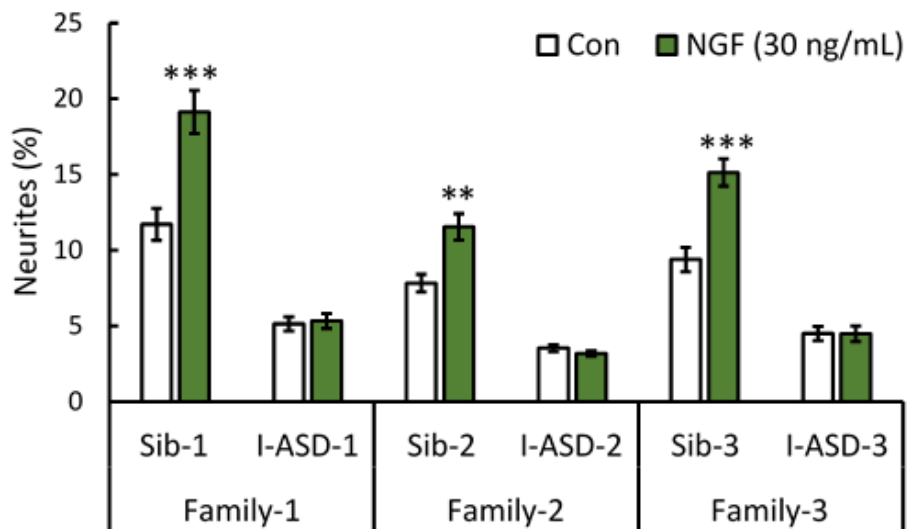
Figure 39: Neurite outgrowth in Sib and ASD in under 10ng/mL NGF in all 3 families from Idiopathic Cohort. In all families, Sib NPCs had an increase in neurite outgrowth under 10 ng/mL NGF while ASD NPCs were unresponsive. Family-1 N: Sib: 13 Expts, 2-3 dishes/ experiment, 4 clones. ASD-1 N=14 expts, 2-3 dishes/expt, 4 iPSC clones. Family-2 N: Sib-2: 11 Expts, 2-3 dishes/expt, 3 clones. ASD-2: 9 expts, 2-3 dishes/expt, 3 clones. Family-3 N: Sib-3: 6 expts, 2-3 dishes/expt, 2 clones. ASD-3: 6 expts, 2-3 dishes/expt, 2 clones

Neurites under 5-HT stimulation

In Family-1, ASD NPCs did not respond to 5-HT at the 100 μg/mL dose but had an increase in neurites at a 300 μg/mL dose. ASD NPCs from both Family-2 &3 showed a similar dose response (though different magnitudes of response) (Figure 40). Sib-2 had a 79% (p=0.0004) and 100% (p<0.0001) increase at 100 μg/mL and 300 μg/mL respectively while ASD-2 had no response at 100 ug/mL (p= 0.255) and a 79% increase in neurites at 300 μg/mL (p<0.0001). Sib-3 had a 41% (p=0.0045) and 30% (p=0.04) increase in neurites

at 100 µg/mL and 300 µg/mL 5-HT respectively while ASD-3 had no response at 100 µg/mL (p=0.99) and a 129% increase in neurites at 300 µg/mL (p<0.0001).
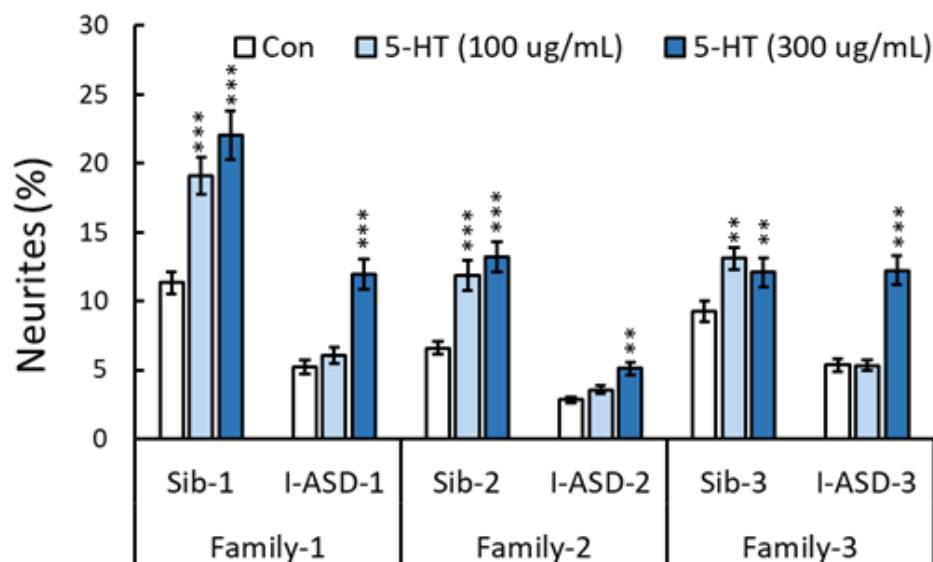
Figure 40: Neurite outgrowth in Sib & ASD in under 100 & 300 ug/mL of 5-HT in all 3 idiopathic families. Sib NPCs had an increase in neurites at both 100and 300 ug/mL of 5HT. ASD NPCs in all families were unresponsive to 100 ug/mL but did exhibit increased neurites at 300 ug/mL of 5-HT. F-1 N: Sib-1: 9 Expts, 2-3 dishes/ expt, 4 clones. ASD-1 N=11 expts, 2-3 dishes/expt, 4 iPSC clones. F-2 N: Sib-2: 9 Expts, 2-3 dishes/expt, 3 clones. ASD-2: 11 expts, 2-3 dishes/expt, 3 clones. F-3 N: Sib-3: 8 expts, 2-3 dishes/expt, 2 clones. ASD-3: 6 expts, 2-3 dishes/expt, 2 clones

Neurite outgrowth under OXT:

In the interest of time, OXT studies were conducted only in Family-2. Here, we found the dose response curve in Sib was a bit more expansive and included responses at 10 nM and 30 nM. Again, NPCs from ASD-2 had no response to OXT, mimicking the results seen in Family-1. As all other EF responses have been similar between the families, I anticipate that Family-3 ASD NPCs would also fail to respond to OXT.

Neurite outgrowth under FGF:

FGF studies were conducted only on NPCs from Family-2. Much like family-1 we found that Sib NPCs responded to FGF with decreases in neurites while ASD NPCs

responded to FGF with an increase in neurites.

Comparison of Idiopathic Cohort to 16p11.2 Deletion & NIMH Controls:

Initially, my studies aimed to analyze developmental processes in three Sib-pairs in our idiopathic ASD cohort. As seen above, these studies showed common defects in neurite outgrowth and EF responses amongst all 3 I-ASD individuals. Later, we received somatic cells from 3 individuals with a genetically defined form of ASD, the 16p11.2 deletion cohort. As most iPSC studies to date have focused on one subtype of ASD, I thought it would be incredibly interesting to compare and contrast developmental phenotypes and EF responses in I-ASD vs 16p11.2 deletion ASD (16pDel) NPCs. Thus, the methods and studies used to characterize I-ASD were applied to 16pDel ASD. For controls, we initially utilized the Sibs from the idiopathic cohort. Later, we acquired unaffected controls (genetically normal newborn cord blood derived cells) from the NIH for additional comparison. While there are 3 individuals with 16p11.2 deletion, our studies mainly focused on the two males. However, studies were also conducted on the female 16p patient, though no female control line was available for study.

Neurite outgrowth in Under Control Conditions:

Neurobiological studies of 16p11.2 have been rare. To date, there is one study in the mouse model of 16pDel and one new iPSC study of 16pDel and duplication in humans [752]. Interestingly, the human iPSC studies found altered dendrite length in the 16pDel neurons. Yet again, no human iPSC study has focused on studying early differentiation in NPCs. Thus, I aimed to study 16pDel NPCs, to understand neurite defects at an earlier developmental stage than the only other 16p iPSC paper.

When compared to a composite average of all 3 Sibs and the average neurites of

NIH Controls (CR) we find that all 3 individuals with the 16p11.2 deletion have lower neurites (Figure 41). For each male, we conducted studies on multiple NPC derivations from 2 iPSC clones. However, the reduced neurite outgrowth is only statistically significant in M-1 ($p < 0.0001$) and the F ($p = <0.0001$). In M-2 neurite outgrowth is at 9.0% which is only slightly lower than the 11 and 12.7% observed in the controls ($p = 0.09$). In this individual there is variation between C-1 and C-2. In clone-1, in both neural inductions studied, there was an increase in neurites with each passage. Thus, while neurites were generally around 2-5% at passage 3, by passage 6 neurites in these NPCs could be as high as 16-20%. In the 2nd clone, of the 4 total neural inductions conducted, 2 inductions showed this initial low neurite percentage and subsequent increases with passage. On the NPCs derived from the other 2 inductions has neurites as high as 10% at P3. Thus, the neurite outgrowth phenotype in this individual is currently unclear, though on average, M-2 NPCs at low passages have on average around 7% neurites (which is statistically lower than the controls). A 3rd clone may need to be studied to get a clearer idea, though, it seems that there is a trend towards decreased neurites (statistically) despite the high variability. Thus, neurite defects are commonly observed in all the ASD patients we have studied!

<u>Neurite outgrowth in 16p11.2 Under EF Stimulation:</u>

NPCs derived from all 3 of our I-ASD patients failed to respond to numerous EFs including PACAP, NGF, 5-HT, and OXT. While both 16pDel NPCs and I-ASD NPCs had defects in neurite outgrowth in control conditions, it is unclear whether impaired EF response would be seen in 16pDel. Thus, the following EFs were tested in our 16pDel NPCs.
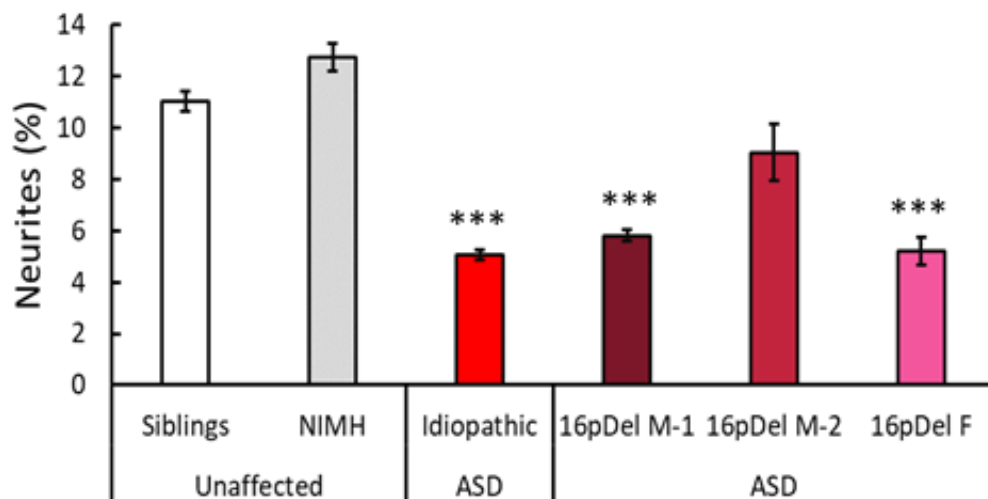
Figure 41: Neurite outgrowth in control conditions in Sibs, NIMH, I-ASD, and 16pdel NPCs. The Sib bar represents an average of control dishes from all 3 Sib patients. Likewise, the NIMH and I-ASD are a compilation of all 2 NIMH individuals and all 3 I-ASD individuals respectively. N for 16pDel: M-1: 21 expts, 2-3 dishes/expt/condition, 2 clones, 4 inductions, M-2: 15 expts, 2-3 dishes/expt/condition, F: 9 Expts, 2-3 dishes/expt/conditions, 1 clone, 2 inductions

Neurite outgrowth under PACAP stimulation:

As discussed previously, Sib NPCs had an increase in neurite outgrowth under numerous

doses of PACAP, while I-ASD NPCs were unresponsive to PACAP. Dose response studies

in all three 16p patients in comparison to all 3 Sibs found that unlike I-ASD, 16pdel NPCs

had typical response to PACAP at multiple doses (Figure 42). Further studies at the 3 nM

dose found that Sib NPCs had a 65% increase in neurites with PACAP ($p<0.0001$).

Likewise, unaffected control cells from the NIH (CR) showed a 59% increase in neurites

under PACAP ($p<0.0001$). This suggests PACAP does indeed stimulate neurite outgrowth

in "typical" NPCs, whether from families with a child with idiopathic autism, or apparently

unaffected individuals. In the 16p11.2 deletion patients, M-1 had a 56% increase in neurites

($p<0.0001$), M-2 had a 58% increase ($p<0.01$), and the female had a 69% ($p<0.01$) increase

under PACAP stimulation. These percent increases were not statistically different than Sib

or CR suggesting that 16p NPCs had normal responses to PACAP. Thus, despite having

common baseline defects in neurites, 16pDel NPCs exhibit a typical PACAP response whereas the ASD NPCs have no response at all to PACAP (Figure 43).
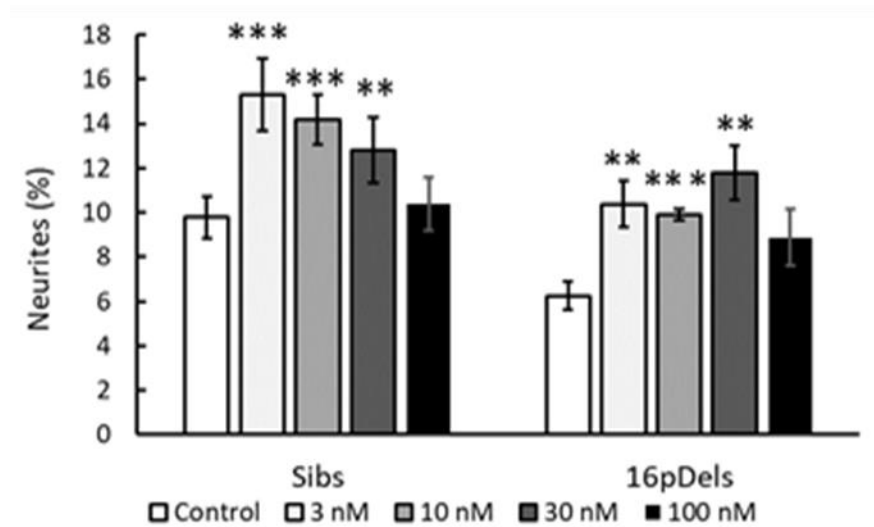
Figure 42 : Dose response studies in neurite outgrowth under PACAP in Sibs and 16pDels. Both Sib and 16p NPCs have increases in neurite outgrowth under 3nM, 10 nM, and 30 nM doses. Sib N= 3 patients, 1 clone each, 3 expts. ASD N= 3 patients, 1 clone each, 4 expts.
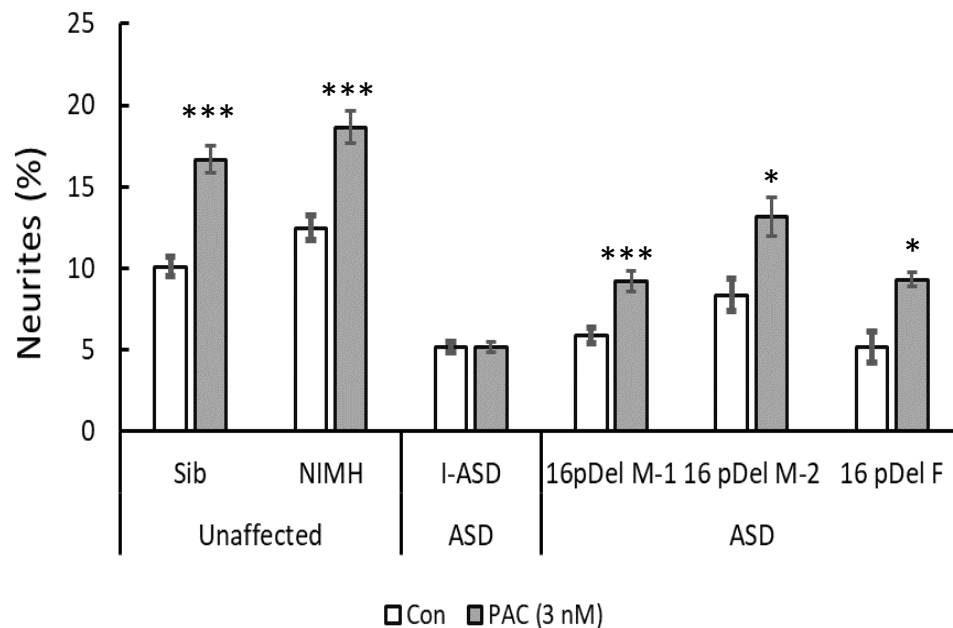
Figure 43: Neurite outgrowth in Sib, NIMH Controls, I-ASD, and each 16pDel under 3 nM PAC. The values for Sib, NIMH, and I-ASD are averages from multiple individuals while the 16p are averages of either multiple clones or multiple inductions. Unaffected individuals have increased neurite outgrowth under PACAP. While I-ASD has no response to PAC, all 3 16p patients show an increase in neurite outgrowth under 3 nM PAC. Sib N= 3 patients, 2-3 clones/

patient, 40 expts, NIMH N= 2 patients, 1 clone/patient, 2-3 inductions /patient, 6 expts, I-ASD N= 3 patients, 2-3 clones/ patient, 40 expts. 16pM-1 N= 2 clones, 2 inductions/ clone, 6 expts, 16pM-2: N= 2 clones, 2 inductions/clone,7 expts. 16p F N= 1 clone, 2inductions, 4 expts.

Neurite outgrowth under NGF Stimulation:

While Sib and CR NPCs had 58 and 53% (p<0.001) increases in neurites with NGF, I-ASD had no response to any dose of NGF. Dose response studies of NGF in 16pdel individuals found neurite responses similar to Sibs. (Figure 44). Further studies found that M-1 had a 60% increase in neurites (p<0.01), Male-2 had a 42% increase (p=0.09), and F (p=0.0049) had a 72% increase (Figure 45). Thus, 16p NPCs had typical responses to NGF. M-2 NGF increase was not significant due to the neurite variability between clones and inductions in this line. However, in almost all experiments NGF increased neurites in M-2.
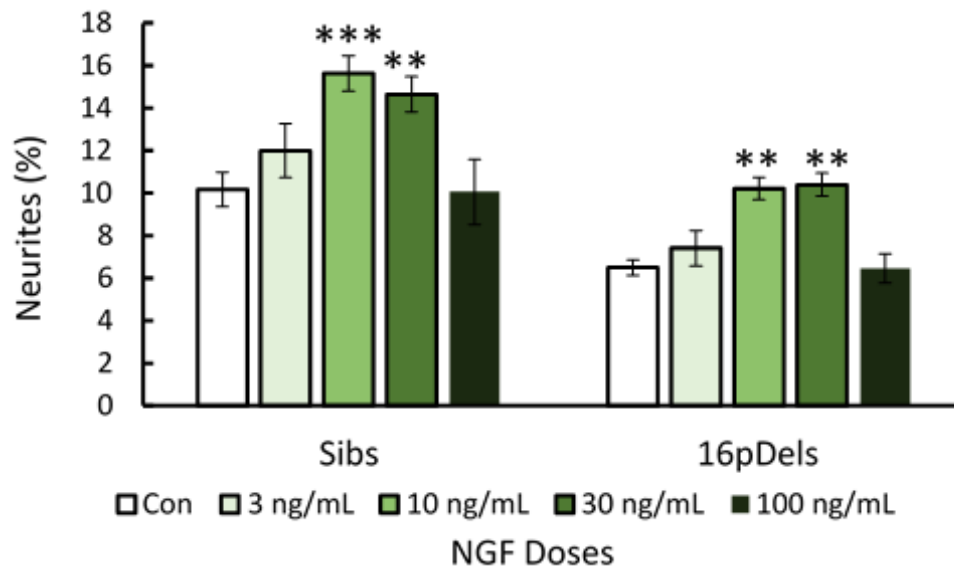
Figure 44: Dose response studies of neurite outgrowth under NGF in Sib and 16p11.2 deletion individuals. Both Sib and 16p NPCs have increases in neurite outgrowth under 10 ng/mL and 30 ng/mL doses of NGF. Sib N= 3 patients, 1 clone each, 3 expts. 16p Del ASD N= 3 patients, 1 clone each, 4 expts.
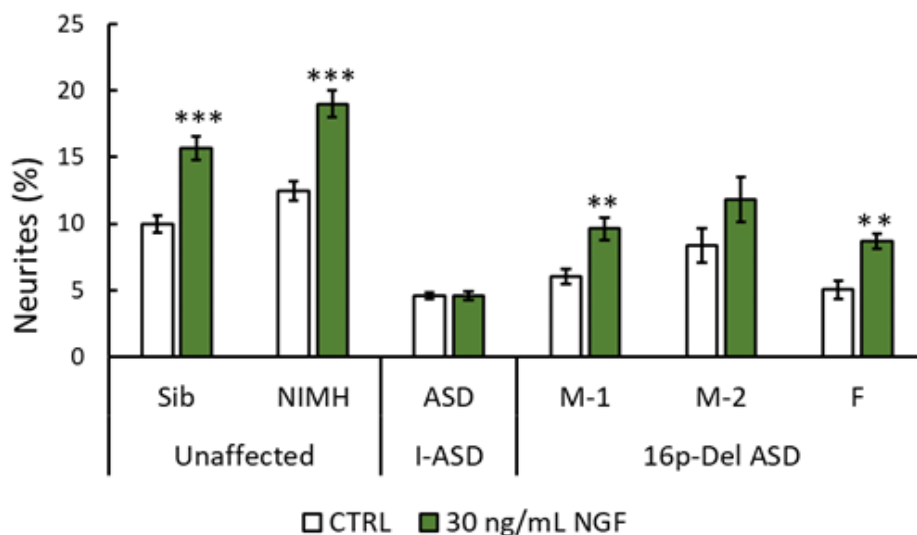
Figure 45: Neurite outgrowth in Sib, NIMH Controls, I-ASD, and each 16p patient under 30 ng/mL NGF. The values for Sib, NIMH, and I-ASD are averages from multiple individuals while the 16p are averages of either multiple clones or multiple inductions. Unaffected individuals have increased neurite outgrowth under NGF. However, while I-ASD has no response to NGF, all 3 16p patients show an increase in neurite outgrowth under 30ng/mL NGF Sib N= 3 patients, 2-3 clones per patient, 35 expts, NIMH N= 2 patients, 1 clone per patient, 2-3 neural inductions per patient, 6 expts, I-ASD N= 3 patients, 2-3 clones per patient, 35 expts. 16pM-1 N= 2 clones, 2 neural inductions/ clone, 6 expts, 16pM-2: N= 2 clones, 2 neural inductions/clone, 6 expts. 16p F N= 1 clone, 2 neural inductions, 4 expts.

Neurite Outgrowth under 5-HT stimulation:

Both Sib and CR NPCs have increases in neurite outgrowth under the stimulation of 100

ug/mL and 300 ug/mL of 5-HT. On the other hand, as reviewed, I-ASD NPCs fail to grow

neurites under 100 ug/mL 5-HT but do show a neurite response at 300 ug/mL 5-HT.

Much like the Sib and CR NPCs, 16pDel NPCs also responded to both doses of 5-HT.

Figure 46 shows response in all patients.
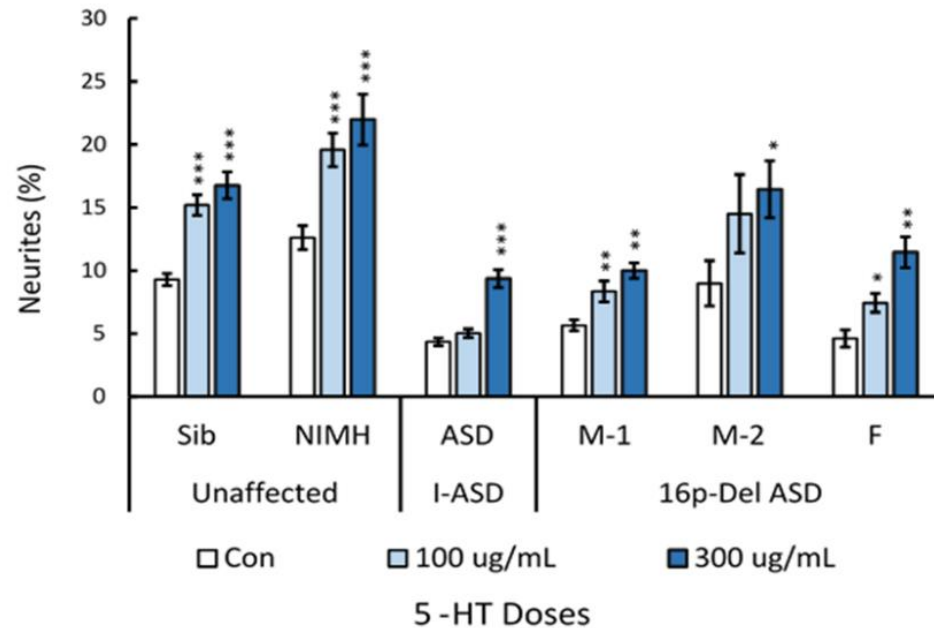
Figure 46: Neurite outgrowth in Sib, NIMH Controls, I-ASD, and each 16p patient under 100 & 300 ug/mL 5-HT. The values for Sib, NIMH, and I-ASD are averages from multiple individuals while the 16p are averages of either multiple clones or inductions. Unaffected individuals have increased neurite outgrowth under both 100 & 300 ug/mL 5-HT. I-ASD has increased neurite outgrowth under 300 ug/mL 5-HT but shows no change to 100 ug/mL of 5-HT. 16p patients generally mimic the Sib and NIMH control dose responses, however, increase in neurites at the 100 ug/mL dose seem a little blunted. Sib N= 3 patients, 2-3 clones per patient, 25 expts, NIMH N= 2 patients, 1 clone per patient, 2-3 neural inductions per patient, 6 expts, I-ASD N= 3 patients, 2-3 clones per patient, 25 expts. 16pM-1 N= 2 clones, 2 neural inductions/ clone, 6 expts, 16pM-2: N= 2 clones, 2 neural inductions/clone, 6 expts. 16p F N= 1 clone, 2 neural inductions, 4 expts.

Neurite outgrowth under OXT stimulation:

OXT was tested in 3 Sib, 2 I-ASD and the three 16pdel NPCs. We found that the Sibs on average had a 62% increase in neurites with 30 nM OXT as seen in the dose response curve in Figure 46. I-ASD NPCs had no response at any dose of OXT, while 16pDel NPCs began to respond to OXT at a lower dose than Sib (3nM, Figure 47). Thus, 16pDel NPCs may be more sensitive to OXT than Sib. Moreover, the percent increase in neurites at the 30 nM dose was also slightly higher in the 16p (77% increase in neurites with OXT).
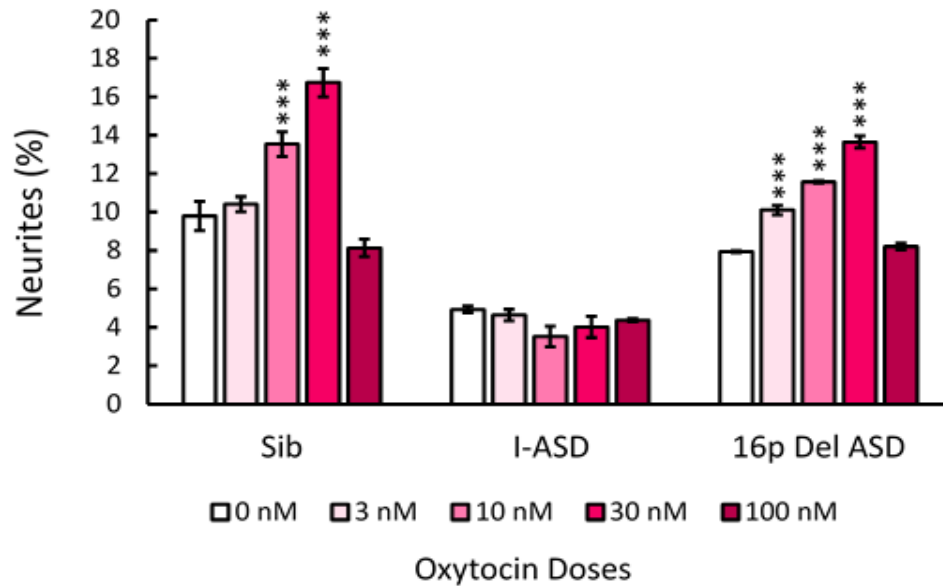
Figure 47: Dose response of neurite outgrowth under OXT in Sib, I-ASD and 16pDel ASD. OXT increases neurite outgrowth under 10 & 30 nM doses in Sib. I-ASD NPCs had no response to any doses of OXT. However, 16p NPCs begin to show a significant increase in neurites at a lower dose than Sib (3 nM) and continue to show increases in neurite at 10 & 30 nM. Sib N= 3 patients, 1 clone/pt, 3 expts, I-ASD= 2 pt,1 clone each, 2 expts, 16p Del ASD 3 patients, 1 clone each, 3 expts.

Neurite outgrowth under FGF stimulation

FGF was tested in all 3 Sibs, 2 I-ASD, and the 2 16pDel. Again, we found that the 16pDel

NPCs responded like Sib NPCs than ASD NPCs as seen in Figure 48
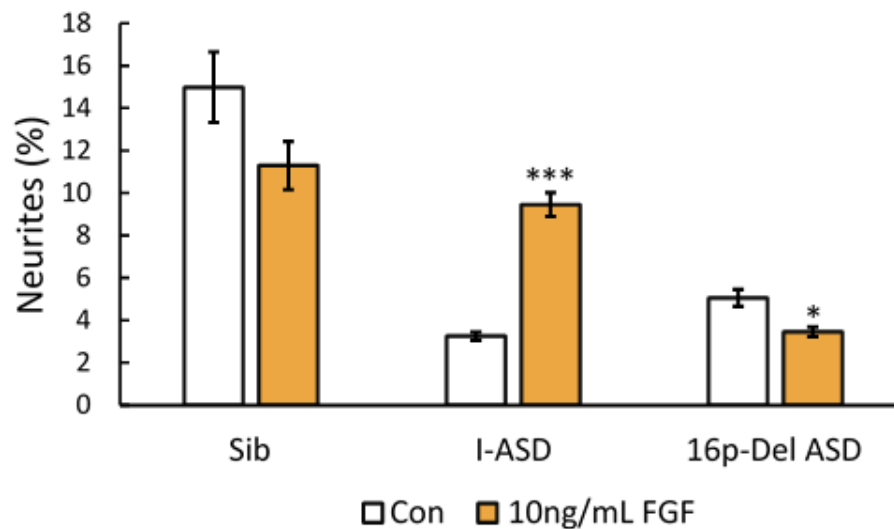
Figure 48: Neurite outgrowth under FGF stimulation in Sib, I-ASD, and 16p Del ASD. In both Sib and 16p Del ASD, FGF decreases neurite outgrowth at 10 ng/mL. In I-ASD FGF increases

neurite outgrowth. Sib N= 3 patients, 1 clone each, 3 expts, I-ASD= 2 patients,1 clone each, 2 expts, 16p Del ASD= 2 patients, 1 clone each, 2 expts.

Summary of neurite studies:

In conclusion, in control conditions, both the 16pDel ASD and the I-ASD had lower percentages of neurites than the Sib and CR NPCs. Firstly, my studies have revealed that impairments in neurite outgrowth manifest before definitive differentiation into neurons suggesting that early differentiation processes may be dysregulated in ASD. Moreover, as this phenotype has appeared in each randomly selected I-ASD individual and in individuals with 16pDel ASD, it is possible that neurite defects are a common phenotype in ASD. Yet, not all the ASD patients we tested were identical in their neurite impairments. Further studies found that I-ASD had impaired or differential responses to all the EFs we tested-PACAP, 5-HT, NGF, OXT, BDNF, NT3, and FGF. This generalized lack of response to numerous EFs- which signal through different receptor systems, suggests that it is unlikely that a defect in a single receptor or a particular G-protein are contributing to these abnormalities. Rather, it is more likely that signaling pathways are commonly dysregulated leading to aberrant EF responses. Despite being derived from individuals with ASD, 16pdel NPCs all responded typically to EFs. This indicates that while there are neurobiological similarities between multiple types of ASD, in depth analyses can reveal that there are indeed subtle differences to these common phenotypes. This indicates that there are indeed "subtypes" of ASD. Moreover, this EF stimulated difference between the two types of ASDs shows the value of conducting studies in more than just control conditions.

Chapter 6- Experimental Results: Cell Migration in ASD NPCs

Family-1 (1072: Sib-1 and ASD-1)

Migration in Control Conditions

As with the neurite assay, my migration studies began with Family-1. There are currently no iPSC-based studies of migration in ASD. However, animal models of ASD have frequently shown migration impairments and post-mortem studies of ASD brains show structural differences that are suggestive of migration impairments. Thus, I hypothesized that migration defects may be present in some of our ASD NPCs. Moreover, as neurite defects were common in all ASD patients studied, it was likely that migration, another cytoskeleton dependent process, could also be commonly dysregulated. Studies in Family-1 found that ASD NPCs migrated 36% less than Sib NPCs (Figure 49). Figure 50 shows that there is little variability in migration between clones derived from the same individual.
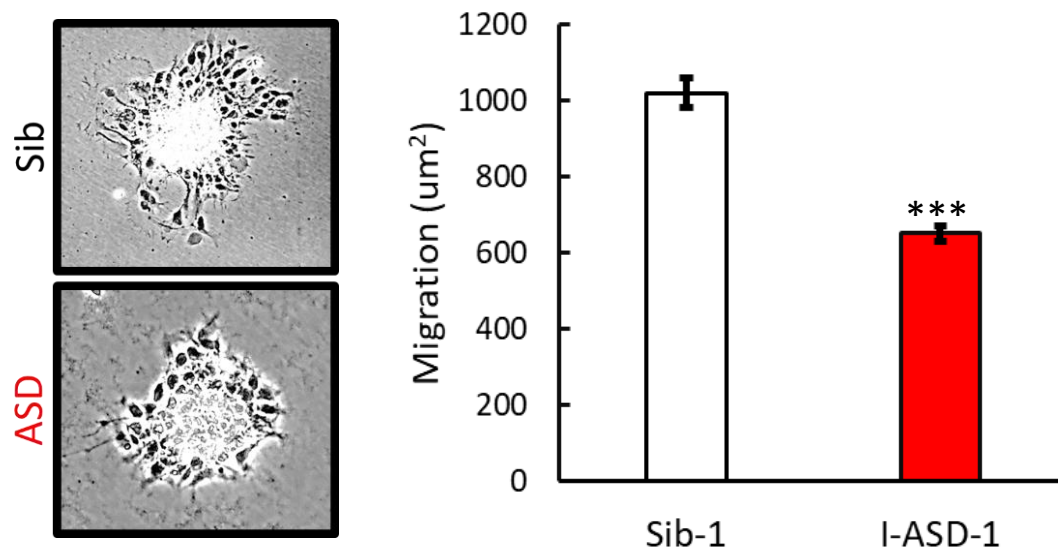
Figure 49: ASD NPCs in Family-1 have reduced cell migration. A) Representative images of neurospheres derived from Sib and ASD. NPCs in Sib neurospheres moved further from the inner cell mass than NPCs in ASD neurospheres. B) Graph quantifying Family-1 NPC migration. Sib-1 N = 13 Expts, 212 spheres, 3 iPSC clones. ASD-1 N=11 expts, 170 Spheres, 4 iPSC clones.
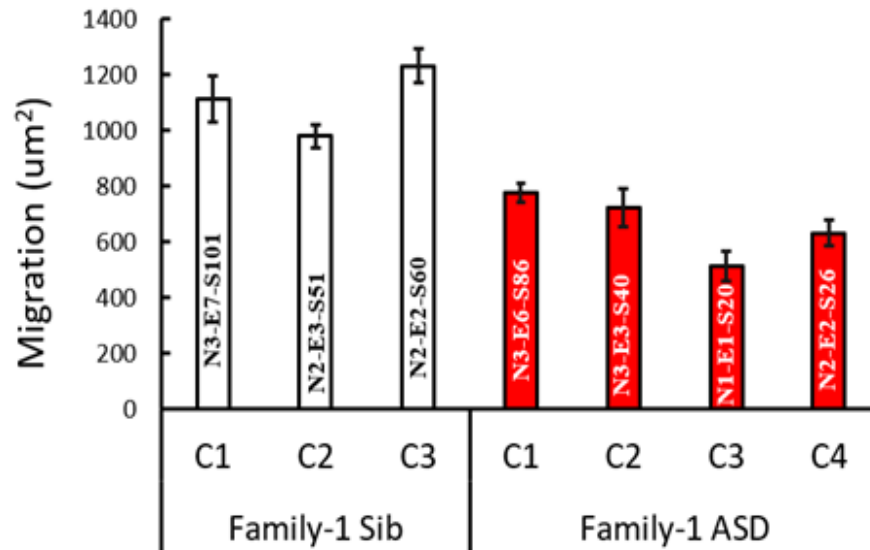
Figure 50: Migration broken down by NPCs derived from 3 clones (C1-C3) of Sib and 4 clones of ASD (C1-C4) in Family-1. In each bar, the "N "signifies the number of distinct neural inductions conducted on the clone. The "E" indicates the number of experiments conducted for each clone. The "S" represents number of spheres. There is not much variation in migration from clone to clone in either Sib or ASD.

Migration under EF Stimulation:

As EF stimulated neurite outgrowth proved to be a useful and informative method, I decided to test the effects of EFs on migration. While numerous EFs had effects on neurite outgrowth, I found fewer EFs were able to stimulate migration. Tests with NGF, BDNF, and NT3 failed to elicit migration in Sib-1 NPCs and thus these EFs were not further tested in ASD NPCs. However, as seen in some of my neurite studies, EFs that fail to stimulate outgrowth in Sib NPCs did, in some cases, elicit neurite outgrowth in ASD. Yet, as my studies had advanced into other families and further aims, I did not test failed EFs in ASD NPCs. However, I did conduct EF stimulated studies with PACAP, 5-HT, and FGF.

Migration under PACAP Stimulation:

As seen with my neurite studies, PACAP elicited migration at multiple doses in Sib NPCs and failed to elicit migration in the ASD NPCs in this family. The dose response curve in the Sib NPCs, however, differed slightly from that seen with neurites indicating

that the two developmental processes are not identical and may be regulated differently. For migration, PACAP had maximal effects at 10 nM and thus, studies with this dose were extended to more clones in both Sib-1 and ASD-1. On average, Sib NPCs have a 51% increase in migration under PACAP (p<0.00001) while ASD NPCs show no change (Figure 51). Indeed, again, all Sib clones were responsive to PACAP while no ASD clones were responsive to PACAP.
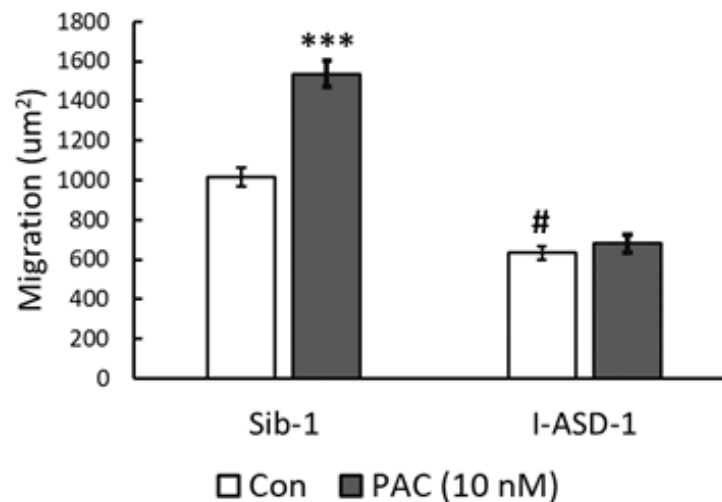
Figure 51: ASD NPCs in Family-1 fail to migrate under PACAP (10 nM) while Sib NPCs have a 51% increase in migration under 10 nM PACAP. Sib N= 9 expts, 157 Spheres/condition, 3 clones. ASD N= 8 Expts, 150 spheres/condition, 4 clones

Migration under 5-HT stimulation:

With neurite outgrowth, high doses of 5-HT were able to stimulate neurite extension in ASD NPCs while lower doses of 5-HT failed to elicit a neurite response. Thus, it was interesting to test whether 5-HT could rescue the migration defects seen in ASD NPCs. Dose response studies in Sib and ASD NPCs from this family found that Sib NPCs began to robustly respond to 5-HT at 30 ug/mL (p<0.0001) doses yet had an inhibition of migration at 300 ug/mL. ASD NPCs responded to 5-HT with increased migration starting at the 100 ug/mL dose (p=0.0021) and continued to respond at the 300 ug/mL (p=0.02) dose unlike Sib (Figure 52). While the dose response curves were different between neurite

outgrowth and migration, we find that ASD NPCs still shows decreased sensitivity to 5-HT when compared to Sib. However, again, migration of ASD NPCs is increased by treatment with 5-HT. These studies were conducted on one clone in Family-1 and were not continued with other Sibs or ASD.
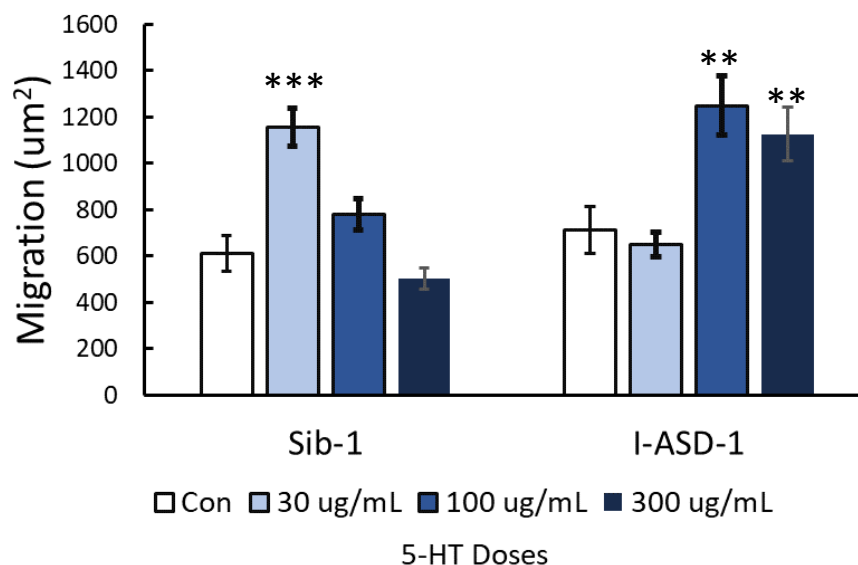
Figure 52: Dose response curve of Sib-1 and ASD-1 NPC migration under 5-HT stimulation. Sib NPCs only had significant migration at 30 ug/mL 5-HT. ASD-NPCs began to respond at later doses of 5-HT and continued to response at 300 ug/mL where Sib-1 had no response. N= 1 expt, 1 clone, 20 spheres/condition per patient.

Migration under FGF Stimulation:

In my neurite studies, FGF successfully stimulated neurite outgrowth in ASD NPCs while many other EFs failed to do so. However, this stimulation of neurite outgrowth was not a "typical" response as all 3 Sibs studied had reduced neurite outgrowth with FGF. On the other hand, studies on proliferation in our lab, found that FGF managed to rescue proliferative defects in Family-1 ASD NPCs. Thus, the effects of FGF were interesting to explore. 10 ng/mL dose of FGF produced huge neurospheres in both Sib and ASD with large increase in migration (Sib: 90% increase P<0.0001, ASD: 360% increase P<0.0001

Figure 53). The increase in migration was greater in ASD NPCs than Sib NPCs. However, as seen in the figure, the size of the spheres was much larger than control spheres or the spheres under any other EF! This suggests that "migration" rescue in this case may partially be due to changes in proliferation induced by FGF. Thus, due to the potential confound with proliferation, further studies were not conducted with FGF.
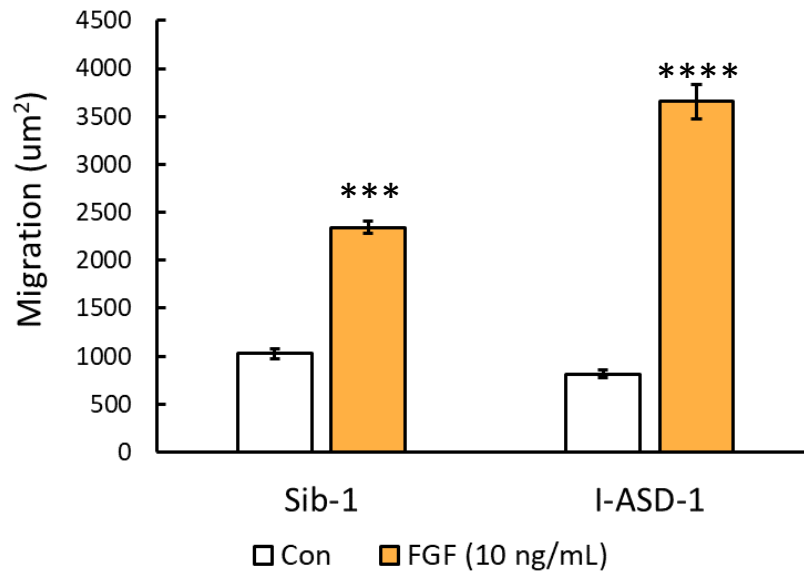
Figure 53: Both Sib-1 and ASD-1 NPCs respond to 10 ng/mL FGF with increased migration (90% and 360% increase respectively). ASD NPCs have a higher percent increase in migration under FGF. Sib & ASD N = 2 expts, 40 spheres/condition, 2 clones

Family-2 (1077) & Family-3 (1012)

With neurite outgrowth, I saw that the defects uncovered in Family-1 were also observed in Family-2 &3. Thus, since ASD-1 NPCs showed defects in migration, it was possible that Family-2 &3 NPCs would also have migration defects. Indeed, my studies found both ASD-2 & ASD-3 NPCs migrated less than their Sib counterparts (37.5%, $p < 0.001$ & 30% $p < 0.001$ less respectively), suggesting migration defects in all 3 ASD patients (Figure 54). In both Family-2 and Family-3, all Sib clones studied had higher migration than the ASD

clones studied, showing the robustness of the migration phenotype (Figure 55, Figure 56).
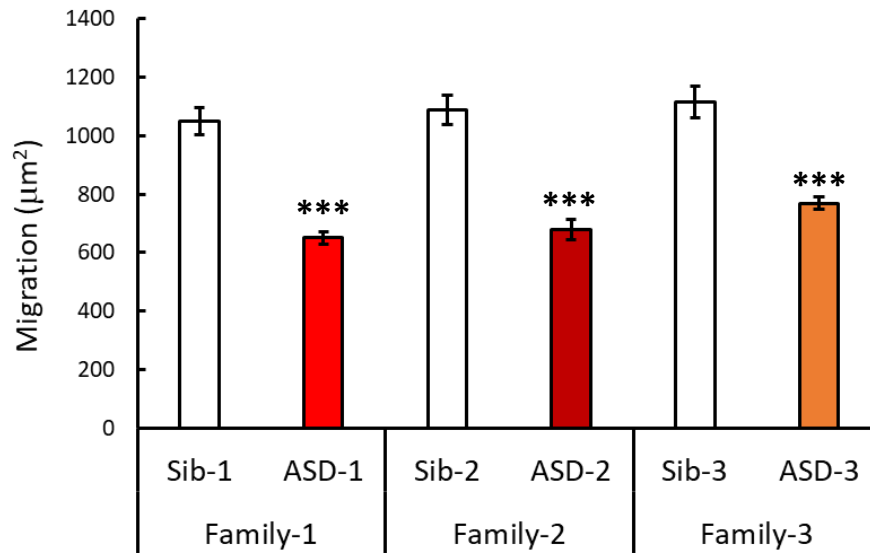
Figure 54: Migration in Sib and ASD in all 3 families from Idiopathic Cohort. In all families, ASD NPCs show reduced migration compared to Sib NPCs. Family-1 N: Sib:13 Expts, 212 spheres total, 3 clones. ASD-1 N=11 expts, 170 Spheres total, 4 Family 2 N: Sib-2: 5 Expts, 110 spheres, 3 clones. ASD-2: 4 expts, 80 spheress, 3 clones. Family-3 N: Sib-3: 4 expts, 84 sphers, 2 clones. ASD-3: 3 expts, 63 spheres, 2 clones .
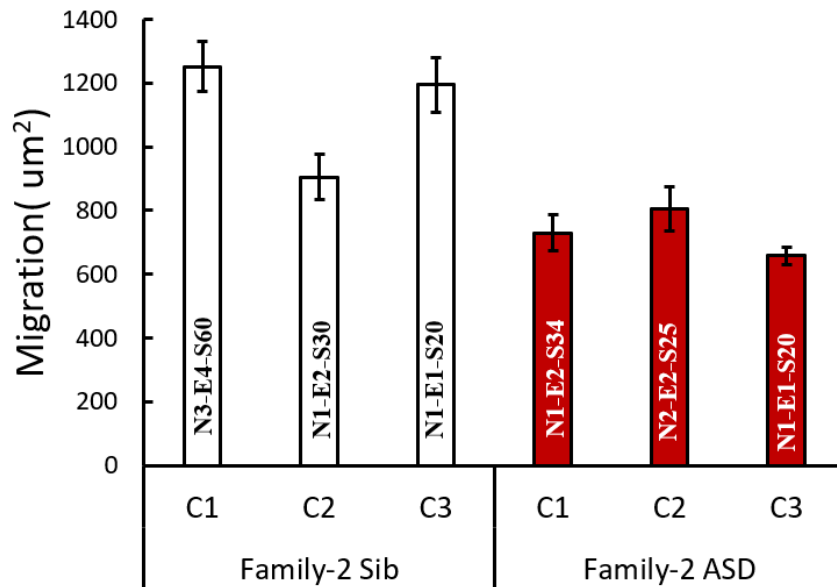
Figure 55: Breakdown of migration in Family-2 by clone. Three clones in Sib and ASD (C1-C3). In each bar, the "N "signifies the number of distinct neural inductions conducted on the clone. The "E" indicates the number of experiments conducted for each clone. The "S" represents number of spheres. In Sib C1 and C3 have similar migration while C2 is a little lower. Migration is very similar between all ASD Clones.
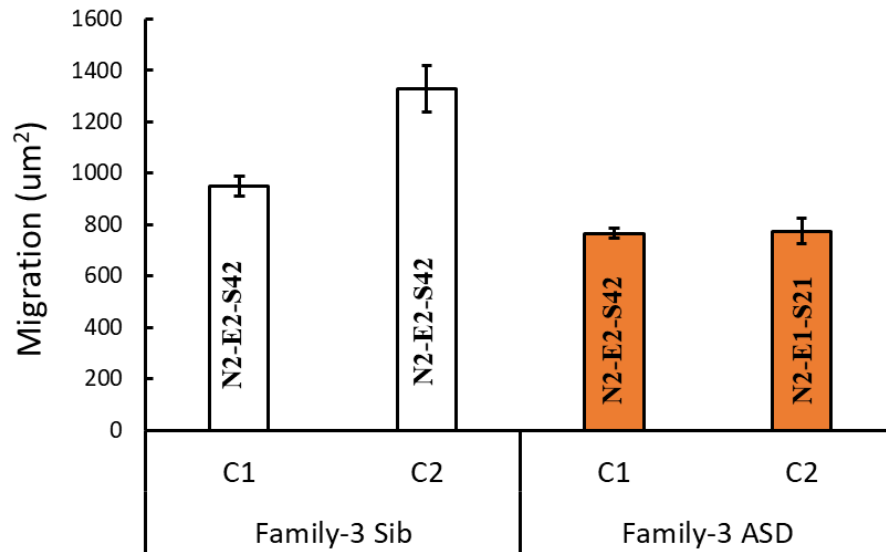
Figure 56: Breakdown of migration in Family-3 by clone. 2 clones in Sib and ASD (C1,C2). In each bar, the "N "signifies the number of distinct neural inductions conducted on the clone. The "E" indicates the number of experiments conducted for each clone. The "S" represents number of spheres. In Sib, there is a statistical difference between migration in C1 and C2. However, both Sib clones have a higher migration than both ASD clones. The migration is statistically identical in the 2 ASD clones.

Migration under PACAP stimulation:

As ASD-2 and ASD-3 NPCs failed to respond to PACAP with increased neurites and ASD-1 NPCs failed to migrate under PACAP stimulation, I expected that PACAP would fail to stimulate migration in the ASD NPCs from these families. Indeed, studies show that while both Sib-2 (40% increase, $p<0.0001$) and Sib-3 (25% increase $p<0.01$) respond to PACAP with increased migration, ASD-2 and ASD-3 NPCS failed to migrate under PACAP stimulation (Figure 57). Again, we see that I-ASD NPCs are exhibiting common neurobiological phenotypes.
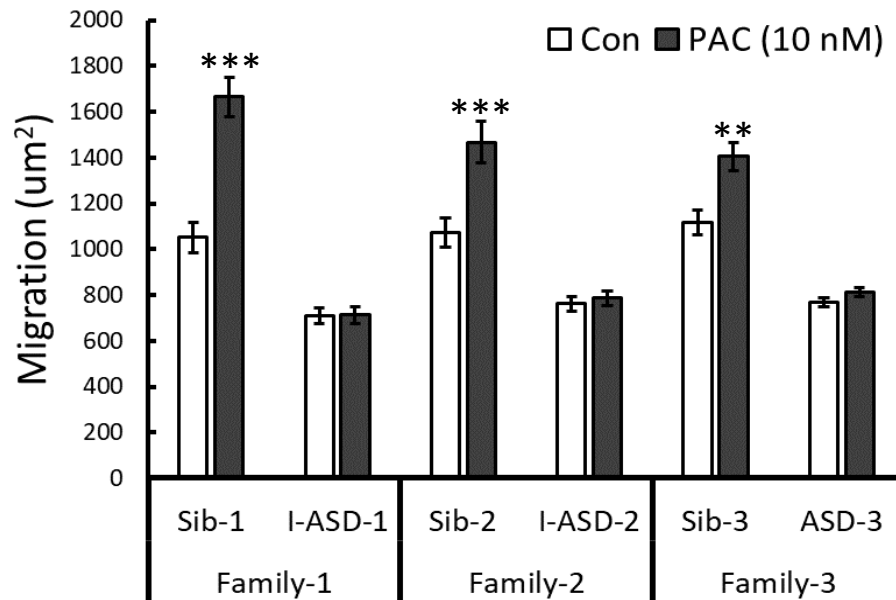
Figure 57: Neurite outgrowth in Sib and ASD in under 10 nM PAC in all 3 families from Idiopathic Cohort.In all families, Sib NPCs had an increase in migration under PACAP while ASD NPCs were unresponsive. Family-1 Sib N: 9 expts, 157 Spheres/condition, 3 clones. ASD N= 8 Expts, 150 spheres/condition, 4 clones. Family-2 N: Sib-2: 6 expts, 3 clones, 101 spheres/condition. ASD-2: 3 expts, 3 clones, 59 spheres/condition. Family-3 N: Sib-3: 4 expts, 84 spheres/condition, 2 clones, ASD-3: 3 expts, 62 spheres, clones

Comparison to 16pDel and NIH Controls

Thus far, my results have shown that neurite defects are common in both ASD cohorts and that migration defects were found in all three I-ASD patients. Thus, it seemed likely that migration defects would also be present in our 16p11.2 deletion cohort. Indeed, studies in a zebra-fish model of 16p found migration defects associated with KCTD1, a gene in the 16p11.2 locus. In our human NPCs, I found that all 3 16p11.2 patients had impaired migration when compared to both Sibs and NIH controls. While average migration in all 3 Sib NPCs was approximately 1078 and CR NPC were 1272, the 16pDel NPCs migrated 642,652, and 772 Thus, like neurite outgrowth, migration impairments were commonly seen in all our ASD patients. Figure 58 shows a comparison of migration in all four groups.
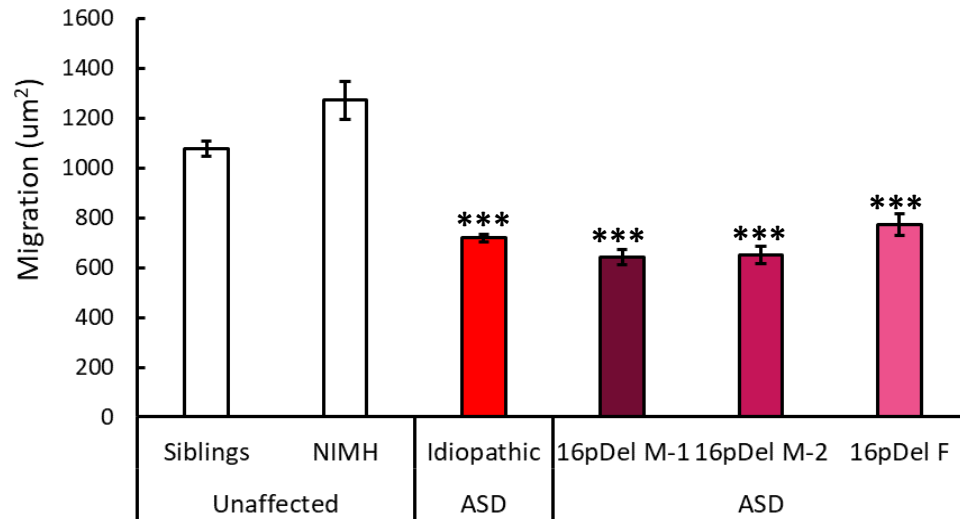
Figure 58: Migration in Siblings, NIMH controls, idiopathic ASD and all three 16p patients

Migration under PACAP stimulation:

For neurite outgrowth, we found that unlike I-ASD NPCs, 16pDel NPCs had no impairment in PACAP response. Thus, I hypothesized that 16pdel NPCs would have increased migration under PACAP stimulation- like Sib and CR. Indeed, my studies found that 16pdel NPCs did respond to PACAP with increased migration (Figure 59). The graph shows comparison of migration response to PACAP in both my control and ASD groups.
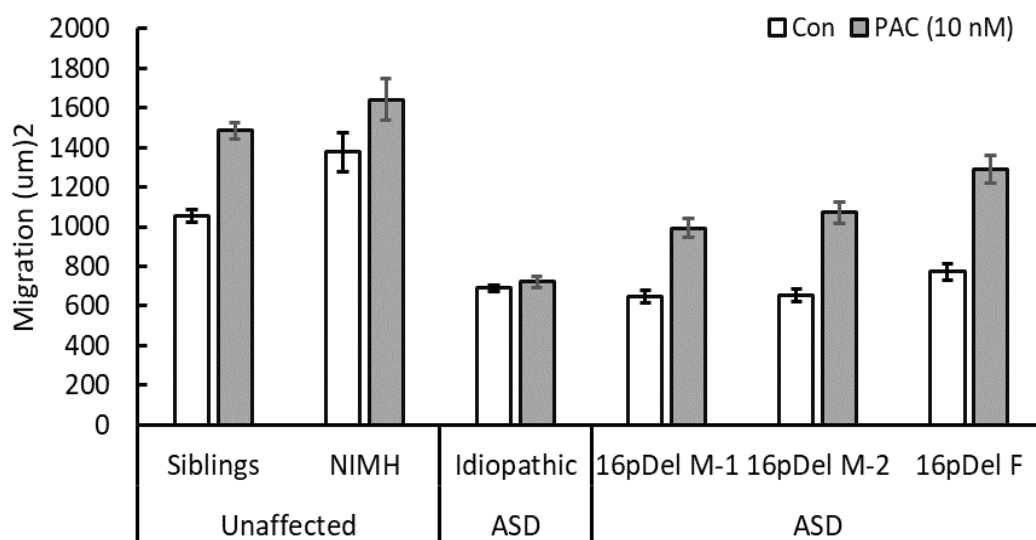
Figure 59: Migration in Sib, NIMH Controls, I-ASD, and each 16p patient under 10 nM PACAP. The values for Sib, NIMH, and I-ASD are averages from multiple individuals while the 16p are averages of either multiple clones or multiple inductions. Unaffected individuals have increased neurite outgrowth under PACAP. However, while I-ASD has no response to PACAP, all 3 16p patients show an increase in neurite outgrowth under 3 nM PACAP. Sib N= 3 patients, 2-3 clones per patient, 40 expts, NIMH N= 2 patients, 1 clone per patient, 2-3 neural inductions per patient, 6 expts, I-ASD N= 3 patients, 2-3 clones per patient, 40 expts. 16pM-1 N= 2 clones, 2 neural inductions/ clone, 6 expts, 16pM-2: N= 2 clones, 2 neural inductions/clone,7 expts. 16p F N= 1 clone, 2 neural inductions, 4 expts

<u>Summary of Migration Results:</u>

In addition to having common impairments in neurite outgrowth, ASD NPCs derived from 3 individuals with I-ASD and 3 individuals with 16p11.2 deletion all showed impaired migration. Much like neurite outgrowth, migration is a process that requires proper regulation and function of the cytoskeleton. Yet, unlike our neurite studies, the use of neurospheres allow us to study NPCs in the context of tightly packed cells that retain cell to cell contact (much like the developing neural tube). Aberrations in both of these processes suggest that the cytoskeleton and its regulation could be commonly dysregulated in autism spectrum disorders. Yet, again, EF studies with neurosphere migration reveal that 16pdel cells are capable of migrating under PACAP stimulation unlike I-ASD individuals. This again illustrates that EFs can be used to subtype or individuals with ASD. Ultimately, our approach of using NPCs and EFs have been able to uncover common defects in migration in ASD and have uncovered differences in these phenotypes between groups.

Chapter 7- Experimental Results: Signaling Pathways in ASD: PKA, MAPK (ERK) and mTOR

Overall, ASD NPCs derived from both I-ASD and 16pdel-ASD showed common defects in neurite outgrowth and migration. Both these processes are known to be regulated by signaling pathways such as PKA, mTOR, MAPK, and WNT. Moreover, studies in rodents and in human iPSCs have shown that these pathways are commonly dysregulated in autism [114, 776-782]. Excitingly, in many cases, defects observed in autism animal models or cell culture models could often be reversed by targeting dysregulated signaling. Thus, it was logical to begin studying signaling pathways in our ASD lines in order to understand the underlying mechanism of the aberrant neurite outgrowth and migration. With the I-ASD lines, we also see failure to respond to numerous EFs. These EFs are known to work through signaling pathways to exert their regulatory effects. In particular, with both neurite outgrowth and migration, my studies showed a failure of ASD NPCs to respond to PACAP. Prior studies in our lab have shown that PACAP acts through the PKA pathway to ultimately phosphorylate CREB (Figure 3). Thus, my initial signaling studies focused on P-CREB levels in control conditions and under the stimulation of 3 nM PACAP. I hypothesized that ASD NPCs would have little or no CREB phosphorylation in response to 3 nM PACAP stimulation while Sib NPCs would have a robust increase in P-CREB.

Development of Method:

First, before analyzing data for the PACAP stimulated conditions, I conducted studies to see what length of PACAP treatment led to maximal P-CREB stimulation. Typically, for neurite outgrowth and migration, cells are incubated in PACAP for 48 hours, however, by this time point, acute signaling with P-CREB is most probably diminished.

Prior literature suggests that P-CREB phosphorylation after treatment with a PKA agonist takes anywhere from 5 minutes to 1 hour. Thus, both Sib and ASD cells were cultured for 48 hours and then treated for the following time points with PACAP: 10 minutes, 30 minutes, and 1 hr. One set of dishes was also cultured for the full 48 hours in PACAP. After collecting proteins and running westerns, I found that P-CREB is increased maximally after 10 minutes of PACAP stimulation, begins to diminish at 30 minutes, and is completely abolished by 1 hour in both Sib and ASD cells. There is also no increase in P-CREB after culturing cells with PACAP for 48 hours. Thus, for all further studies, cells were treated for 10 minutes with vehicle (DMEM/F12) or 3 nM PACAP for 10 minutes.

PKA-P-CREB Pathway

Family-1:

In Family-1, studies in multiple clones at multiple passages found that Sib NPCs had about a 4-fold increase in P-CREB levels when stimulated by PACAP ($p<0.0001$) (Figure 60). ASD NPCs, which don't respond to PACAP with increased neurite outgrowth and migration, only had a 1.5-fold increase in P-CREB when stimulated with PACAP ($p=0.049$). Overall the P-CREB response to PACAP was 2.5-fold higher in the Sib than the ASD ($p=0.0124$). In control conditions, the P-CREB levels were identical in both Sib and ASD. Thus, this suggests that the signaling response to PACAP is diminished in ASD cells and could be contributing to impaired PACAP response seen in these lines.
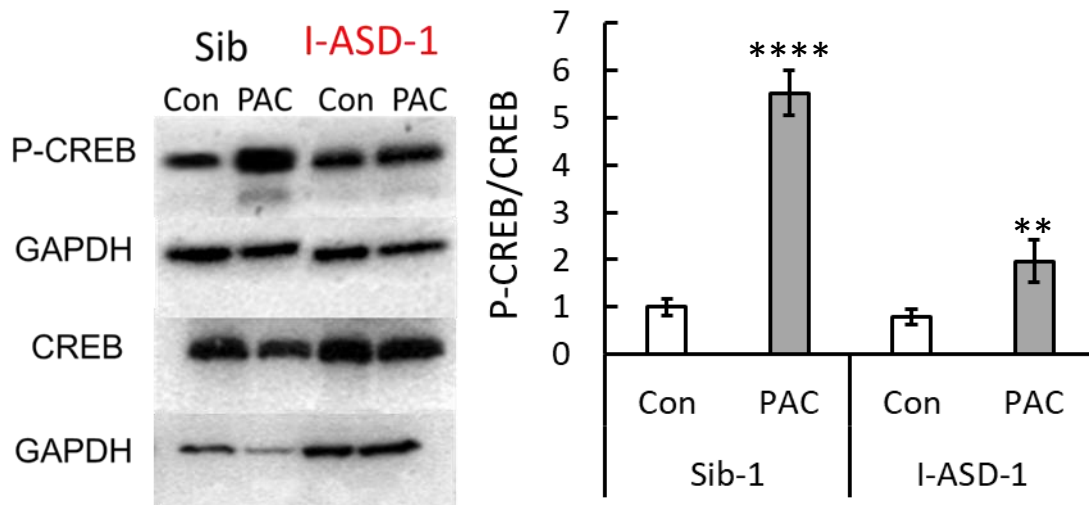
Figure 60: PACAP Stimulated P-CREB in Family-1. A) Representative image of western blot in Family-1 Sib and ASD under Control and 3 nM PAC (10 minute). Sib NPCs on average have a 4-fold increase in P-CREB under PACAP stimulation while ASD NPCs only had a 1.5-fold increase in P-CREB under PACAP. On average ASD had a 2.5-fold lower P-CREB response than Sib. Sib N= 5 Expts, 2 clones, ASD N = 6 expts, 3 clones.

Family-2:

In terms of neurite, migration, and EF responses, Family-1 & Family-2 NPCs were nearly identical. In Family-1, as hypothesized, increases in PACAP stimulated P-CREB level were reduced in the ASD NPCs. Thus, I postulated that similar blunting would be observed in Family-2. Surprisingly, despite having impaired response to PACAP, PACAP stimulated P-CREB levels were not significantly different between Sib and ASD NPCs in Family-2. Both Sib-2 and ASD-2 NPCs had about a 1.9-fold increase in P-CREB under PACAP ($p<0.0001$) (Figure 61). Thus, we see that common defects in neurobiology can be correlated with different underlying mechanisms. Moreover, in Family-2, it is possible that impaired PACAP response is caused by defects in other signaling pathways. Indeed, while PACAP is well known to signal through the PKA pathway, other pathways such as PKC and ERK are also commonly stimulated by PACAP. Likewise, the impairment in PACAP response could be due to alterations in an effector downstream of P-CREB.
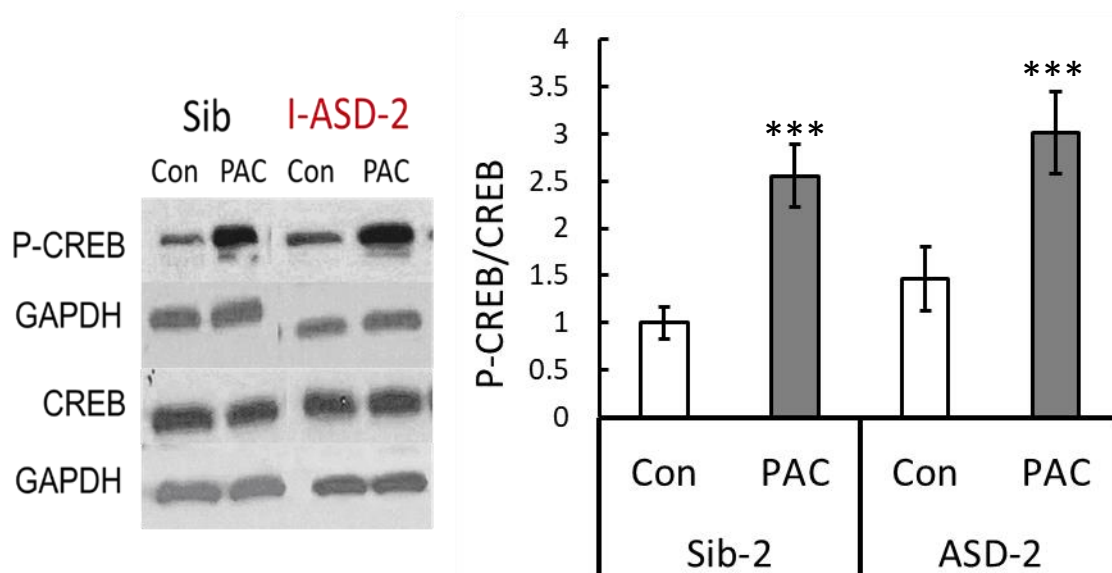
Figure 61: PACAP Stimulated P-CREB in Family-2. A) Representative image of western blot in Family-2 Sib and ASD under Control and 3 nM PAC (10 minute). Blot shows bands for P-CREB and GAPDH and CREB and GAPDH. Both Sib-2 and ASD-2 NPCs had a 1.9-fold increase in P-CREB. Thus, there were no differences between Sib and ASD in P-CREB response. Sib N = 2 clones 5 expts. ASD N = 3 clones, 5 expts.

Family-3

As seen, Family-1 ASD NPCs showed defects in the PKA pathway, while Family-2 ASD NPCs had normal PKA pathway function when stimulated by PACAP. Thus, it was difficult to predict PKA pathway impairments in the 3rd family. Interestingly, preliminary data from 1 western blot study in 1 clone each from Sib and ASD from Family-3 found that much like Family-1, Family-3 ASD NPCs had significantly lower increase in P-CREB levels under PACAP than Sib-3. While Sib-3 NPCs showed a 105% increase in P-CREB with PACAP stimulation, ASD-3 NPCs showed only a 26% increase in P-CREB with PACAP (Figure 62). This is almost a 2-fold difference in stimulated P-CREB levels between the Sib and the ASD. Unlike Family-1 however, in this preliminary study, ASD NPCs had about a 40% lower level of P-CREB in control conditions when compared to Sib. It is unclear if this more minor difference will replicate.
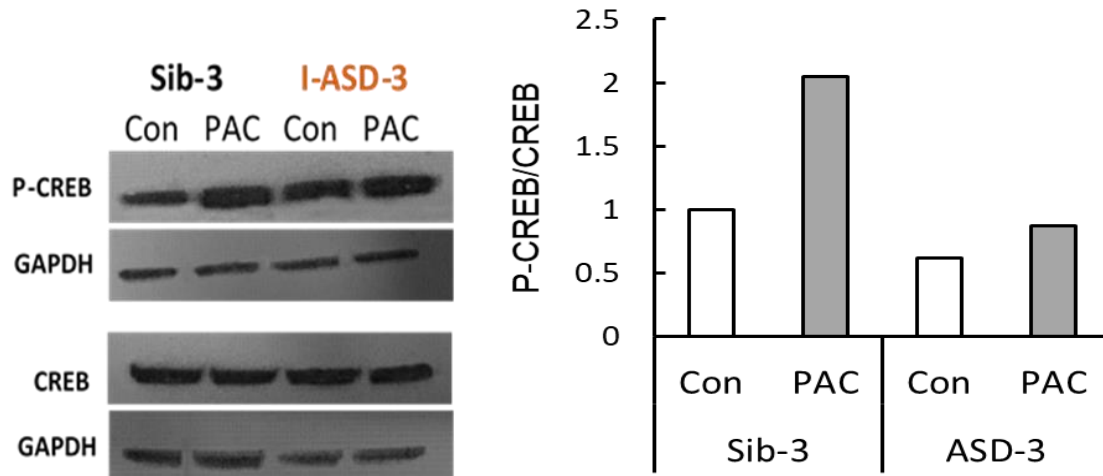
Figure 62: PACAP Stimulated P-CREB in Family-3. A) Representative image of western blot in Family-3 Sib and ASD under Control and 3 nM PAC (10 minute). Blot shows bands for P-CREB and GAPDH and CREB and GAPDH. Almost a 2 fold difference was observed in stimulated P-CREB levels between Sib-3 and ASD-3.

NIMH Controls and 16p11.2

As the 16pdel NPCs have no impairments in PACAP response with neurite outgrowth or migration, I predicted that PKA pathway function would be normal in all our 16pdel NPCs. Indeed, western blot analysis (1 expt) confirmed that NIMH controls, Sibs, and 16pdel NPCs have highly similar baseline P-CREB and stimulated P-CREB levels (Figure 63).
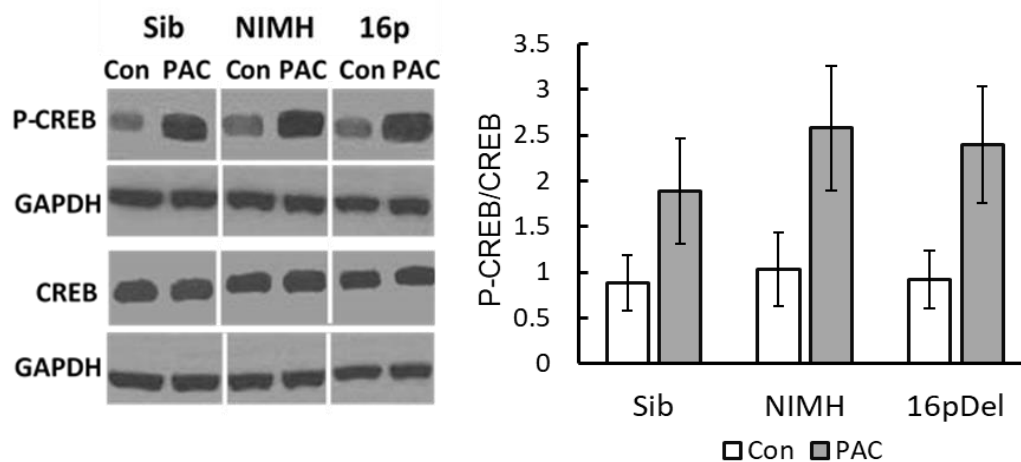
Figure 63: PACAP stimulated P-CREB levels in Sib, NIMH, and 16p11.del patients. Left) Representative image of western blots of P-CREB, CREB and GAPDH loading control in Sib, NIMH, and 16pDel NPCs. There are no statistical differences in PACAP stimulated P-CREB levels between Sib, NIMH, and 16pdel NPCs as seen in graph on right. Sib N= 2 patients, 1 clone/patient,

1 expt, NIMH N= 2 patients, 1 clone/patient, 16pdel N = 3 patients, 2 clones/ male patient, 1 clone of female patient, 1 expt.

Summary:

While there is common neurobiology in the I-ASD patients, there may be differential mechanisms contributing these defects. Despite all I-ASD patients not responding to PACAP, only NPCs in Family-1 and Family-3 show diminished P-CREB response to PACAP stimulation. The 16p Del NPCs as expected did not show changes in this pathway. Thus, I began to study other commonly dysregulated pathways in ASD.

The MAPK signaling pathway

As reviewed, the ERK pathway has been shown to be dysregulated in multiple models of ASD including BTBR mice and the 16p11.2 deletion mice. Indeed, MAPK3, which codes for ERK1, is one of the genes found in the 16p11.2 locus and one copy is missing in the 16p11.2 deletion patients. Thus, levels of ERK1 and P-ERK1 were of particular interest for signaling studies in our 16pDel NPCs. Experiments initiated by Dr. Robert Connacher in our lab found that the 16p11.2 deletion NPCs had about a 30-40% reduction in total ERK, as expected since the MAPK3 gene is deleted in the CNV interval, but a normal ratio or slightly elevated (30%, not significant) of P-ERK/Total ERK (T-ERK) when compared to unaffected controls. The absolute levels of P-ERK (not normalized to total ERK), however, were reduced in the 16p11.2 cells. These results were confirmed by further westerns that I conducted (Figure 63). As seen, P-ERK/T-ERK levels were similar to unaffected individuals (top panel) while P-ERK and T-ERK (normalized to GAPDH) were reduced (40% and 50% respectively, $p < 0.01$) in 16pDel NPCs.

16p11.2 & NIMH Controls
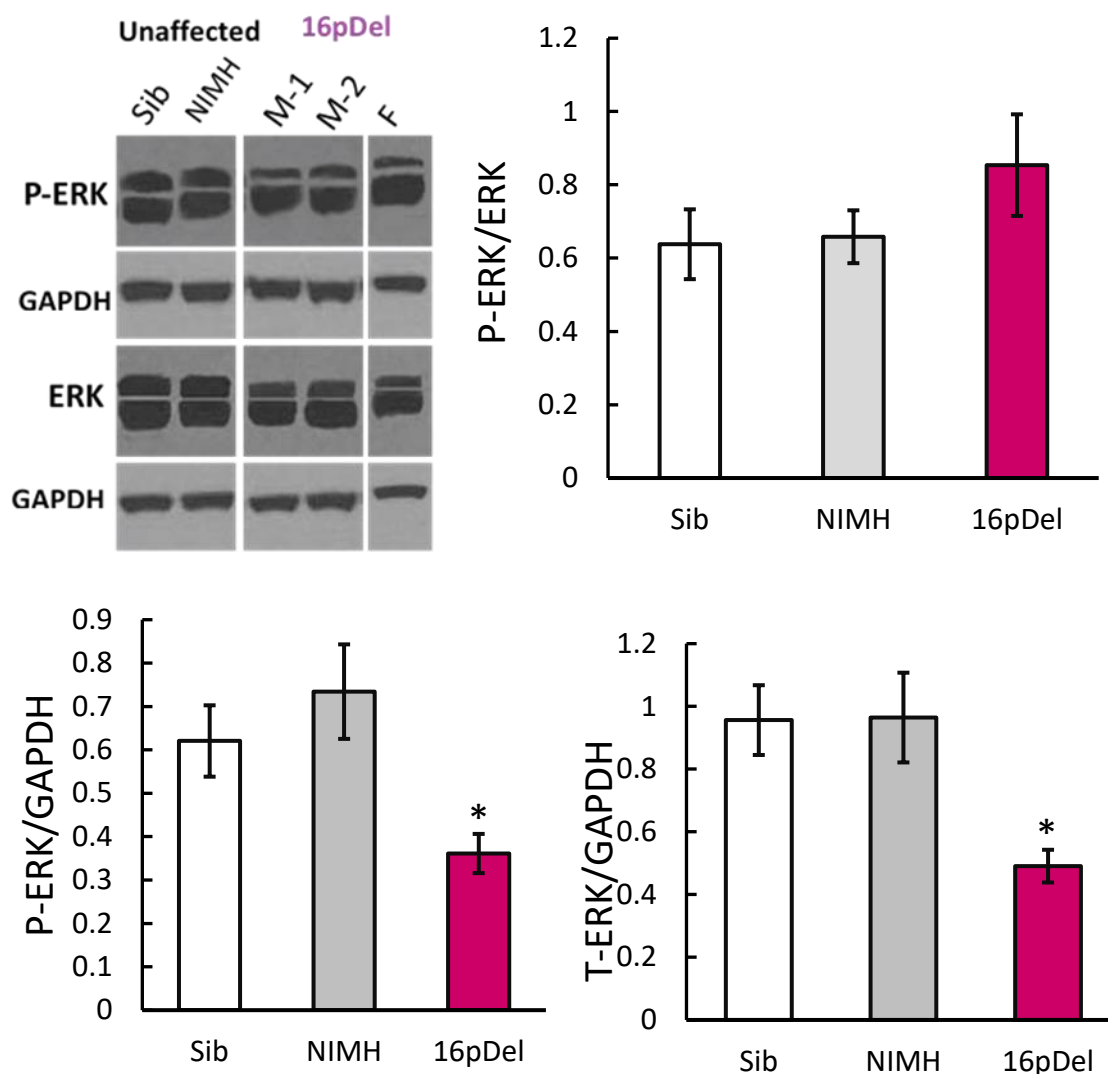
Figure 64: P-ERK levels in Representative Sib, NIMH, and all 3 16pDel patients A) Western blot images of P-ERK, GAPDH and ERK. Note, P-ERK and T-ERK levels are lower in all 3 16pdel NPCs when compared to both Sib and NIMH B) Graph quantifying: P-ERK/T-ERK ratio, P-ERK, and T-ERK in 16pdel NPCs Sib N= 3 patients, 2 clones each, 2 experiments. NIMH N= 2 patients, 2 neural inductions, 2 expts, 16pdel M N= 2 patients, 2 clones each, 2 inductions/ clone, 2 expts, 16p F N = 1 patient, 1 clone, 4 neural inductions, 2 expts

As all three 16pDel individuals had low neurites, low migration, hyperproliferation, and ERK defects, I thought it would be interesting to study the ERK pathway in our idiopathic NPCs, particularly in Family-2, where the I-ASD individual had very similar phenotypes to the 16pDel in terms of neurites, migration, and hyperproliferation.

Family-2

Analysis of P-ERK and Total ERK levels in Family-2 ASD NPCs found, that like the 16pDel NPCs, the I-ASD individuals had a lower level of P-ERK (Figure 65, bottom panel) than Sib-2 NPCs. However, unlike the 16pDel NPCs, the I-ASD-2 NPCS had no change in total ERK levels compared to Sib-2 NPCs (Figure 65, bottom). Thus, the ratio of P-ERK to Total ERK was also reduced in the ASD-2 NPCs (Figure 65, top).
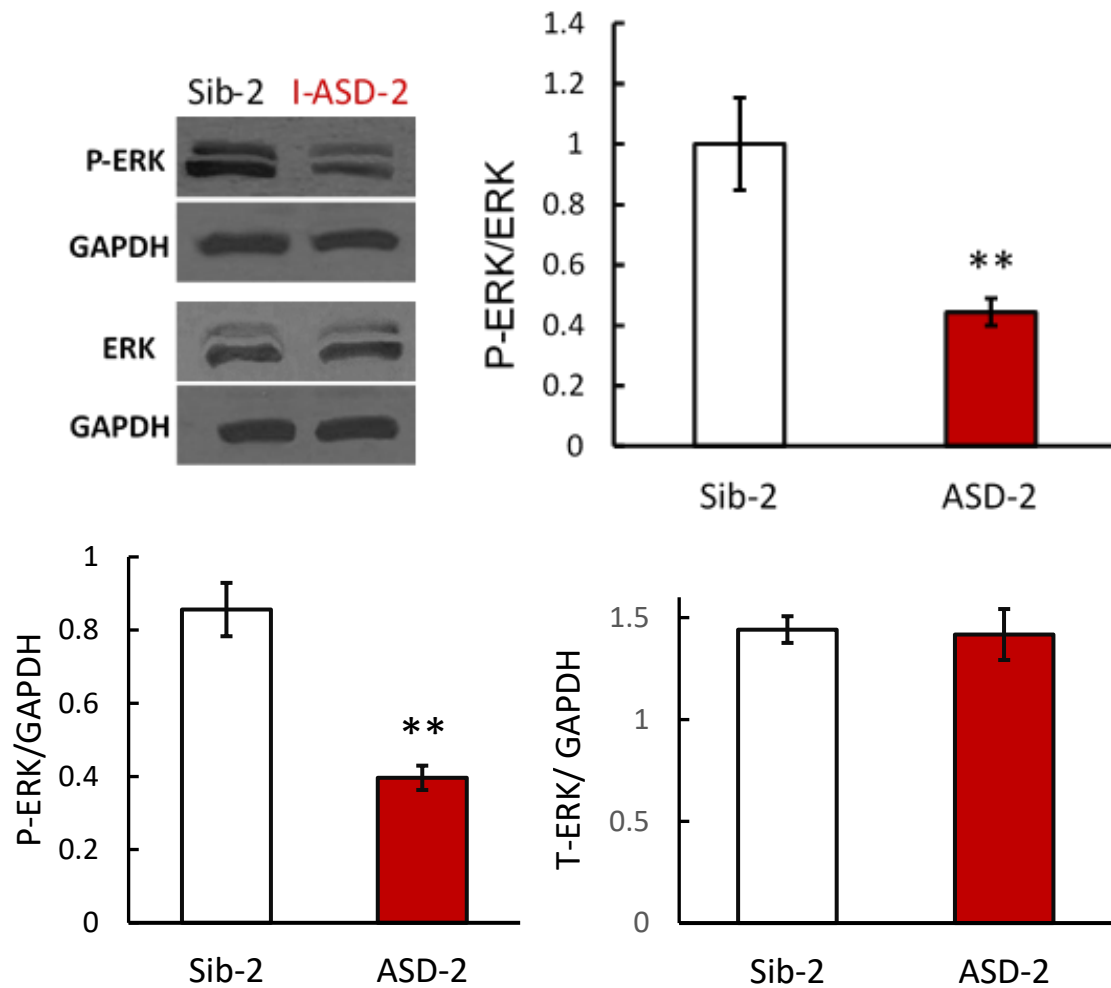
Figure 65: P-ERK levels in Family-2. Top) Representative image of western blot in Family-2 Sib and ASD. Image shows P-ERK, ERK, and GAPDH loading controls in control conditions. ASD-2 NPCs have a 50% reduction in P-ERK/ERK compared Sib-2 NPCs. Bottom) Graphs looking at P-ERK and T-ERK levels. Unlike 16pdel NPCs, T-ERK levels were not different in I-ASD-2 compared to Sib. P-ERK levels were reduced. Sib N= 2 clones 3 Expts, ASD N = 3 clones, 3 expts

Family-1 & 3:

      Much like 16pdel and I-ASD-2 NPCs, I-ASD-1 and I-ASD-3 NPCs have defects in neurite outgrowth in migration. However, unlike the 16pdel and I-ASD-2 NPCs, both I-ASD-1 and I-ASD-3 NPCs were hypo-proliferative. Thus, it was interesting to assess the activity of the ERK pathway in Family-1 &3. Unlike I-ASD-2 and 16pdel NPC, P-ERK levels were elevated in I-ASD-1 NPCs (Figure 66, image) and in I-ASD-3 NPCs (Figure 66 image). Total ERK levels were identical in Sib-1 and ASD-1 and total ERK levels were also identical between Sib-3 and ASD-3 (Figure 66,67 image). Thus, P-ERK/T-ERK levels were increased both Family-1&3 ASD NPCs (Figure 66, 67 graph). Therefore, while the 16pDel and I-ASD patients have common defects in neurite outgrowth, migration and EF response, they have patient-specific differences in P-ERK levels and P-ERK/Total ERK ratio. Thus, we see that P-ERK is indeed dysregulated in all of the patients we have studied. However, I-ASD-1 and I-ASD-3 have higher P-ERK (Figure 66, 67) while all the other patients (I-ASD-2, 16pdel M-1, M-2 and F) exhibit lower P-ERK (Figure 64,65)
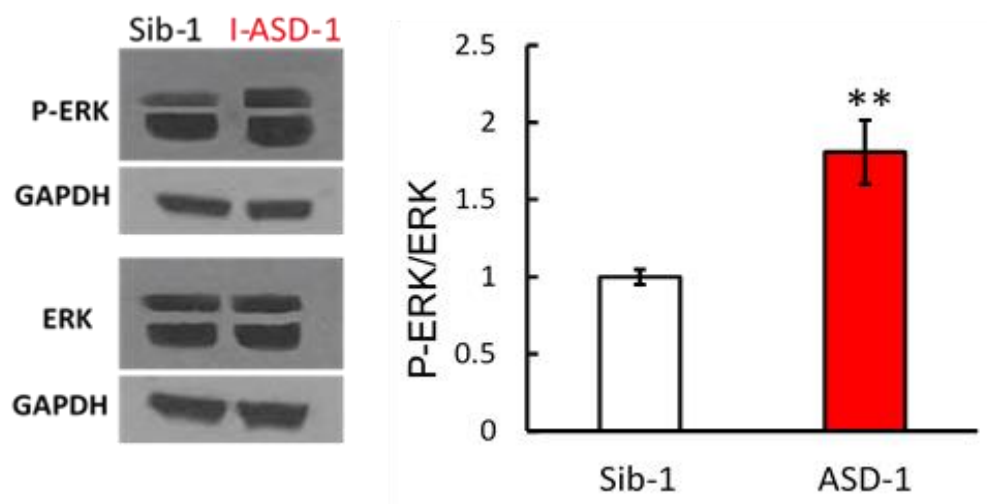
Figure 66: P-ERK levels in Family-1. A) Representative image of western blot in Family-1 Sib and ASD. Image shows P-ERK, GAPDH, ERK, and GAPDH levels in control conditions. ASD-1 NPCs have an 80% higher level of P-ERK than Sib-1 NPCs. Sib N= 2 clones 3 Expts, ASD N = 3 clones, 3 expts
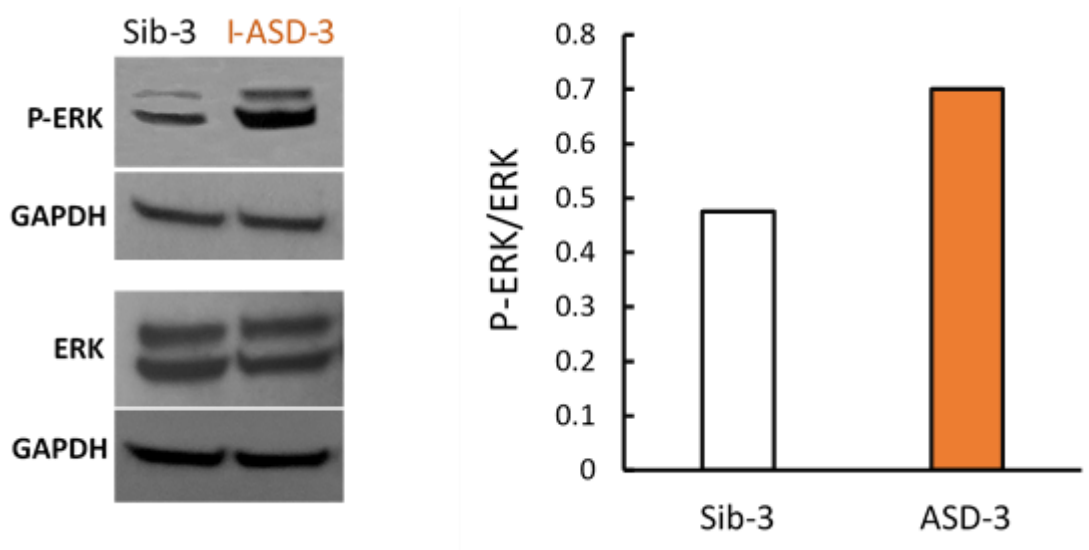
Figure 67: P-ERK levels in Family-3. A) Representative image of western blot in Family-3 Sib and ASD. Image shows P-ERK, GAPDH, ERK, and GAPDH levels in control conditions. ASD-3 NPCs have an 40% higher level of P-ERK than Sib-3 NPCs. Sib N= 1 clone 1 Expt, ASD N = 1 clones, 1 expt

The mTOR Pathway

The canonical mTOR pathway is thought to begin with the activation of P13K which ultimately through PDK1 leads to the phosphorylation of AKT. AKT then phosphorylates TSC1/2 and activates mTOR which then activates S6K which phosphorylates S6 ribosomal protein. This cascade of events leads to the regulation numerous cellular functions including developmental processes like proliferation, migration, and neurite outgrowth. Many syndromic or genetic forms of ASD are associated with mutations in the mTOR pathway including Cowden's syndrome (PTEN), Tuberous sclerosis. Furthermore, dysregulation in this pathway has also been associated with other forms of ASD including Fragile-X, NF1, and idiopathic ASD. Thus, due to this pathway's importance in regulating development and the high prevalence of alterations in mTOR in ASD, I chose to explore P-AKT and P-S6 levels in our ASD patients to understand both upstream and downstream mutations in this pathway. Despite having common aberrations

in neurite outgrowth, migration, and EF response, signaling pathway defects have not been common in all our patients. Yet, due to the commonality of mTOR defects in ASD, I hypothesized that alterations would be found in P-AKT or P-S6 in all our ASD patients. However, I anticipate that some individuals would have upregulation in the mTOR pathway while others would have down-regulation in mTOR pathway activity.

Family-1:

In Family-1 studies in multiple clones derived from Sib and I-ASD we found that ASD NPCs had a 3-fold level lower P-AKT ($p<0.0001$) (Figure 68) and 4-fold lower P-S6 than Sib NPCs ($p<0.001$) (Figure 69). Thus, in control conditions, I-ASD-1 NPCs have a lower mTOR pathway activity than the Sib. These baseline defects could be contributing to the neurite outgrowth and migration defects observed in these NPCs in control conditions. As the I-ASD-1 NPCs had such low levels of P-AKT and P-S6 in some instances, longer exposure times needed to visualize the I-ASD-1 bands led to oversaturation of Sib-1 bands. Thus, the differences in P-AKT and P-S6 are potentially even higher than illustrated through densitometry studies.
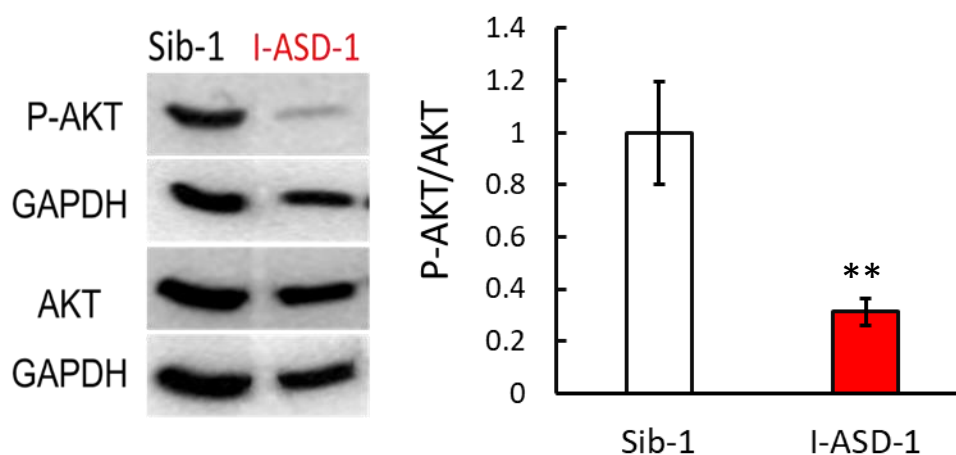
Figure 68: P-AKT levels in Family-1. A) Representative image of western blot in Family-1 Sib and ASD. Image shows P-AKT, GAPDH, AKT, and GAPDH levels in control conditions. Sib-1 NPCs on average have 4-fold higher P-AKT than ASD NPCs. Sib N = 6 Expts, 2 clones, ASD N= 8 expts, 4 clones
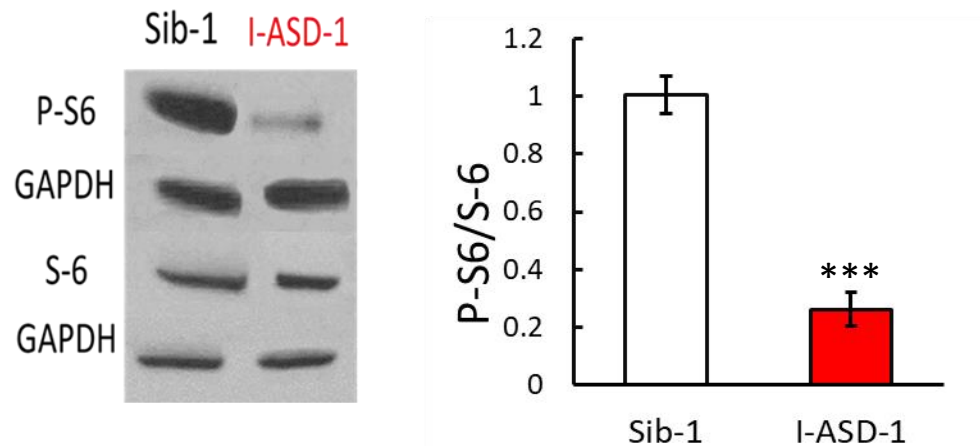
Figure 69: P-S6 levels in Family-1. A) Representative image of western blot in Family-1 Sib and ASD. Image shows P-S6, GAPDH, S6, and GAPDH levels. Sib-1 NPCs on average have 4-fold higher P-AKT than ASD NPCs. Sib N = 5 Expts, 2 clones, ASD N= 7 expts, 5 clones

Family-2 (1077):

Unlike Family-1, I-ASD-2 NPCs did not show defects in PACAP stimulated P-CREB levels. Moreover, while I-ASD-1 NPCs had higher levels of P-ERK, I-ASD-2 NPCs had lower levels of P-ERK when compared to the respective Sib NPCs. Thus, I hypothesized that the mTOR defects found in Family-1 and Family-2 may be different. Indeed, while P-AKT levels were lower in Family-1 ASD NPCs, I-ASD-2 and Sib-2 NPCs had statistically similar P-AKT levels (p=0.36) (Figure 70). Moreover, unlike ASD-1 NPCs, ASD-2 NPCs had higher levels of P-S6 (4.5x higher, p=0.006) than its respective Sib NPCs (Figure 71). Thus, while the mTOR pathway is dysregulated in Family-2, the direction of the aberrant P-S6 levels is different between the two families.
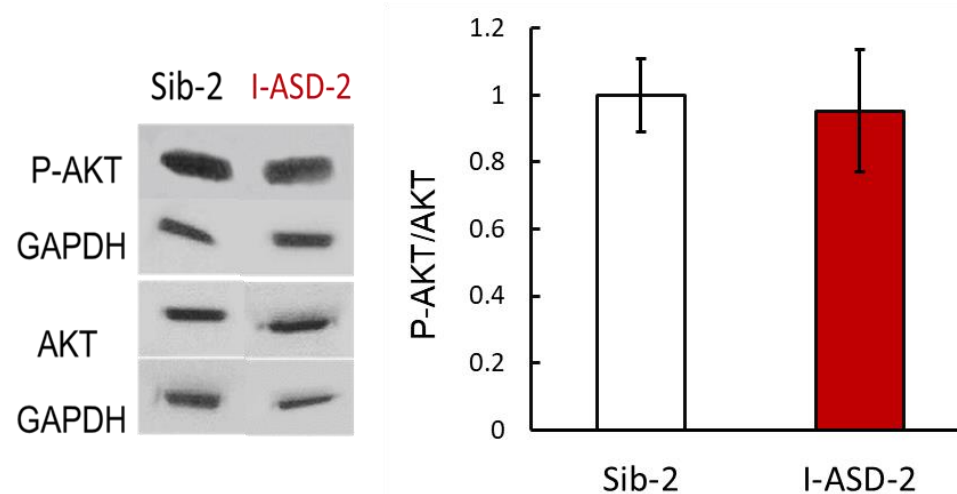
Figure 70: P-AKT levels in Family-2. A) Representative image of western blot in Family-2 Sib and ASD-2. Image shows P-AKT, GAPDH, AKT, and GAPDH levels. Sib-2 NPCs and ASD-2 NPCs have no statistically significant differences in normalized P-AKT levels. Sib N= 3 expts, 2 clones, ASD N= 3 expts, 2 clones
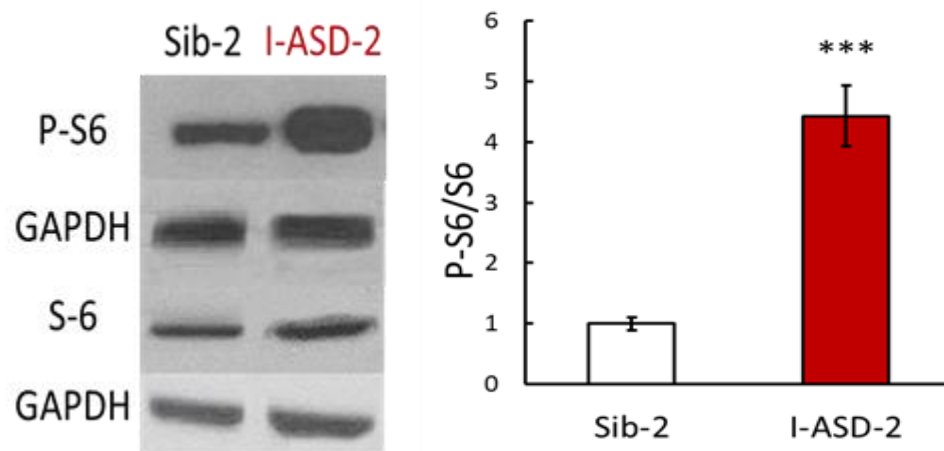
Figure 71: P-S6 levels in Family-2. A) Representative image of western blot in Family-2 Sib and ASD-2. Image shows P-S6, GAPDH, S6, and GAPDH levels. ASD-2 NPCs had almost a 4.5 times higher levels of P-S6 than Sib-2 NPCs. Sib N= 3 expts, 2 clones, ASD N=3 expts, 2 clones

Family 3:

Based on studies in Family-1 and Family-2, despite common neurite outgrowth and migration defects we find differential signaling pathway defects between these two families. However, thus far, Family-3 had similar PKA and MAPK dysregulation to Family-1. Thus, I hypothesized that Family-3 I-ASD NPCs would have lower P-AKT and lower P-S6 like Family-1 I-ASD. Indeed, preliminary studies in 1 clone each from Sib and

ASD found that like Family-1 NPCs, Family-3 ASD NPCs had lower P-AKT (Figure 72) and lower P-S6 (Figure 73) compared to Sib NPCs. The magnitude of difference however was much smaller than that seen in Family-1. Sib-3 NPCs had 75% higher P-AKT and 98% higher P-S6 than ASD-3 NPCs.
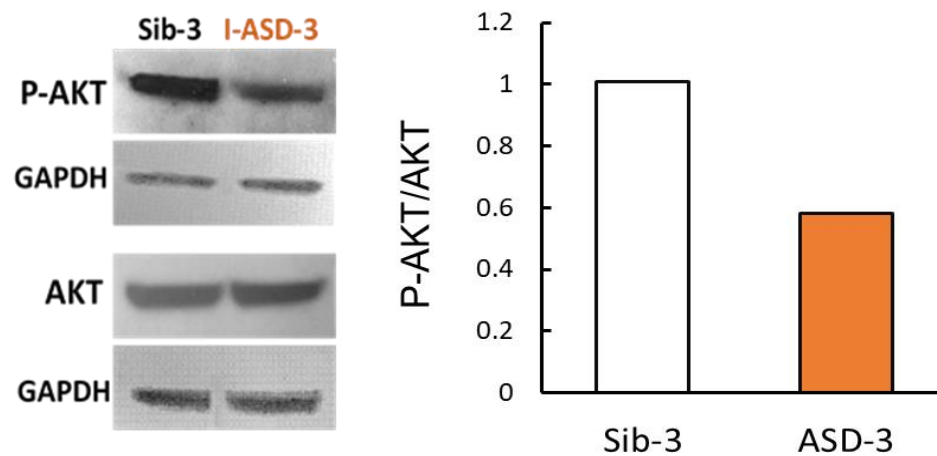
Figure 72: Normalized P-AKT levels in Family-3. A) Representative image of western blot in Family-3 Sib and ASD-3. Image shows P-AKT AKT, and GAPDH loading control levels. ASD-3 NPCs have ~50% reduction in P-AKT levels compared to Sib-3 NPCs. B) Graph quantifying P-AKT/AKT levels. Sib and ASD N = 1 clone, 1 Expt
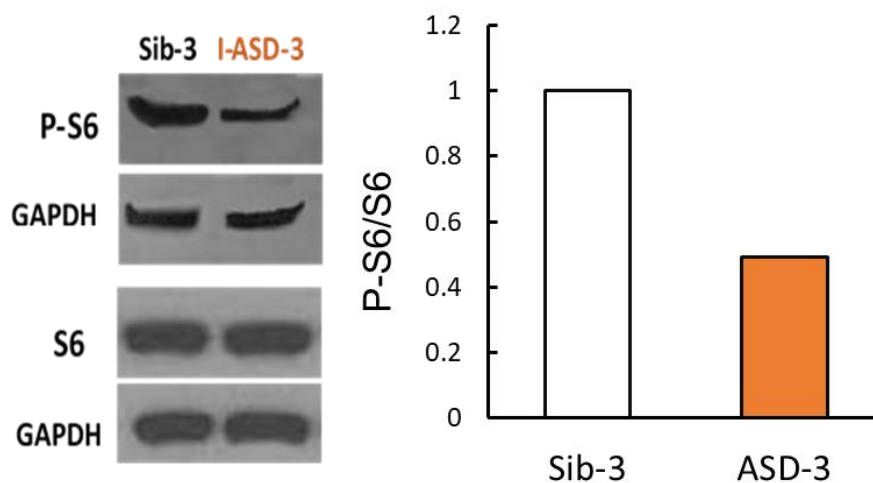
Figure 73: Normalized P-S6 levels in Family-3. A) Representative image of western blot in Family-3 Sib and ASD-3. Image shows P-S6, S6, and GAPDH loading control levels. ASD-3 NPCs have ~50% reduction in P-AKT levels compared to Sib-3 NPCs. B) Graph quantifying P-S6/S6 levels. Sib and ASD N = 1 clone, 1 Expt

16p11.2 Deletion and NIH Controls:

Thus far, we find that the signaling profile of the 16p11.2 deletion patients have been similar to the profile of I-ASD-2. Indeed, we find that both I-ASD-2 and 16pDel males show normal P-CREB levels under PACAP stimulation and have lower levels of P-ERK. Thus, I hypothesized that 16pdel, like I-ASD-2 would have normal P-AKT levels and elevated P-S6 levels. Incredibly, studies by myself and Dr. Robert Connacher found that all three 16pDel NPCs had normal P-AKT levels (Figure 74) and higher P-S6 levels when compared to both CR (p=0.029) and Sibs (p=0.049) (Figure 75). While all three 16pDel patients had elevated P-S6 levels the magnitude of difference between the 1each individual and the average of all unaffected individuals varied. 16pM-1 had a 110% higher P-S6 compared to a composite of all 3 Sib and CR while 16PM-2 and F had 73% and 55% higher P-S6 respectively.
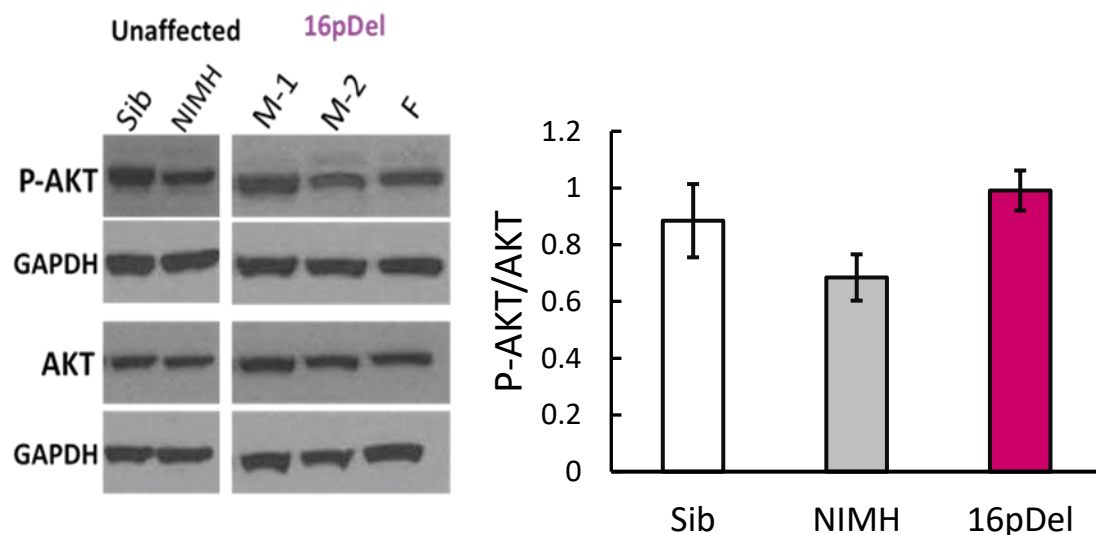
Figure 74: P-AKT levels in Representative Sib, NIMH, and all 3 16pDel patients A) Western blot images of P-AKT, GAPDH and AKT P-AKT levels are variable and across unaffected and affected groups. Thus, there are no significant differences between unaffected and 16pdel ASD NPCs in relative P-AKT levels. B)Graph quantifying: P-AKT/AKT levels Sib N= 3 patients, 2 clones each, 2 experiments. NIMH N= 2 patients, 2 neural inductions, 2 expts, 16pdel M N= 2 patients, 2 clones each, 2 inductions/ clone, 2 expts, 16p F N = 1 patient, 1 clone, 4 neural inductions, 2 expts
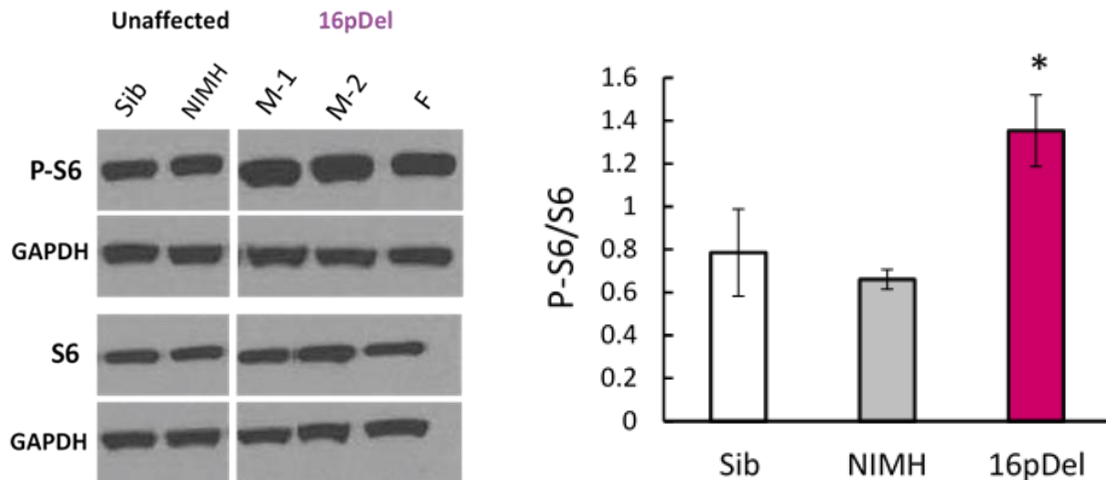
Figure 75: P-S6 levels in Representative Sib, NIMH, and all 3 16pDel patients A) Western blot images of P-S6, GAPDH and S6. Overall, P-S6 levels are higher in NPCs derived from all 3 16pdel Patients. B) Graph quantifying: P-S6/S6 levels Sib N= 3 patients, 2 clones each, 2 experiments. NIMH N= 2 patients, 2 neural inductions, 2 expts, 16pdel M N= 2 patients, 2 clones each, 2 inductions/ clone, 2 expts, 16p F N = 1 patient, 1 clone, 4 neural inductions, 2 expts

Summary of all signaling studies:

In summary, my studies show that there are two predominant signaling profiles in our ASD cohorts. I-ASD-1 and I-ASD-3 NPCs both have blunted PACAP stimulated P-CREB levels, higher P-ERK levels, and lower P-AKT and P-S6 levels. On the other hand, I-ASD-2 and the 16pDel-M-1 & M-2 have no defects in P-CREB with PACAP stimulation, lower P-ERK levels, no difference in P-AKT levels and higher P-S6 levels. In the ERK pathway, there was a minor difference between the I-ASD-2 and the 16P NPCs while P-ERK levels were lower in both, due to the reduced total-ERK levels found in the 16pdel NPCs, P-ERK/Total ERK ratio was comparatively normal in the 16pDel NPCs while it was lower in the I-ASD-2 NPCs. Fascinatingly, despite two different signaling "subtypes", all ASD individuals characterized have common/similar defects in neurite outgrowth, migration, and responses of these processes to EFs. Of course, all the signaling pathway defects uncovered are merely correlative. Thus, my next aim was to target and manipulate these pathways and study the effects on migration, neurite outgrowth, and EF response.

Chapter 8- Experimental Results: Rescue Studies

In the previous chapter, I identified numerous signaling pathways that were dysregulated in our ASD NPCs. Yet, without gain or loss of function studies, it is difficult to ascertain whether these pathways are indeed contributing to the neurite outgrowth, migration, and EF response defects that we see in our cells. Thus, I chose agonist or antagonist drugs targeted towards multiple pathways to test whether correcting abnormal signaling would rescue the neurobiological phenotypes observed in our cells.

Rescue: The PKA-P-CREB Pathway

Family-1:

I began my studies with Family-1, where the impaired PACAP response was correlated with a blunted PACAP stimulated P-CREB level in I-ASD. Thus, I postulated that increasing P-CREB levels in I-ASD NPCs could rescue the neurite outgrowth, migration, and EF responses seen in the NPCs. At this point in my studies, I was unaware that Family-2 NPCs did not have a P-CREB impairment. Thus, my main hypothesis was that PKA pathway defects could be contributing to the many phenotypes seen in Family-1. To test this hypothesis, I selected db-cAMP, a PKA pathway agonist to test on neurite outgrowth and neurosphere migration assays. Db-cAMP is a cell permeable version of cAMP, a signaling molecule essential for the activation of PKA and ultimately the phosphorylation of P-CREB. First, I conducted a dose response study of Db-cAMP in both my Sib and ASD cells (Figure 76). Interestingly, higher concentrations of db-cAMP were needed to stimulate neurite outgrowth in the ASD, suggesting that this pathway's baseline activity is indeed diminished in I-ASD-1 NPCs. Yet, we see that 3mM concentrations of db-cAMP were able to increase neurite outgrowth (by 265 % p <0.0001) in the I-ASD-1

NPCs to the level of Sib NPCs treated with PACAP (Figure 76). However, this "rescue" was not specific to the ASD NPCs as Sib NPCs also had increased neurite outgrowth (by 320%, p<0.0001) with 3 mM db-cAMP stimulation. The percent increase in neurites and the absolute levels of neurites was higher in Sib-1 under db-cAMP when compared to ASD-1 (p=0.02). Likewise, 3 mM db-cAMP increased migration in I-ASD NPCs by 84% (p< 0.0001) (Figure 77). However, again, the increase in migration was not specific to I-ASD and Sib NPCs also showed an increase (30% p=0.001) Thus, while db-cAMP, unlike PACAP, did manage to increase neurite outgrowth and migration in I-ASD-1, these effects were not specific to diagnosis.
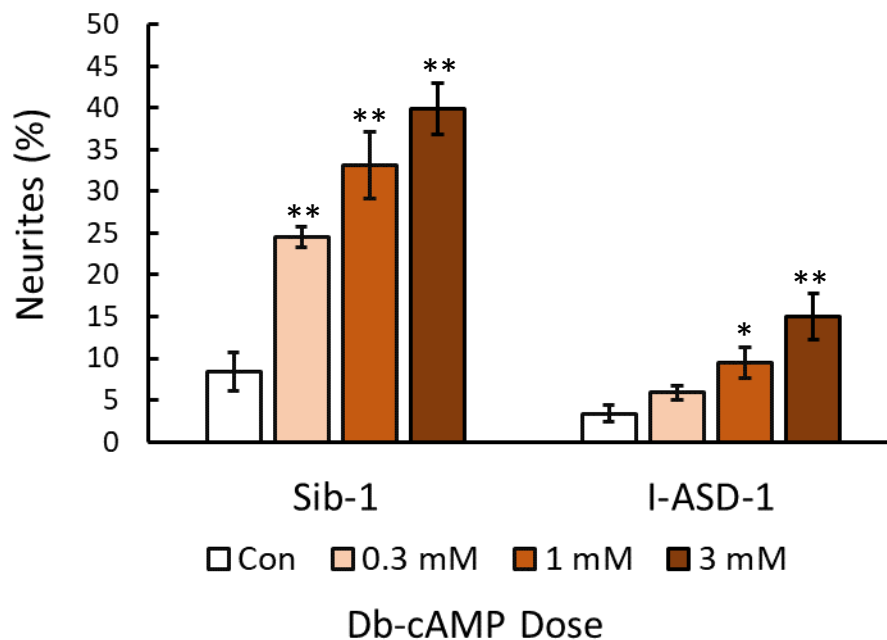
Figure 76: Dose response of Neurite outgrowth in Family-1 NPCs under PKA pathway agonist db-cAMP. While both Sib and ASD NPCs respond to db-cAMP, ASD NPCs only begin responding to db-cAMP at 1 mM while Sib NPCs have robust neurite outgrowth at lower doses. N = 2 expts, 2 dishes/condition/expt, 2 clones per patient. There is a statsitcally significant difference between Sib and ASD from 0.3mM-3mM (p<0.001)
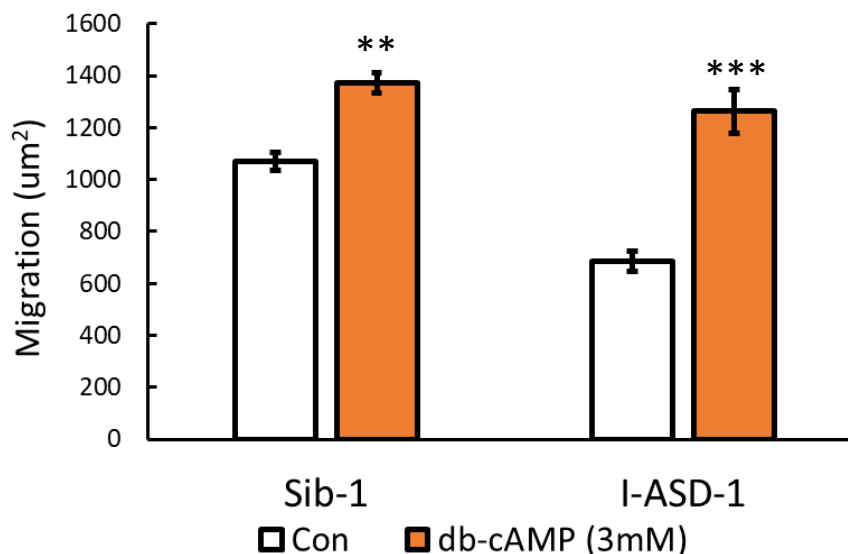
Figure 77: Db-cAMP increases migration in both Family-1 Sib and ASD NPCs. Unlike neurite outgrowth studies, db-cAMP does increase migration to the level of Sib NPCs under db-cAMP in this case. Sib N = 4 expts, 3 clones, 75 spheres/condition. ASD N= 3 expts, 3 clones, 55 spheres/condition.

Of course, as the studies above demonstrate, the PKA pathway is important for regulating neurite outgrowth and migration. Our ASD cells show defects in these processes as well as an inability to respond to important EFs like PACAP, 5-HT, and NGF. Canonically, we know that PACAP and 5-HT are thought to signal through the Gs G-protein system which leads to a signaling cascade that causes an upregulation in cAMP, activation of PKA, and ultimately phosphorylation of CREB. Thus, I was interested to see if "restoring" signaling by the PKA pathway, by adding a sub-threshold dose of db-cAMP with typical doses of PACAP, 5-HT, and NGF would now allow unresponsive I-ASD cells to respond to these signals. I defined a sub-threshold dose based on neurobiological response rather than in pharmacological terms. That is, based on dose response studies, I selected a dose in which neither Sib nor ASD cells showed increased neurite outgrowth as "sub-threshold". As seen in the graph, when this lower dose of db-cAMP (0.01 mM) was used, it did not lead to increased neurite outgrowth (Figure 78). Moreover, as seen in Chapter 5, PACAP, NGF,

and 5-HT were unable to stimulate neurite outgrowth in the I-ASD but did stimulate neurite outgrowth in the Sib (Figure 78). Amazingly, when the sub-threshold db-cAMP was combined with PACAP and 5-HT in the I-ASD, it prompted a neurite outgrowth response (Figure 77A)! However, db-camp + NGF still did not increase neurites. Combining low dose db-cAMP with PACAP, 5HT, or NGF had no extra stimulatory effect on Sib neurite outgrowth. Thus, this suggests that low levels of cAMP or underactivity of the PKA pathway could be contributing to the lack of response to EFs that use Gs stimulatory pathways in this Family. However, it does not completely rescue all EF responses.
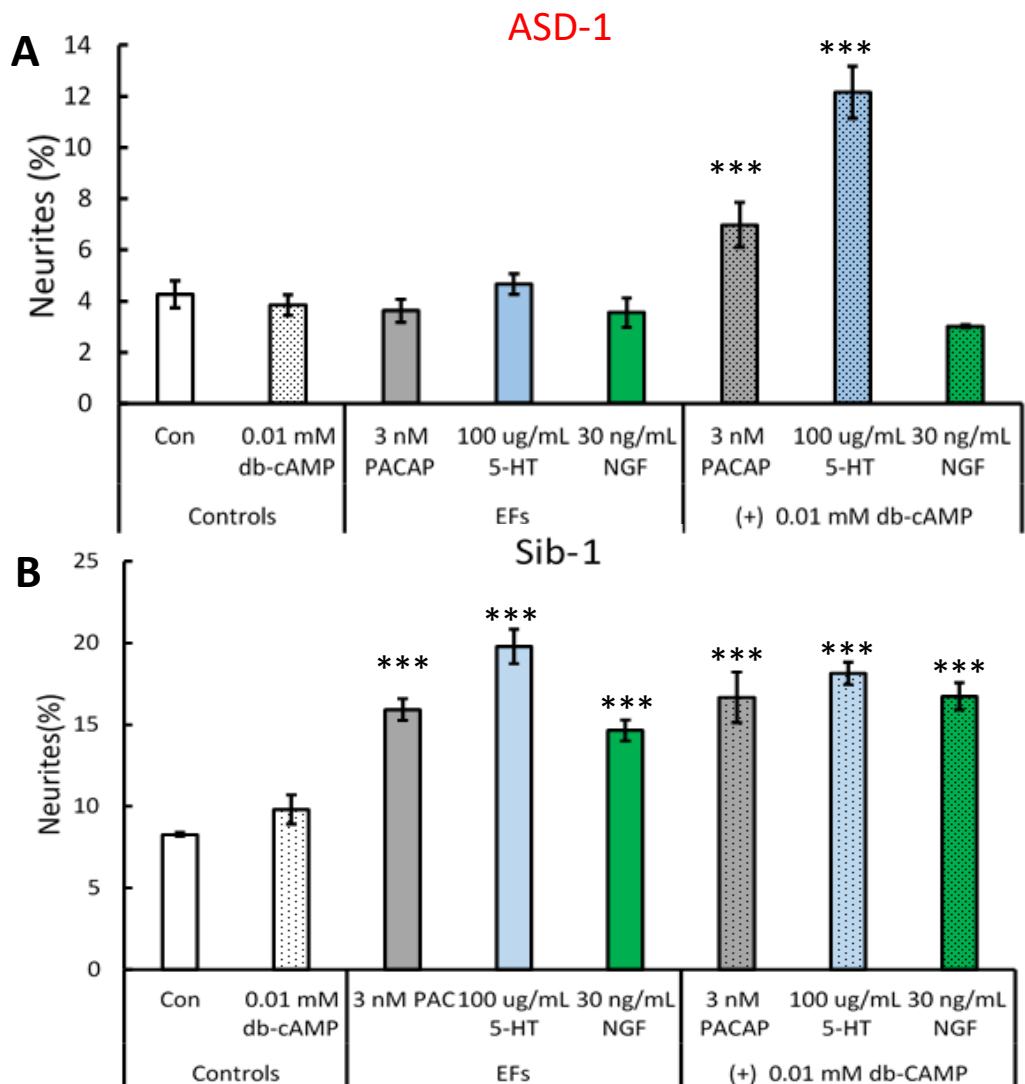
Figure 78: Combination studies of db-cAMP + EFs in Family-1. Graph A) Shows db-cAMP + EF studies in ASD-1 NPCs. PACAP, NGF, and 5-HT failed to elicit neurite outgrowth in ASD NPCs. Combining a dose of db-cAMP that does not stimulate neurite outgrowth with EFs allows ASD NPCs to respond to PACAP and 5-HT but not NGF. B) Db-cAMP + EF studies in Sib-1 NPCs. PACAP, NGF, and 5-HT all lead to increased neurite outgrowth in the Sib. Combining these EFs with sub-threshold db-cAMP does not change EF response in Sib-1 NPCs. Sib N = 2 expts, 2 dishes/condition /expt, 2 clones. ASD N= 2 expts, 2 dishes/condition/expt, 2 clones.

Family-2, 3, and 16p11.2 deletion

As shown above, in Family-1 higher doses of db-cAMP increase neurites and migration in both Sib and ASD. However, at a very low dose, it is specifically able to rescue cellular responses to PACAP and 5-HT in the ASD-1 NPCs. However, ASD-2 NPCs do not have diminished P-CREB levels when stimulated by PACAP. This indicates that the PKA pathway is normal in ASD-2 and suggests that PKA dysfunction is not contributing to the lack of PACAP response in ASD-2. Therefore, in this Family, I expected higher doses of db-cAMP to stimulate neurite outgrowth in the Sib and ASD. However, I did not expect sub-threshold doses of db-cAMP to rescue PACAP response in the ASD-2 NPCs. Indeed, Figure 79 (A and B) shows that 3 mM db-cAMP increases neurites in both the Sib-2 and ASD-2 NPCs as expected. Moreover, the PACAP response was not rescued in the ASD, showing that PKA is not contributing to the defective EF response in this family.

Like ASD-2, 16p NPCs have no P-CREB defects and also are able to respond to PACAP and 5-HT. Moreover, since db-cAMP's ability to increase neurites and migration are non-specific to defect or diagnosis, this agonist was not tested in 16p. In 1012, PACAP stimulated P-CREB deficits were similar to those seen in Family-1. Thus, we would expect db-cAMP results to be similar to Family-1, however, these studies were not conducted.
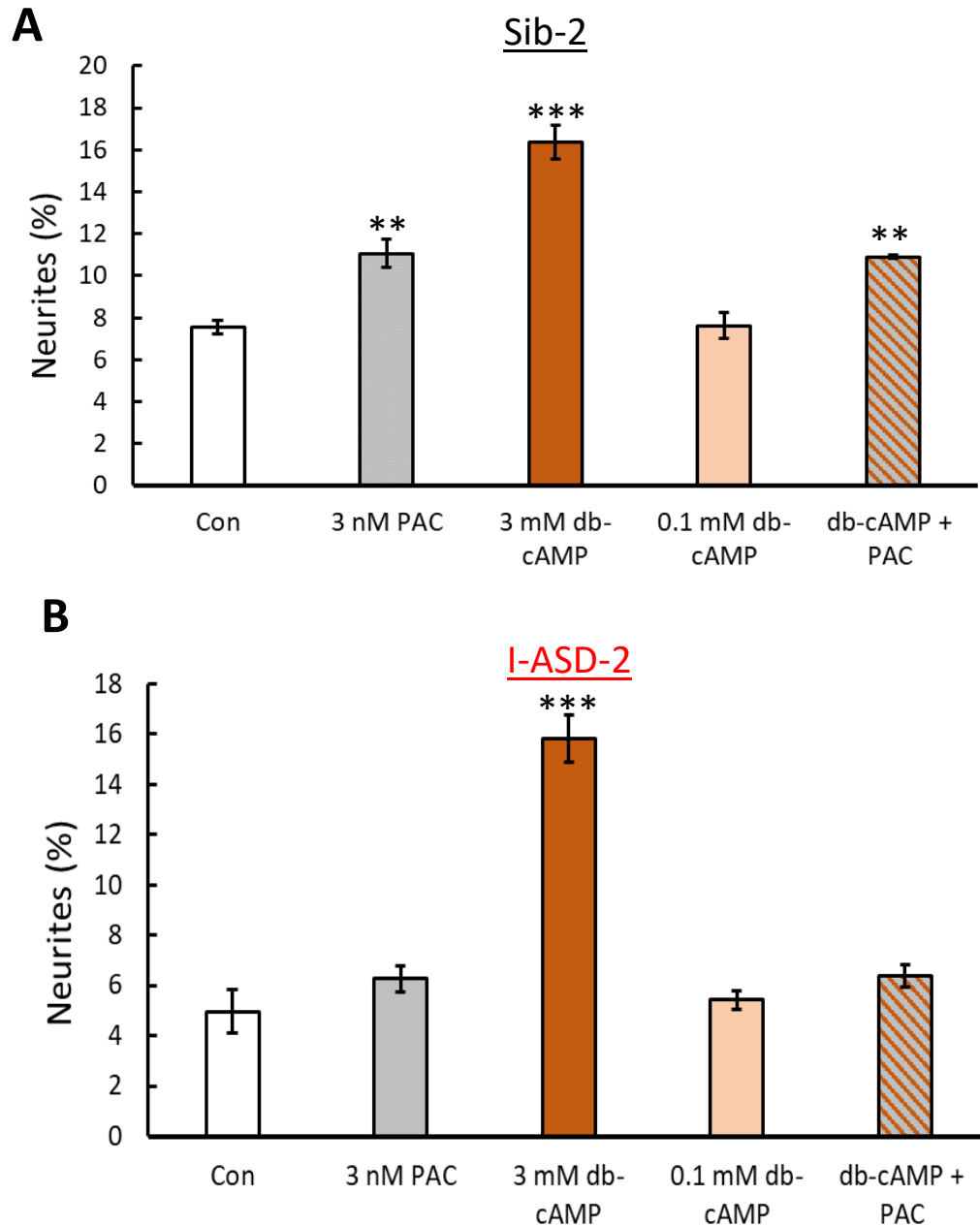
Figure 79: db-cAMP + EF studies in Family-2. A) Sib NPCs in Family-2 respond to both PACAP and db-cAMP with increased neurite outgrowth. Low doses of db-cAMP do not elicit neurite outgrowth in Sib-2 and addition of low dose db-cAMP with PACAP does not enhance PACAP response. B) ASD-2 NPCs fail to respond to 3 nM PACAP but show increases in neurite outgrowth with 3 mM db-cAMP. Low doses of db-cAMP + PACAP does not facilitate PACAP response in this ASD patient. Sib N = 1 expt, 2 dishes/condition /expt, 1 clone. ASD N= 1 expts, 2 dishes/condition/expt, 1 clones.

Rescue: The MAPK pathway

After conducting studies on the PKA pathway, I determined that under-activity of PKA may contribute to defects in the response to some EFs but that this pathway was not central to the developmental defects seen in our cell lines. Our cells also exhibited dysregulations in the ERK pathway-with Family-1 & 3 showing higher P-ERK and Family-2 and the 16pDel showing lower P-ERK levels. Therefore, it is possible that both low and high levels of P-ERK could lead to reduced neurites and migration in NPCs. To determine if this is true, gain and loss of function studies must be conducted on the ERK pathway. Interestingly, as detailed in Chapters 5 &6, ERK pathway agonist FGF was tested on NPCs. If both higher and lower P-ERK are contributory to neurite and migration defects, we would expect, in Family-1 NPCs, where P-ERK is already elevated, that FGF would exacerbate neurite and migration defects. On the other hand, we would expect FGF to rescue neurites and migration in Family-2 where P-ERK is reduced. However, as seen in Chapters 5 and 6, FGF increased neurites and migration in both I-ASD patients, indicating that decreases and increases in the ERK pathway are not necessarily causing the reduced migration and neurites seen in our patients. However, these studies show that modulation of ERK does lead to changes in both migration and neurite outgrowth.

Rescue: The mTOR pathway

In chapter 7 I reviewed data showing that the mTOR pathway is commonly dysregulated in all our I-ASD patients. There seemed to be two sub-types of mTOR dysregulations in our cohort. One group, which includes I-ASD-1 and I-ASD-3 showed lower levels of both P-AKT and P-S6 while the 2nd group which includes I-ASD-2 and all three 16pDel NPCs have no change in P-AKT and higher levels of P-S6. Yet, when I began

my rescue studies, our lab had primarily been working on Family-1 NPCs and had just received NPCs from the 2nd family. Thus, my rescue studies primarily focus on Family-1 and knowledge gleaned from in-depth studies in this Family.

Family-1:

Gain of Function:

Phosphorylation activates AKT which ultimately leads to activation of mTOR and phosphorylation and activation of S6. Thus, in Family-1 where P-AKT and P-S6 levels are lower in ASD NPCs (Figures 68& 69), I chose to use AKT agonist drug, SC-79, to increase activity in this pathway. A majority of drugs that target the mTOR pathway are designed for the treatment of cancer or for use in cancer cell lines, where cells are hyperproliferative and generally have higher mTOR pathway activation. Thus, most drugs for this pathway are inhibitors and SC-79 is the only agonist for the mTOR pathway that is currently commercially available. SC-79 works by increasing the likelihood of phosphorylation of AKT through a configurational change in the protein that makes it easier for kinases to access the phosphorylation domain. Indeed, western analysis showed that 2ug/mL dose of SC-79 increased levels of P-AKT and P-S6 in the ASD to that of Sib in control conditions (Figure 80). The drug had no effect on P-AKT or P-S6 levels in Sib NPCs.
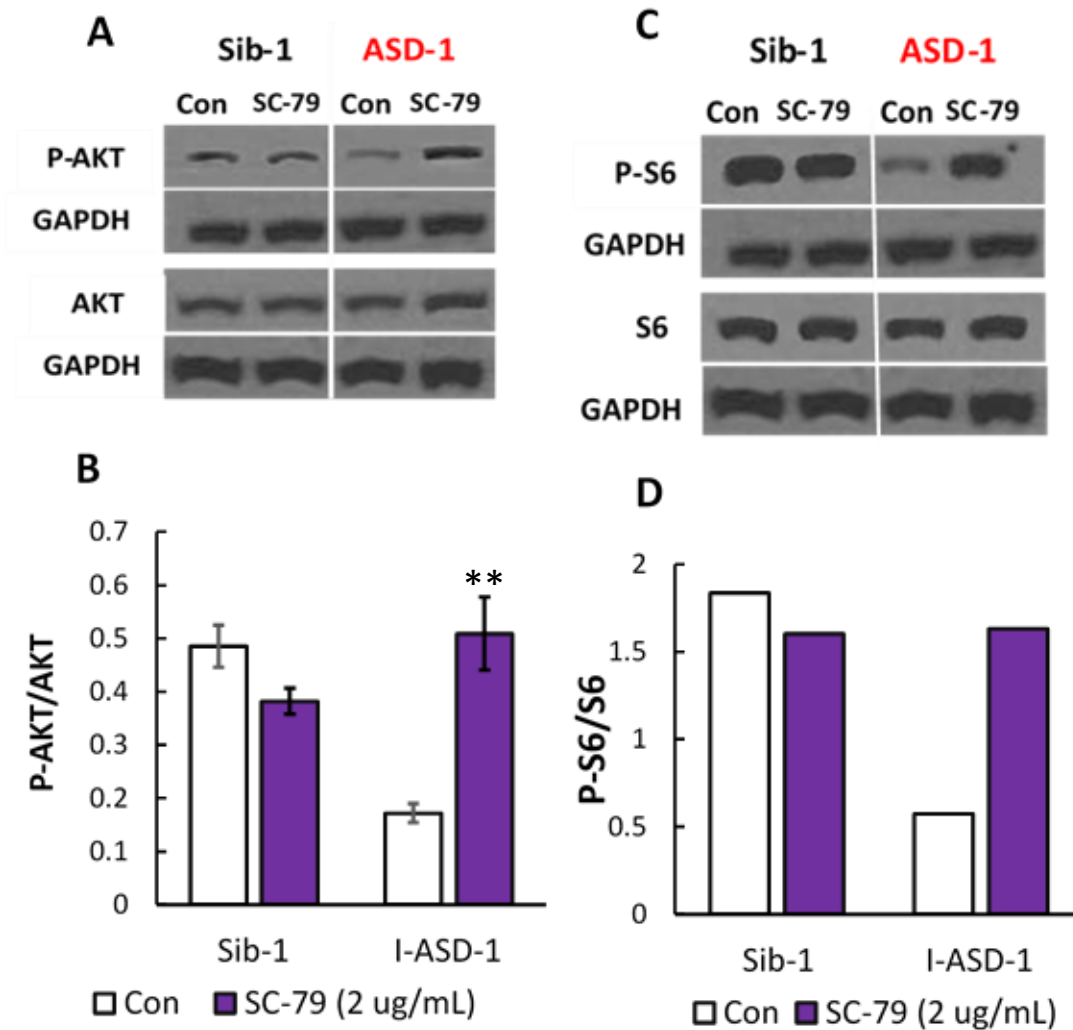
Figure 80: SC-79 Rescues P-AKT and P-S6 levels in I-ASD-1 NPCs. A)P-AKT and P-S6 levels in Sib-1 and ASD-1 NPCs in control conditions and under the stimulation of SC-79, AKT activator. As seen in the figures, P-AKT levels are significantly reduced in ASD NPCs. Treatment with SC-79 increases these P-AKT levels in the ASD- NPCs to the level of Sib NPCs. Similar results are seen for P-S6. B) Graphs quantifying P-AKT/AKT and P-S6/S6 levels in Sib and ASD under control and SC-79. P-AKT Sib & ASD N= 2 clones, 2 expts. P-S6 Sib and ASD N= 1 clone, 1 expt. **NOTE:** P-AKT/P-S6 and AKT/S6 data presented is from the same blot. Thus, GAPDH loading control is identical for the phosphor antibodies and GAPDH for total antibodies are also identical

After ensuring the drug rescued P-AKT and P-S6 levels in ASD (Figure 79), I tested the effects of SC-79 on neurite outgrowth by conducting dose responses in both Sib and ASD NPCs in Family-1. Strikingly, while multiple doses of SC-79 increased neurite outgrowth in the ASD NPCs, no doses of SC-79 were able to increase neurites in the Sib (Fig 80).

Interestingly, the 10ug/mL of SC-79 did decrease neurite outgrowth in Sib NPCs by 40% (p=0.005, Figure 81) indicating that excess mTOR activation leads to decreased neurites. Maximal neurite outgrowth in the ASD was observed at the 2ug/mL dose. Furthermore, in a study by Jo et al 2012 found that the 2ug/mL dose was also optimal in increasing neuronal survival in a cell culture model of ischemic stroke in hippocampal neurons. Thus, this dose was selected for further studies which confirmed that SC-79 increased ASD-1 NPC neurite outgrowth (by 90% p <0.0001) to the level of Sib NPCs without changing neurite outgrowth in the Sib (Figure 82). Applying the 2ug/mL dose o migration studies showed that once again, SC-79 increased migration in the ASD NPCs (50 % p< 0.0001) while leading to no change in the Sib NPCs (Figure 83). Thus, increasing phosphorylation of AKT specifically rescues both neurite outgrowth and migration in I-ASD-1 NPCs.
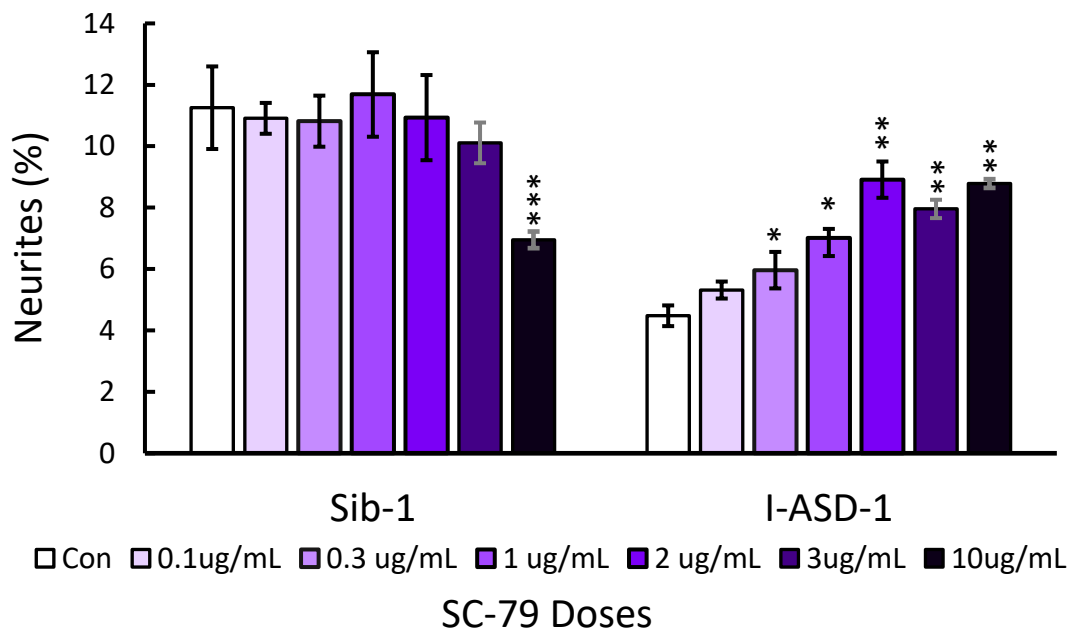
Figure 81: Dose response studies of SC-79 in Family-1. P-AKT agonist SC-79 does not change neurite outgrowth in Sib from 0. 1ug/mL to 3 ug/mL doses. However, at the 10 ug/mL dose, SC-79 decreases neurites in Sib. In I-ASD-1 SC-79 increases neurites at 1 ug/mL all the way till 10 ug/mL. Peak neurite outgrowth was found at 2 ug/mL doses. N= 2 expts, 2 clones, 2 dishes/condition/expt per patient.
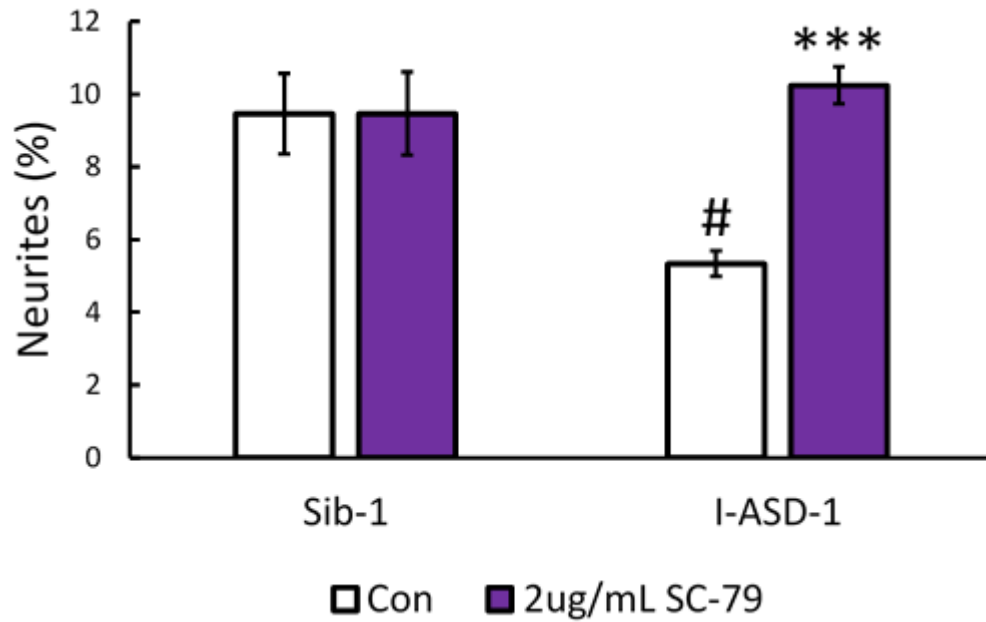
Figure 82: In Family-1, SC-79 rescues neurite outgrowth in I-ASD-1 NPCs. Sib-1 had no change in neurites under SC-79, while ASD-1 had a 90% increase in neurites at the 2ug/mL dose. Sib N = 5 expts, 2-3 dishes/expt/condition, 3 clones. ASD N = 5 Expts, 2-3 dishes/expt/condition, 2 clones.
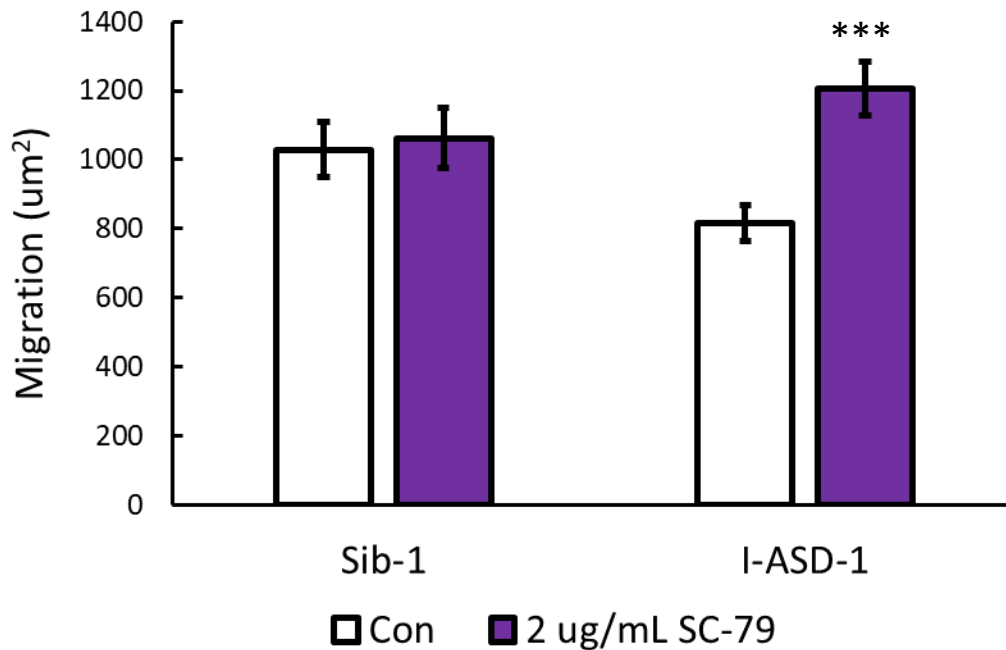
Figure 83: In Family-1, SC-79 rescues migration in I-ASD-1 NPCs. Sib-1 had no change in neurites under SC-79, while ASD-1 had a 50% increase in neurites at the 2ug/mL dose. N = 2 expts, 2 clones, 40 spheres/condition per patient.

Of course, these NPCs also had defects in response to EFs. Earlier, I showed that using sub-threshold doses of db-cAMP, a PKA pathway agonist, allowed ASD NPCs to respond to 5-HT and PACAP but did not restore an NGF response. Thus, to test if mTOR defects are indeed contributing to all 3 major defects I have uncovered in these cells, I conducted similar experiments with SC-79. Essentially, a sub-threshold dose of SC-79 where no neurite outgrowth and migration were observed was selected. This low dose was then combined with normal doses of PACAP, NGF, and 5-HT. Stunningly, in I-ASD-1, use of this lower dose of SC-79 to "prime" the cells led to a robust response of these I-ASD-1 cells to all three EFs ($p < 0.0001$ for SC+ EF) (Figure 84)! When the same experiment was conducted in Sib NPCs, no enhancement was seen in the Sib NPCs' response to PACAP, NGF, or 5-HT. Thus, targeting of the mTOR pathway was able to successfully reverse all three phenotypes in I-ASD-1 cells. This suggests that mTOR dysregulation is central to the neurodevelopmental phenotypes observed in this family.
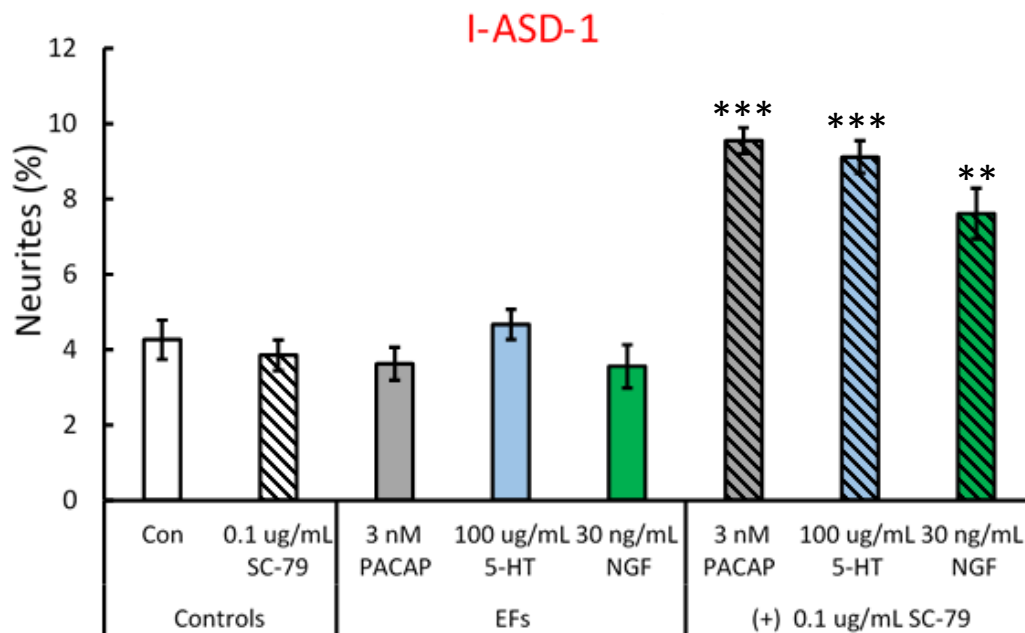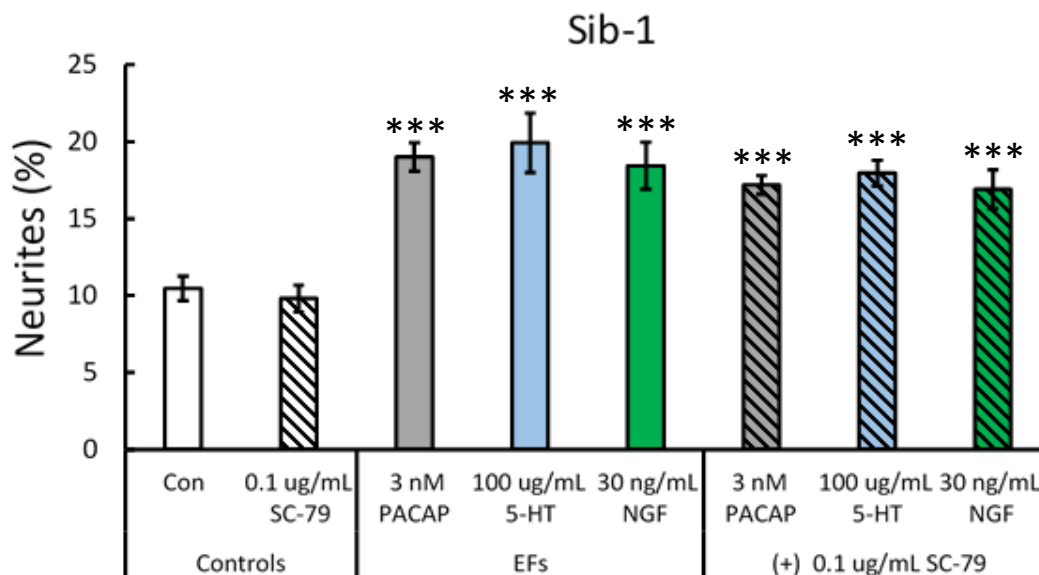
Figure 84: SC-79+ EF neurite outgrowth studies in Family-1 A) Shows SC-79 + EF studies in ASD-1 NPCs. As seen before, PACAP, NGF, and 5-HT failed to elicit neurite outgrowth in ASD NPCs. Combining a dose of SC-79 that does not stimulate neurite outgrowth with EFs allows ASD NPCs to respond to PACAP, NGF, and 5-HT B) SC-79 + EF studies in Sib-1 NPCs. PACAP, NGF, and 5-HT all lead to increased neurite outgrowth in the Sib. Combining these EFs with sub-threshold SC-79 does not change EF response in Sib-1 NPCs. N = 2 expts, 2 dishes/condition /expt, 2 clones per patient.

As we see, low doses of SC-79 are able to rescue EF responses in the ASD-1 NPCs. In the PKA section of Chapter 7, I showed that ASD-NPCs had blunted PACAP stimulated P-CREB. I hypothesized that these "blunted" P-CREB levels were contributing to the lack of PACAP response in the ASD patient. Thus, as priming ASD-1 NPCs with SC-79 facilitated PACAP response in these cells, I wanted to see if P-CREB levels were normalized to that of Sib treated with PACAP. Thus, I conducted western blot studies where both ASD and Sib cells were treated with the following: Vehicle, 3 nM PACAP, 0.1 ug/mL SC-79, and 0.1 ug/mL + PACAP. In Sib, PACAP treatment led to a large increase in P-CREB levels. Treatment with SC-79 did not change P-CREB levels. The combination of SC-79 + PAC in the Sib was no different than PACAP alone (Figure 85). In ASD NPCs, treatment with PACAP lead to a small increase in P-CREB levels (as per usual). SC-79

alone led to no change in the P-CREB levels. However, the combination of SC-79 and PACAP led to great increases in the P-CREB levels in ASD (same as Sib treated with PACAP). Thus, this suggests that "normalizing" the mTOR pathway in ASD NPCs allows for PACAP to now stimulate the PKA pathway appropriate and increase P-CREB levels. This suggests that the mTOR has interactions with PKA or that dysregulations in mTOR are also affecting the PKA pathway.
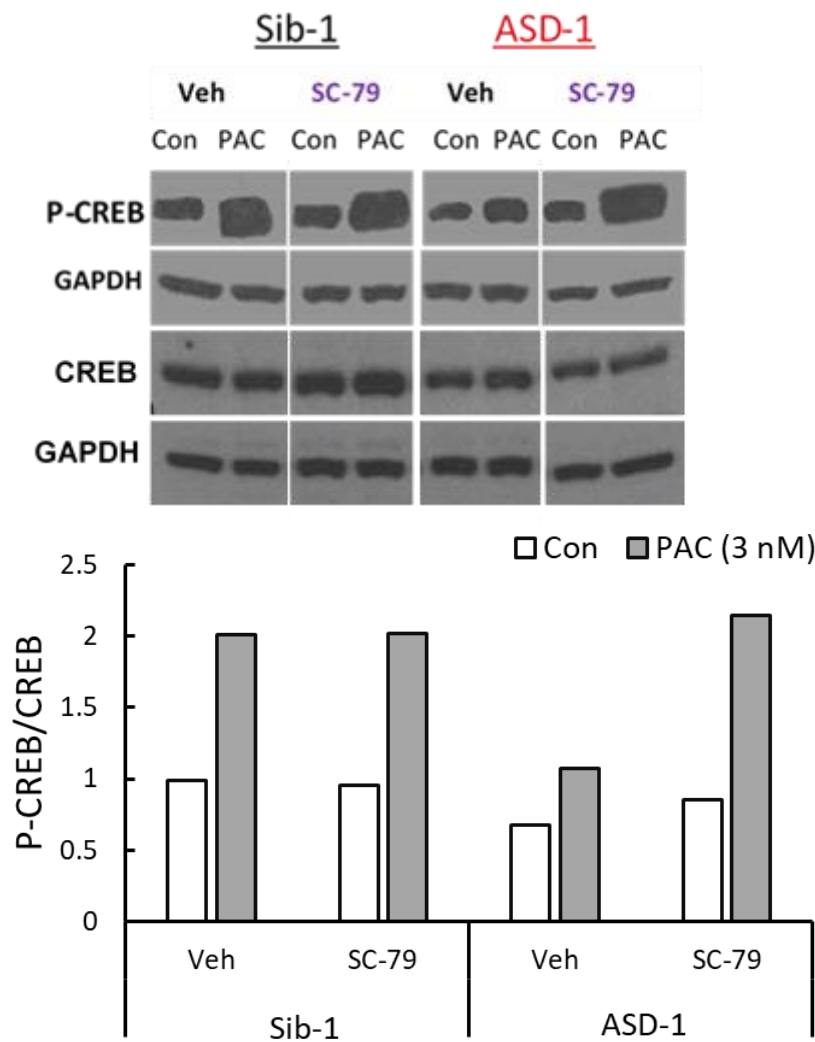
Figure 85: SC-79 rescues PACAP stimulated P-CREB responses in I-ASD-1 NPCs. Top) Images of P-CREB levels under Control and PACAP in either Vehicle or SC-79 conditions in both Sib-1 and ASD-1 NPCs. ASD-1 NPCs, under vehicle, as seen before has diminished P-CREB response to PACAP. SC-79 increases the P-CREB response under PACAP stimulation in ASD. SC-79 leads to no changes in PACAP response in Sib-1. Sib and ASD N= 1 clone 1, Expt

Loss of Function Studies:

To confirm the necessary and sufficient role of mTOR in the phenotypes observed in ASD-1 NPCs, in addition to rescuing the ASD cells with an mTOR pathway agonist, I designed studies to reduce P-AKT & P-S6 in Sib, to determine whether these reductions would phenocopy in the Sib, the ASD phenotype. I employed the cancer therapeutic MK-2206, which acts on the same phosphorylation site as SC-79, to reduce P-AKT levels. In Sib-1, MK-2206 reduced P-AKT and P-S6 levels to that of ASD in control condition (Figure 86). Yet, the drug had no effect on P-AKT or P-S6 levels in the ASD-1 NPCs.
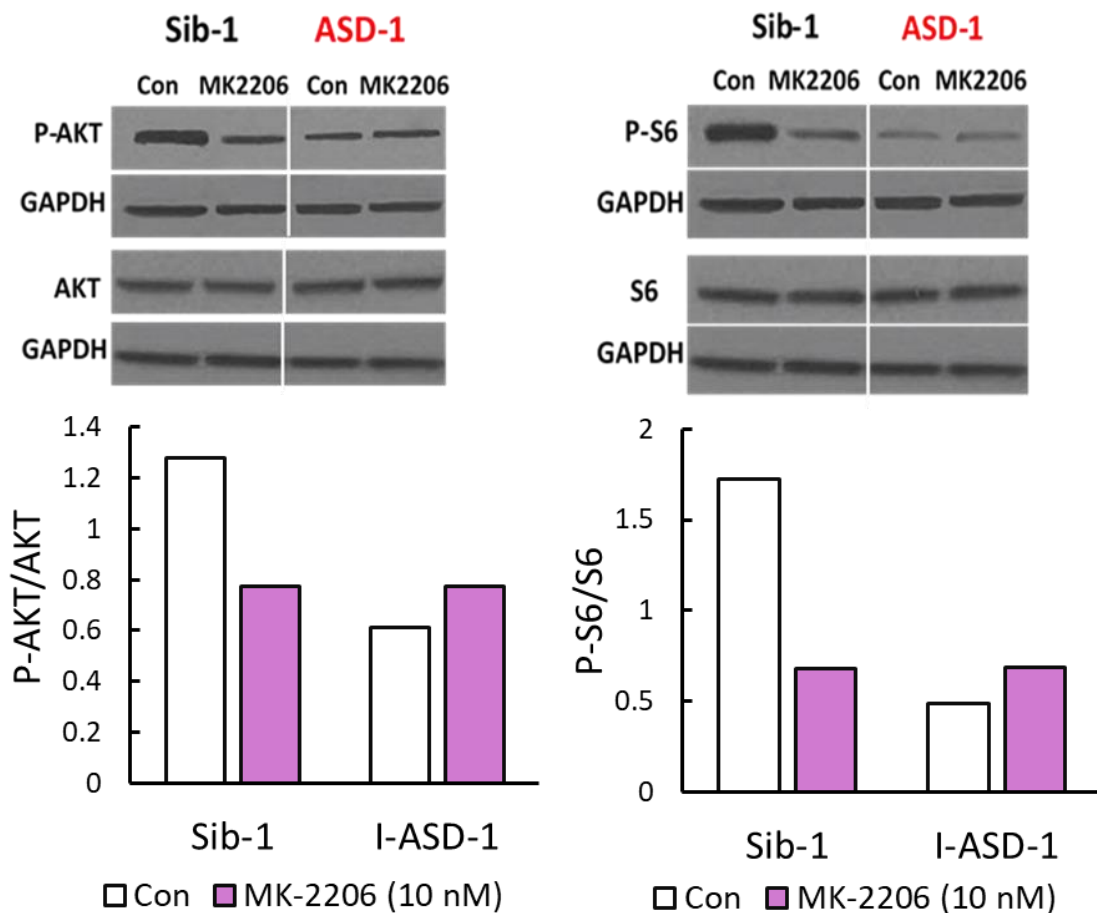
Figure 86: MK-2206 diminished both P-AKT and P-S6 levels in Sib-1 NPCs. Top) Figures of westerns showing P-AKT, AKT, P-S6, S6 and loading control GAPDH. MK-2206 brings down Sib P-AKT and P-S6 to ASD NPC levels. Bottom) Quantifications of westerns showing (left) P-AKT/AKT levels and (right) P-S6/S6 levels in Sib-1 and ASD-1 NPCs under control or MK-2206 stimulation. Sib & ASD N = 1 clone, 1 Experiment

 Dose response studies of MK-2206 on neurite outgrowth found that Sib NPCs exhibited reduced neurites at 3 doses (p= 0.002, 0.01, p=0.003), but that theses doses had no impact on ASD NPCs (Figure 87). Interestingly, the neurite outgrowth in MK treated Sib NPCs were statistically identical to the percentage of cells with neurites in control conditions for ASD NPCs (p=0.89). Likewise, with 30 nM of MK2206, we see that migration in Sib NPCs was reduced to the level seen in the ASD NPCs in control (Figure 88). Thus, by inhibiting the mTOR pathway through an AKT antagonist, I showed that Sib NPCs now had impaired migration and impaired neurite outgrowth similar to the ASD NPCs.
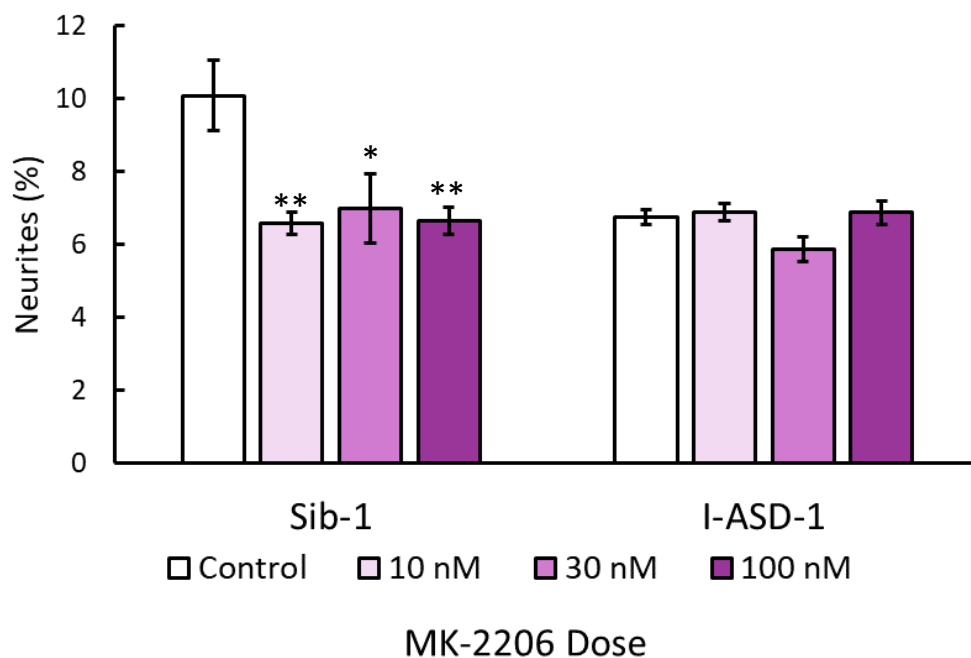
Figure 87: Dose response studies of MK-2206, P-AKT inhibitor, on neurite outgrowth in Family-1. While Sib NPCs had statistically significant decreases in neurite outgrowth under multiple doses of MK-2206, ASD NPCs had no change with MK-2206. MK-2206 treatment reduced neurites in Sib to the level of ASD NPCs. N= 2 expts, 2 clones, 2-3 dishes/ condition/ expt per patient.
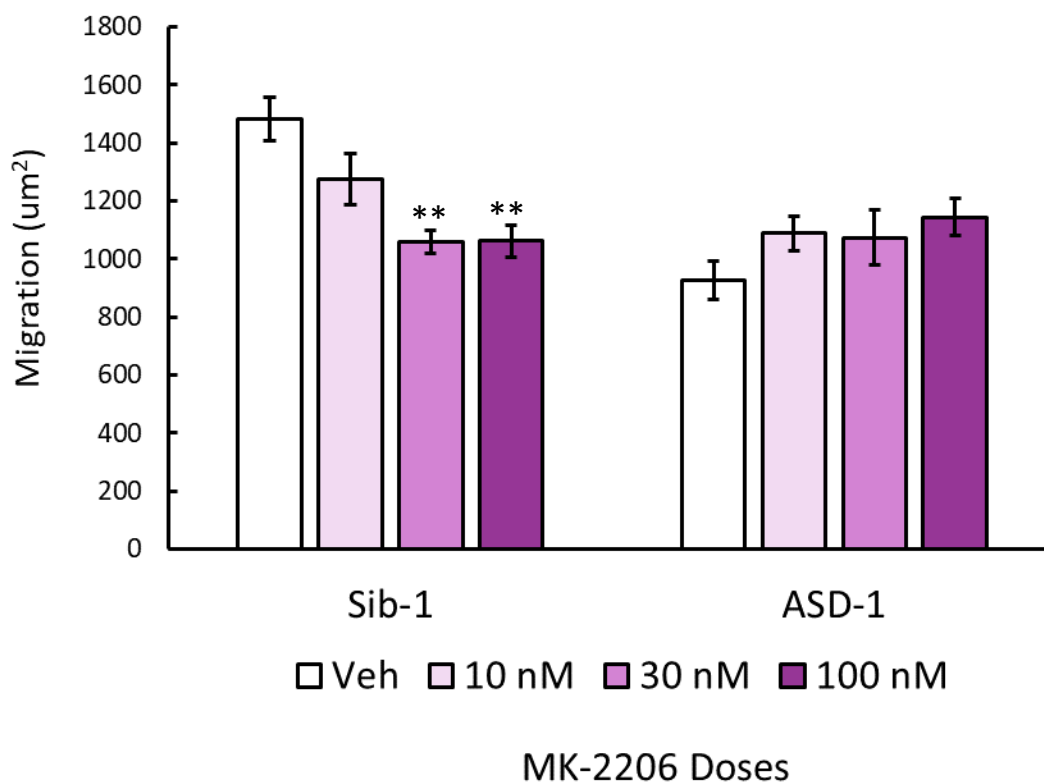
Figure 88: Dose response studies of MK-2206, P-AKT inhibitor, on migration in Family-1. While Sib NPCs had statistically significant decreases in migration under multiple doses of MK-2206, ASD NPCs had no change with MK-2206. MK-2206 treatment reduced migration in Sib to the level of ASD NPCs. Sib N= 2 expts, 20 spheres/condition. ASD N= 1 expt 20 spheres/condition.

Lastly, as the I-ASD NPCs had defects in responding to EFs, I wanted to see if AKT inhibition would abolish EF responses in the Sib. Thus, I again combined sub-threshold doses of the drug, in this case MK-2206, with typical doses of PACAP, NGF, and 5-HT. As shown in Figure 89, Sib cells usually respond to NGF, PACAP, 5-HT with increased neurites. However once treated with 1 nM MK, Sib cells no longer respond to the EFs, thereby recapitulating the EF defects found in the I-ASD!
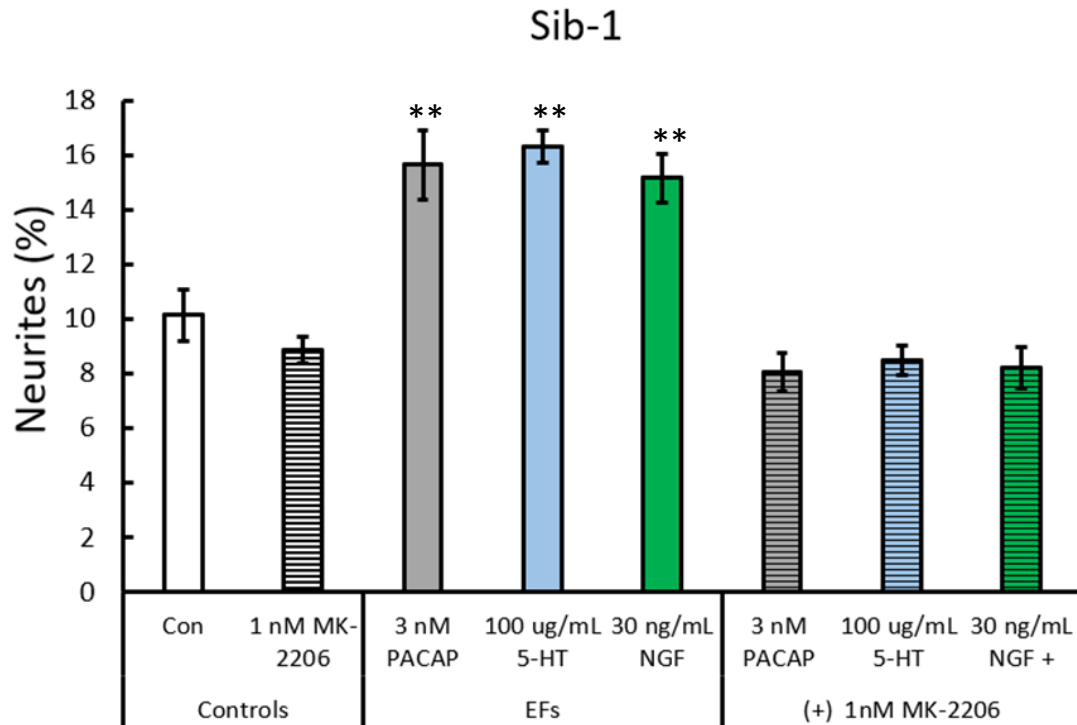
Figure 89: MK-2206 + EF studies in Sib-1. PACAP, NGF, and 5-HT all lead to increased neurite outgrowth in the Sib. Combining these EFs with sub-threshold MK-2206 abolished Sib-NPC response to PACAP, 5HT, and NGF. N= 2 expts, 2 dishes/condition/expt, 2 clones.

Again, as antagonizing the mTOR pathway abolishes EF response in Sib, I wanted to see how PACAP stimulated P-CREB levels looked like in the Sib under stimulation of MK-2206. Interestingly, treatment of Sib NPCs with sub-threshold doses of MK-2206 did not alter P-CREB levels in control conditions (Figure 90). However, under the stimulation of PACAP, Sib-1 NPCs that were treated with MK-2206 showed a large reduction in the levels of P-CREB. Thus, the Sib-1 NPCs when treated with MK-2206, recapitulated the blunted P-CREB levels seen in I-ASD-1 NPCs under PACAP stimulation!
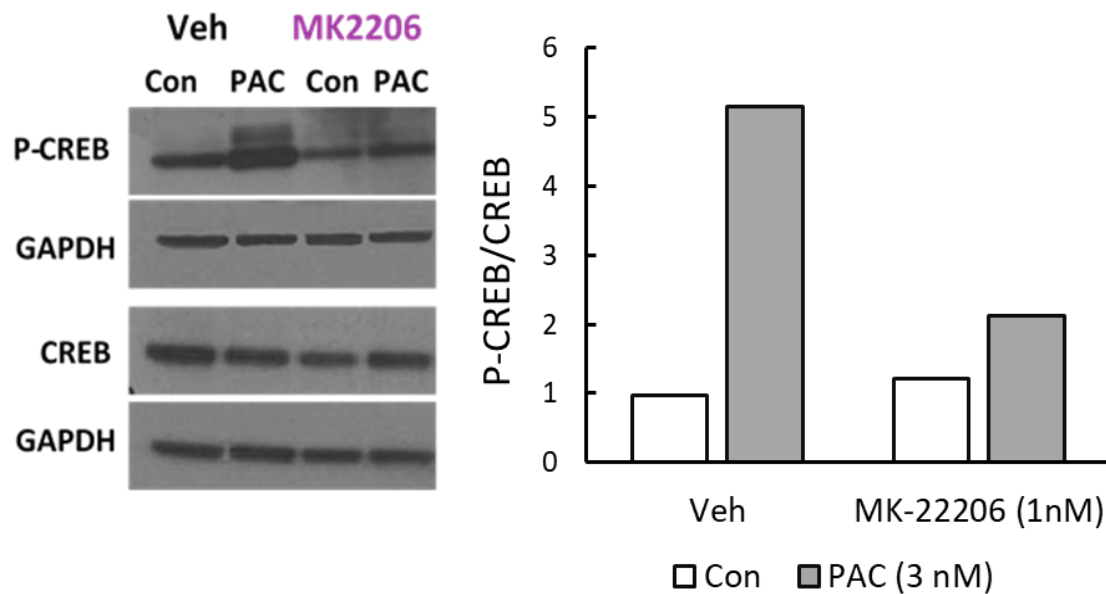
Figure 90: MK-2206 reduces the PACAP stimulated P-CREB response in Sib. Left) Westerns showing P-CREB, CREB and GAPDH loading controls in Sib-1 NPCs under Control and PACAP pre-treated with either Veh or MK-2206. In vehicle conditions Sib-1 NPCs have a large increase in P-CREB levels under PACAP. Pre-treatment with MK-2206 diminishes this P-CREB response. Right) Graph quantifying P-CREB/CREB levels in these conditions. N=1clone 1 Expt

In summary, in Family-1, neurite outgrowth, migration, and EF response defects in the I-ASD NPCs could be rescued by targeting and increasing P-AKT with the agonist SC-79. Likewise, all three of these defects could be induced in the Sib NPCs by reducing P-AKT levels with MK-2206. Thus, AKT and the mTOR pathway seem to be central to the defects seen in I-ASD-1.

Family-2 (1077):

In Family-2, the neurobiological defects observed in the I-ASD-2 were similar to that of I-ASD-1. However, unlike ASD-1 NPCs, P-AKT was normal in ASD-2 NPCs and P-S6 was elevated. Thus, the mTOR pathway activity levels were higher in the ASD-2 NPCs while they were lower in ASD-1 NPCs. MK2206 which acts to reduce P-AKT would also reduce P-S6 (as seen in Family-1, Figure 86). Therefore, Family-2, I tested whether MK-2206 could reduce P-S6 levels and thereby rescue the neurite and migration defects

seen in ASD-2 NPCs. Western blot analyses showed that in I-ASD-2 NPCs MK-2206 reduced P-S6 levels (Figure 91). In Sib-2, surprisingly, no changes were seen in P-S6 levels at this dose of MK-2206. However, preliminary studies did show that MK-2206 did reduces both P-AKT and P-S6 levels in Sib-2 NPCs at 100 and 300 nM.
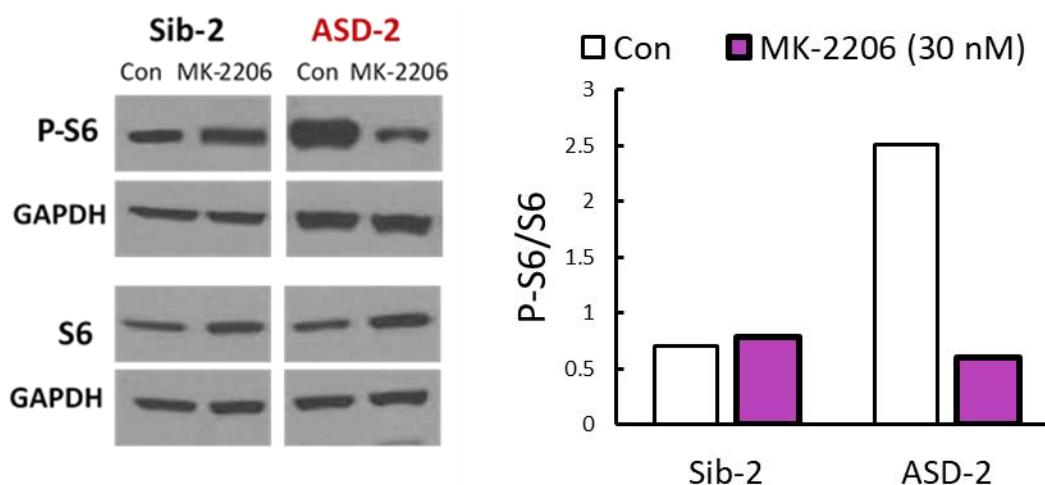
Figure 91: MK-2206 reduces the elevated P-S6 levels in ASD-2 NPCs. Right) Westerns showing P-S6, S6 and GAPDH loading control in Sib-2 and ASD-2 NPCs under Control or MK-2206 conditions. Left) Graph quantifying the changes in P-S6/S6 levels caused by MK-2206 drug. MK2206 reduces the P-S6 levels only in ASD-2 NPCs and leads to no change in Sib-2 NPCs. Sib and ASD N= 1 clone, 1 Expt

Once the MK-2206 was shown to reduce P-S6 in I-ASD-2, I conducted dose response studies on the neurite outgrowth assay in Family-2. Figure 92 shows that ASD NPCs have an increase in neurite outgrowth beginning at the 10nM dose (62% increase p=0.054). and becoming statistically significant at 30 nM and 100 nM (180% increase in both conditions p=0.001). At 30 and 100 nM ASD-2 NPC neurite outgrowth was statistically identical to that of the Sib-2 in Control. Sib NPCs had no change in neurites at the 10 nM or 30 nM dose. However, they did show a reduction in neurites (much like Sib-1) at the 100 nM dose of MK2206 (47% reduction p<0.001) (Figure 92). Interestingly, western studies in Sib-2 also showed that inhibition of P-S6 did not occur until the 100 nM dose suggesting that reductions in P-S6 parallel neurites. Moreover, these studies indicate

that a delicate balance of P-AKT and P-S6 (not too high and not too low) are needed for proper neurite outgrowth. Dose response studies on neurospheres showed similar results. ASD spheres began migrating at the 10 nM dose and reached maximal migration at 30 nM. On the other hand, Sib spheres had no change in migration at 10 or 30 nM but had a reduction in migration at 100 nM (Figure 93). Thus, in Family-2 where P-S6 was higher in ASD-NPCs, reducing P-S6 levels with an antagonist successfully rescued neurite outgrowth and migration in ASD NPCs.
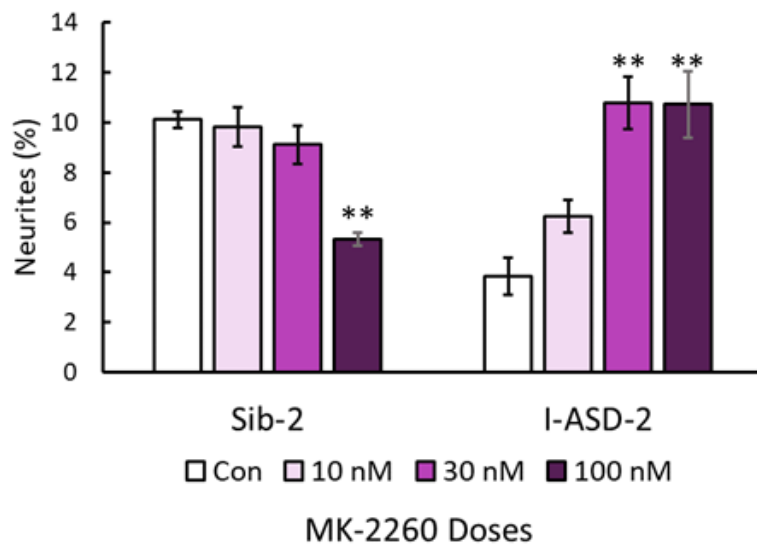
Figure 92: Dose response studies of MK-2206, P-AKT inhibitor in Family-2. While Sib NPCs showed 47% reductions in neurite outgrowth at 100 nM dose. ASD NPCs had increased neurite outgrowth at the 30 and 100 nM dose. N= 2 expts, 2 clones, 2-3 dishes/ condition/ expt per patient.
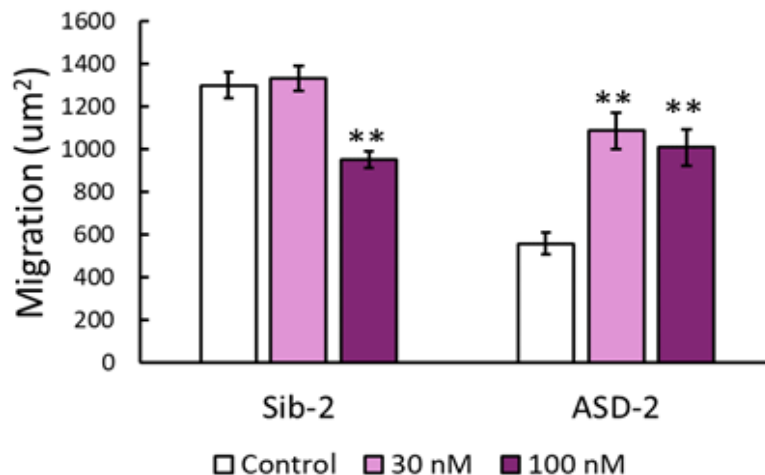
Figure 93: Dose response studies of MK-2206 P-AKT inhibitor on Neurosphere migration in Family-2. In ASD-2, inhibiting AKT levels led to an increase in migration. In the Sib, at 30 nM, MK-2206 had no effect on migration. However, at 100 nM Sib NPCs showed reduced migration with P-AKT inhibition. N= 2 expts, 15 spheres/condition/expt, 2 clones/ patient

In addition to rescuing the neurite defects in the ASD NPCs, I aimed to increase P-S6 levels in Sib-2 to see if neurite defects could be induced. SC-79, which increases P-AKT should also elevate P-S6 (as seen in Family-1 figure 80). Indeed, a 6ug/mL dose of SC-79 increased P-S6 levels in Sib-2 to that of ASD-2 (Figure 94). Interestingly, a higher dose of SC-70 (10 ug/mL) also reduced neurite outgrowth in Sib-1 NPCs. Likewise, SC-79 dose responses in Family-2 again showed that high doses of SC-79 decreases neurite outgrowth in Sib-2 (36% decrease, p=0.002) while it did not change neurites in ASD-2 (Figure 94). Again, it seems that over- or underactivity in the mTOR pathway are leading to similar neurite phenotypes.
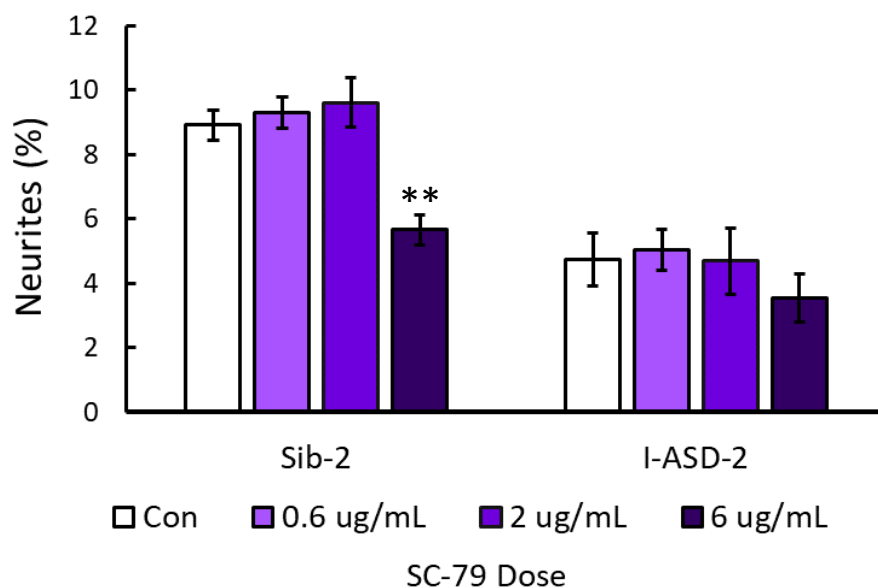
Figure 94: Dose response studies of SC-79, P-AKT activator in Family-2. Increasing P-AKT in Sib-2 with high doses of SC-79 led to a 36% reduction in neurites. ASD-2 NPCs had no response to SC-79. 6ug/mL dose of SC-79 made neurite outgrowth in the Sib-2 and ASD-2 NPCs statistically identical. N= 2 expts, 2 clones, 2-3 dishes/ condition/ expt per patient.

16p11.2 Deletion:

The 16p11.2 deletion cells, much like the Family-2 ASD NPCs had higher levels of P-S6. Neurite defects were seen in both severely affected individuals with 16pdel ASD (16pdel M-1 and 16pdel F). Neurite reductions were observed in the 16pdel M-2 but this reduction was not statistically significant. Migration defects, however, were seen in all 3 16pdel patients. Preliminary studies in 16p11. Del M-1 showed that MK-2206 does indeed rescue neurites (Figure 95). Thus, by targeting dysregulated mTOR signaling neurites were also rescued in 16pdel NPCs.
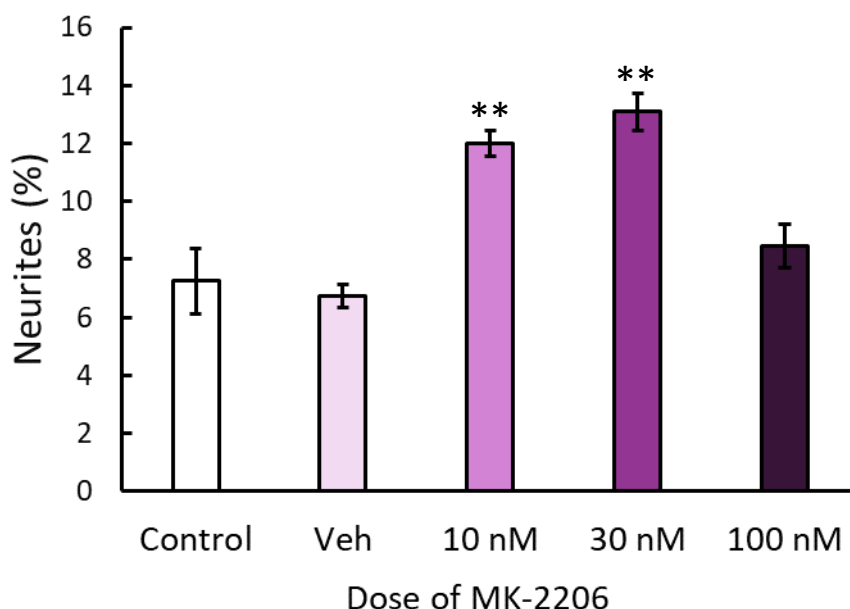
Figure 95: Dose response studies of MK-2206 in 16pDel M-1. MK-2206 increases neurite outgrowth in the 16pdel at 10 nM and 30 nM doses thereby rescuing neurite outgrowth. N= 1 expt, 1 Patient

Summary 2:

As mentioned in Chapter 7, I found that there were two main profiles of signaling defects in our ASD cohorts. One group of individuals including I-ASD-1 and I-ASD-3 with blunted P-CREB, higher P-ERK and lower P-AKT and P-S6 and the other group including I-ASD-2 and the 16pDel with no PKA defects, lower P-ERK, normal P-AKT, and higher

S6. In the studies I conducted above, I selected 1 patient pair from each group- (I-ASD-1 from group 1 and I-ASD-2 from group-2) and tested agonists and antagonists for these dysregulated pathways on these cells which were representatives of the two groups. Ultimately, I found that while manipulating PKA and P-ERK did change neurites and migration, these changes were often not specific to ASD patients nor did they necessarily match the dysregulation seen in the patient. For example, in Family-1 ASD NPCs, P-ERK levels were found to be increased while in Family-2 P-ERK levels were decreased. Yet, FGF which is known to increase P-ERK levels increased neurites in both patients, suggesting FGF or ERK may not be specifically regulating the abnormalities seen in our patients. On the other hand, by appropriately manipulating the mTOR pathway, neurite, migration, and EF defects were ameliorated in our patients. Moreover, both too high and too low levels of mTOR seemed to contribute to similar neurobiological abnormalities. In Family-1, where ASD NPCs had lower mTOR activity, application of an agonist to ASD-NPCs rescued all defects. Likewise, in Family-1 Sib, antagonizing mTOR recapitulated all the ASD-1 defects. On the other hand, Family-2 ASD NPCs had higher mTOR pathway activity. Reducing mTOR activity with an antagonist rescued neurite and migration defects in the ASD NPCs. Likewise, increasing mTOR activity in Sib-2 led to neurite defects similar to that of ASD-2. Thus, in both individuals, the mTOR pathway was central to neurobiological defects seen. Moreover, mTOR levels need to be tightly regulated to a "middle ground" to have normal cellular function. Preliminary studies in 16pdel NPCs also shows that targeting mTOR can rescue neurites in this other sub-type of ASD.

Chapter 9-Experimental Results: Metabolome and Proteome

Metabolic Abnormalities in Family-1

Individuals with disorders of metabolism such as Lesch Nyhan Syndrome or phenylketonuria often exhibit neurodevelopmental or neuropsychiatric symptoms. This suggests that alterations in important metabolic pathways or disruptions in metabolism may contribute to brain abnormalities. In ASD, a multitude of studies have suggested disrupted metabolism. Often, however, these metabolic studies are conducted in blood or urine samples which do not necessarily reflect or represent the metabolic state of the brain. Thus, with the advent of iPSC technology we now have the ability to study and compare the metabolism of neural cells derived from individuals with disorders like ASD. Thus, we aimed to study the metabolism of our Sib and ASD NPCs using metabolomics approach. These studies were conducted only in Family-1 and multiple samples of I-ASD-1 and Sib-1 NPCs were sent to Metabolon for analyses.

The data from Metabolon showed that of 243 metabolites measured, 130 were altered in ASD NPCs suggesting wide-spread metabolic dysregulation. The largest differences were noted in lipid, amino acid, purine, and pyrimidine metabolism. However, the most striking differences were seen in the purine and pyrimidine metabolic pathways. In the purine metabolic pathway, adenine and guanine, which are nucleotide bases (nucleobases) that are in molecules such as ATP and GTP, were approximately 9 and 11-fold higher respectively in ASD cells (Table 2). On the other hand, the di and monophosphate molecules (AMP/GMP, ADP/GDP) containing these bases were 40-60% lower in the ASD NPCs. Likewise, in the pyrimidine pathway the base uridine is higher in ASD NPCs while the di and tri-phosphate (UDP, UTP) molecules were lower in ASD

(Table 2). This suggest two potential possibilities 1) the enzymes that make di and triphosphates from nucleotide bases were underactive causing a build-up of nucleobases and a dearth of di-and tri-phosphates or 2) the enzymes that degrade di-and-tri phosphate molecules to nucleobases could be over-active rapidly converting these molecules into an excess of nucleobases.

	Biochemical Name	Fold of Change	
		ASD/ SIBCTRL (cell # normalized)	ASD/ SIBCTRL (protein normalized)
Purine Metabolism, (Hypo)Xanthine/Inosine containing	inosine	0.76	0.96
	hypoxanthine	4.06	5.04
	xanthine	3.12	3.9
Purine Metabolism, Adenine containing	adenosine 5'-diphosphate (ADP)	0.53	0.66
	adenosine 3',5'-diphosphate	0.39	0.49
	adenosine	8.78	10.73
	adenine	8.88	10.9
Purine Metabolism, Guanine containing	guanosine 5'-triphosphate	0.28	0.39
	guanosine 5'- diphosphate (GDP)	0.55	0.69
	guanosine	4.84	6.47
	guanine	10.27	12.61
Pyrimidine Metabolism, Orotate containing	N-carbamoylaspartate	0.17	0.21
	dihydroorotate	0.21	0.23
	orotate	0.48	0.58
	orotidine	1.07	1.35
Pyrimidine Metabolism, Uracil containing	uridine 5'-triphosphate (UTP)	0.4	0.51
	uridine 5'-diphosphate (UDP)	0.5	0.63
	uridine	2.66	3.51
	uracil	4.18	4.67
	beta-alanine	1.2	1.44
Pyrimidine Metabolism, Cytidine containing	cytidine triphosphate	0.43	0.54
	cytidine diphosphate	0.56	0.7

Table 2: Purine and Pyrimidine metabolite levels in Sib-1 and ASD-1 NPCs.

Metabolon provides pathway maps which show metabolites and the enzymes that act to produce and degrade them. Using these maps, I searched for enzymes that either produced di-and-triphosphates or degraded them. Interestingly, there are 3 unique enzymes that are

involved in making each nucleobase (adenine, guanine, uridine) into their mono-phosphate and ultimately di-and tri-phosphate forms. However, it seemed unlikely that there were separate dysregulations in 3 separate enzymes that are responsibe for producing ATP, GTP, and UTP. Fascinatingly, for all three di/tri-phosphate molecules, degradation into the nucleobases had once central pathway with 2 enzymes in common- ribonucleotide reductase (RNR) and purine nucleotide phosphorylase (PNP) (Figure 96). Thus, I hypothesized that the nucleotide degradation pathway was overactive in ASD-1 NPCs.
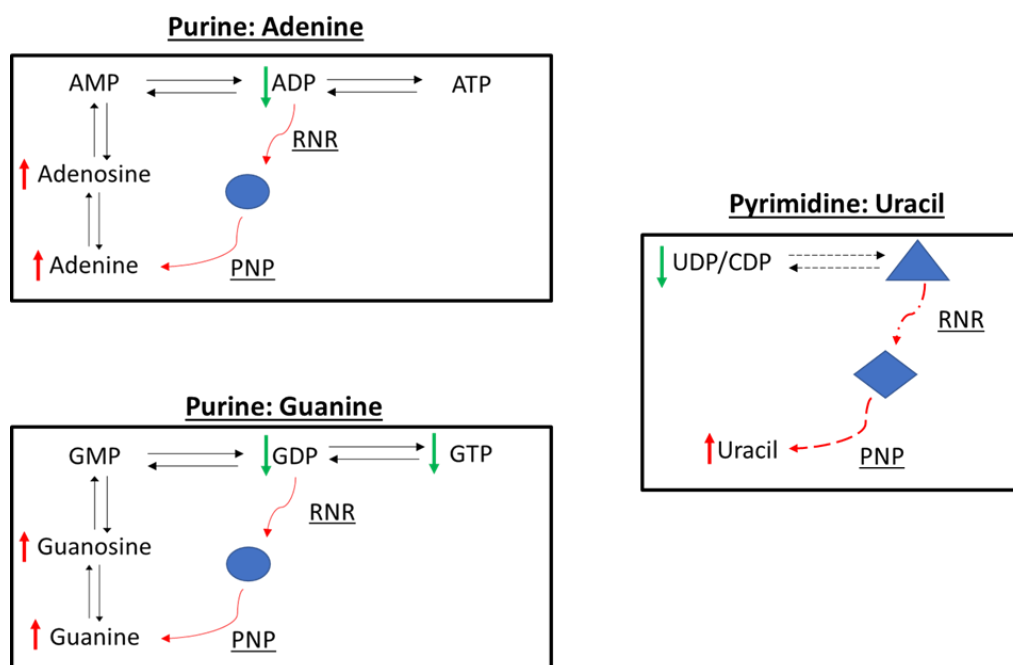
Figure 96: Schematic of metabolites and enzymes involved in the degradation pathways of purine and pyrimidine nucleotides.

As I postulated that both RNR and PNP were overactive, I aimed to find antagonists to these enzyme in order to determine whether neurite and migration behavior could be rescued. RNR is a well-established enzyme that produces deoxyribonucleotides from ribonucleotides for the synthesis of DNA. A very well-known antagonist to this enzyme, hydroxyurea, is often utilized for the treatment of sickle cell disease. Thus, I tested the effects of HU on neurite outgrowth and migration in both Sib-1 and ASD-1. Dose response

studies found that there was a dose-dependent decrease in neurites in Sib-1 (Figure 97). However, ASD-1 NPCs had an increase in neurites at the 10 and 100 uM doses. As HU blocks an enzyme essential for producing DNA, it is likely it would cause cell death. Analysis of cell numbers on the neurite dishes found that there were decreases in cell numbers at the 100 uM dose indicating it was not optimal for use. Thus, further studies were conducted at the 10 uM where neurite rescue was seen in ASD NPCs without any decrease in cell numbers. As shown in (Figure 98), neurites were increased in the ASD (50%, p<0.0001) at this dose while Sib had no change. Cell numbers remained unchanged in both groups. Migration was also increased by 60% (p<0.0001) I-ASD-1 with 10 uM HU. HU also slightly increases migration in Sib-1 NPCs (15% increase, p=0.075), though this increase was not significant (Figure 99).
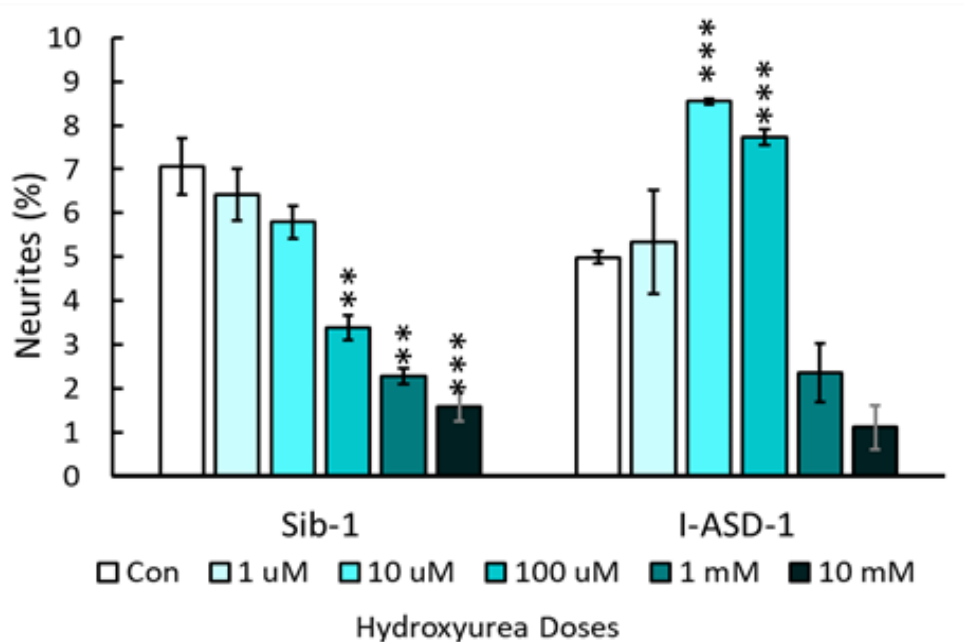
Figure 97: Dose response studies of HU in Sib-1 and ASD-1 NPCs. HU induces cell death and decreases neurites in both Sib and ASD starting at 1 mM. At 100 uM Sib NPCs had reduced neurites. At 1uM and 10 uM Sib NPCs showed no change in neuries. ASD NPCs had increased neurites at 10 uM and 100 uM doses.
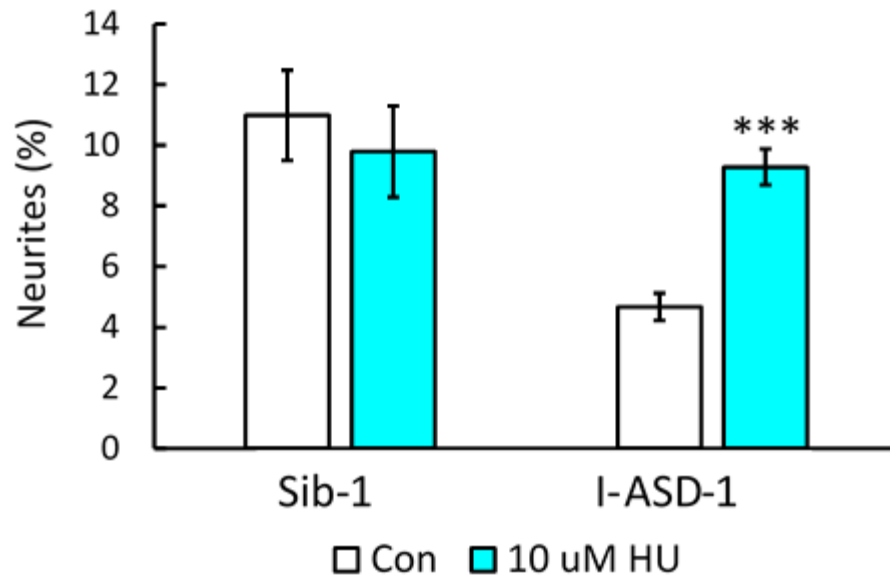
Figure 98: Effect of 10 uM HU on neurite outgrowth on Sib and ASD in Family-1. Sib NPCs have no change in neurite outgrowth under 10 uM HU while ASD NPCs have a 50% increase in neurites with 10 uM HU. Sib N = 3 expts, 3 clones, 2-3 dishes/condition/expt. ASD N =4 expts, 3 clones, 2-3 dishes/condition/expt
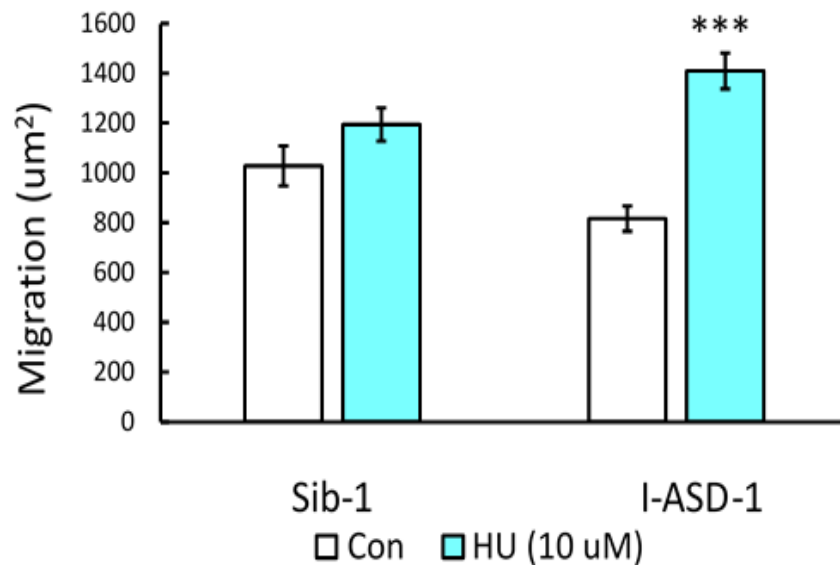
Figure 99: Effect of 10 uM HU on Migration in Sib and ASD in Family-1. Sib NPCs have a slight non-significant increase in in migration but ASD NPCs show a significant increase migration N= 2 experiments, 2 clones, 30 spheres/condition per patient.

While HU is a well-established well studied antagonist of RNR, it can be non-specific. Moreover, as I mentioned, RNR has an incredibly important role of making deoxy-ribonucleotides for DNA synthesis. Thus, altering RNR could alter DNA synthesis

and other deoxy-ribonucleotide dependent processes in the cell. Thus, to confirm that the neurite rescue was caused by blocking the purine degradation pathway I selected a drug against PNP. PNP, as mentioned, also works to degrade purines and pyrimidines but unlike RNR has no direct influence on DNA synthesis. Thus, I tested PNP antagonist forodesine on the neurite assay. I found that like with HU, antagonizing PNP with forodesine led to increases in neurite outgrowth in the ASD NPCs (Figure 100). Thus, my experiments suggest that blocking the enzymes that ultimately produce adenosine, guanosine, and uridine ameliorates the neurite and migration defects in ASD NPCs. However, metabolomic studies were not conducted to show that levels of these metabolites were decreased with HU and forodesine treatment. Moreover, neither rescue drug could rescue the proliferative defect found in the ASD cells in this family. This suggests that while correcting metabolism of purine and pyrimidine may ameliorate the defects in ASD NPCs, they are not contributing to all the defects like we see with the mTOR pathway.
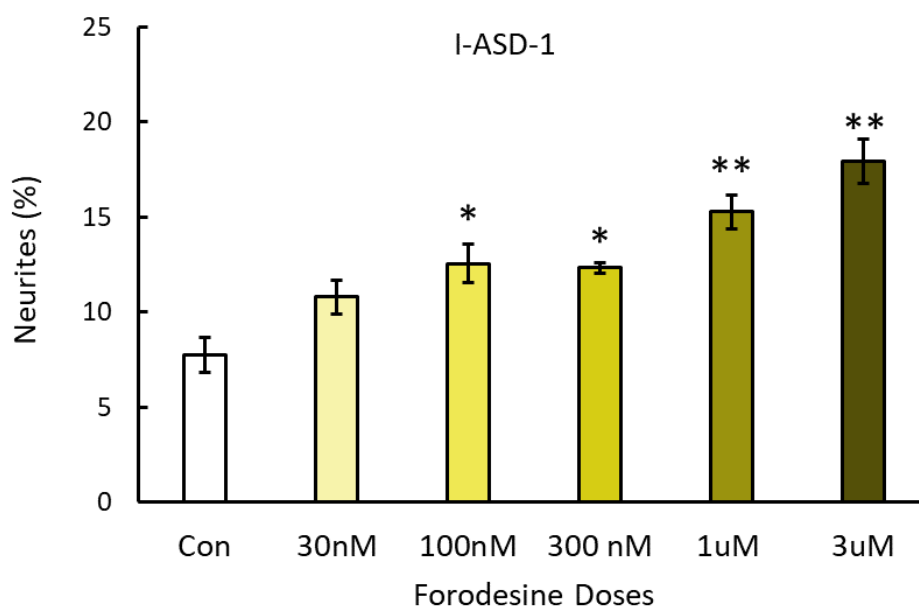
Figure 100: Dose response of PNP agonist forodesine in I-ASD-1 NPCs. I-ASD NPCs have a dose-dependent increase in neurites with forodesine.

Proteomics:

While select analysis of individual cellular signaling pathways was conducted and reviewed in Chapter 7 we decided to take an unbiased approach and review all of the known signaling pathways in Family-1, 2 & 3 and the two 16pDel males. Specifically, we conducted a phospho-proteome analyses to assess the relative levels of phosphorylated proteins in our Sib and ASD NPCs in each of these groups (Table 3). Sample collection is complete, and analysis is pending!

	Control	PACAP
Family-1 (1072)		
Sib- 2139	X	X
ASD- 2004	X	X
Family-2 (1077)		
Sib- 07c06	X	
ASD- 1861	X	
Family-3 (1012)		
Sib- 2166	X	
ASD 2187	X	
16p11.2 (Males)		
Individual 252-89852	X	
Individual 799-80991	X	

Table 3: Samples submitted for proteomic analyses.

Chapter 10: Discussion

Summary of Results:

Autism is a complex and heterogeneous neurodevelopmental disorder with uniquely human features. Due to its heterogeneity, our limited ability to directly study human brain development, and difficulty modeling the disorder in animals, uncovering the mechanisms that contribute to ASD have been challenging. Yet, decades of studies utilizing human post-mortem, imaging, and genetic techniques along with experimental data from mouse models have found that alterations in neurodevelopment and the regulation of neurodevelopment are key to the pathogenesis of ASDs. Despite this, an overwhelming number of ASD studies have focused on ASD as a disorder of "altered synapse function and formation". While there is no doubt that synapses are altered in ASD, there are a host of developmental processes that occur before synapse formation that are essential for the normal architecture and function of the brain. Not only that, the fidelity of these early processes are also necessary for normal synapse formation! Indeed, newer genetic studies have begun to suggest that altered neurodevelopment in ASDs occur even before the differentiation of NPCs into post-mitotic neurons [117]. Thus, studying NPCs and developmental processes that NPCs undergo are essential to understanding ASDs. However, until recently, the major way to study early developmental neurobiology and the behavior of NPCs has been in mouse models. In addition to these studies being sparse, mouse models cannot entirely recapitulate human neurodevelopment. Moreover, most mouse models of ASD are made by altering a single ASD associated gene- usually a rare variant gene or a gene associated with monogenic forms of ASD. These models therefore

are not representative of the 80% of cases of human ASD which are idiopathic. In addition, mouse models are unable to capture cases of ASD that are polygenic. With the advent of iPSC technology, we are now able to derive NPCs and neurons from individuals with genetically complex disorders like ASD. Using this new technology, studies have begun to uncover differences in human neurons of individuals with ASD. As the iPSC field is still in it's infancy, there are still technical hurdles to overcome and many facets of ASD biology that are yet unexplored. Many studies are characterizing the biology of post-mitotic neurons weeks after their generation and leaving earlier neurodevelopmental features unstudied. Moreover, studies regarding the factors that regulate neurodevelopment such as signaling pathways and EFs remain largely unexplored in the human iPSC field.

When I first began this project, hardly any studies were looking into the developmental neurobiology of NPCs. The few papers that studied NPCs took a small cursory look at proliferation using FACs and no further characterizations were done. Moreover, at the inception of my project, there were no publications of iPSC studies of idiopathic ASD. Thus, the goal of my project was to utilize iPSC technology to study the development of NPCs in 3 patients with idiopathic ASD compared to their unaffected Siblings. Specifically, I wanted to study migration and early neurite outgrowth in these NPCs as these facets of development have remained largely unexplored in both human and murine models, particularly in the case of ASD. Secondly, to understand how these processes were regulated, I aimed to understand how developmentally relevant EFs influenced neurite outgrowth and migration. However, to do so, I first needed to develop methods that could rapidly assess neurodevelopmental phenotypes in multiple clones and iterations of NPCs in control conditions and under the stimulation of relevant EFs. I

hypothesized that multiple patients in our ASD cohort would have defects in either one or both processes. As I had no genetic information to work off and no studies showing homogeneity of phenotypes in idiopathic disorders, I did not anticipate similar phenotypes in all our patients nor did I expect that every patient would show a neurite or migration defect. Likewise, as signaling aberrations are a major point of convergence for many ASD risk genes, I hypothesized that aberrations in signaling would be found in ASD NPCs and would contribute to any abnormal developmental phenotypes observed.

My studies initially began with a deep look into Family-1 NPCs, which were the focus of my analyses for more than a year and a half. Initially, the most striking difference noted between Sib and ASD NPCs was a lack of PACAP stimulated neurite outgrowth in ASD NPCs. As PACP signals through G-proteins, I wanted to see if alterations in the G-protein pathway were leading to this aberrant PACAP response. Thus, I tested another EF, 5-HT that works through G-protein coupled systems. Moreover, 5-HT dysregulation has commonly been seen in ASD making it an interesting molecule to study. Interestingly, ASD NPCs failed to respond to low doses of 5-HT but were responsive to higher doses, indicating an altered 5-HT sensitivity. The ASD NPCs also failed to respond to Oxytocin, another EF that signaled through G-protein coupled receptors. This lack of response suggested that perhaps, ASD NPCs had a defect in a protein or 2nd messenger molecule important for G-protein coupled signaling. However, it was unclear if ASD responses to other EFs, which did not utilize this receptor system, were altered. Thus, I tested NGF, which uses receptor tyrosine kinase system on our NPCs. Again, while Sib NPCs showed an increase in neurite outgrowth with NGF, ASD NPCs showed no changes in neurites. This showed that the EF response defects were not constrained to one particular receptor

or one receiver family. It is more likely that a common second messenger system or signal transduction system is altered leading to changes in responses to multiple EFs. ASD NPCs also showed unusual responses to FGF and BDNF. Counterintuitively, FGF, a known mitogen, increased neurite outgrowth in ASD NPCs. In some cell types, FGF has been shown to increase differentiation. However, in the ASD cells, both proliferation and neurite outgrowth were increased. The mechanism for both a differentiation and proliferation phenotype being increased is currently undefined. In Sib NPCs, as expected, FGF increases proliferation and decreases neurite outgrowth. With BDNF, ASD NPCs showed an increase in neurite outgrowth. Yet, Sib NPCs remained unresponsive to BDNF. The underlying reason for this differential neurotrophin profile also remains undefined

The fact that ASD NPCs were able to grow neurites under certain stimulation conditions showed that the systems required for neurite outgrowth were still functioning in these NPCs. However, as ASD NPCs had a differential response to almost every EF tested indicates altered regulation of cellular processes. As more clones and lines were tested, it became evident that ASD NPCs also had a lower percentage of neurites in control conditions when compared to Sib NPCs. Thus, overall, neurite outgrowth and EF responses are dysregulated in ASD NPCs. Of course, our neurite studies are conducted in conditions where NPCs are dissociated into single cells. This loss of cell-to-cell contact could alter developmental signals and is not entirely representative of the developing neural tube or brain where NPCs are tightly packed. Thus, it was important to study NPCs development in a high density model that preserved cell to cell contact. Once the neurosphere assay was optimized, I was able to conduct migration studies in Family-1 NPCs. Much like the results from the low density neurite cultures, the ASD NPCs grown in high density spheres showed

reduced migration and lack of response to PACAP. Thus, altered neurodevelopment and EF response were seen our cells in two culture model systems.

As a lack of PACAP response was seen our ASD NPCs, I hypothesized that the PKA pathway, through which PACAP signals, could be altered in ASD-1. Thus, I assessed levels of P-CREB in Sib-1 and ASD-1 under control and PACAP stimulated conditions. This revealed that ASD NPCs had a 3-fold lower P-CREB response to PACAP than Sib NPCs. This blunted response could contribute to the PACAP stimulated neurite outgrowth effect seen in ASD-1 NPCs. Therefore, I next treated my cells with db-cAMP, a cell permeable agonist of the PKA pathway known to increase P-CREB levels. Drug treatment increased neurites and migration in Sib and ASD NPCs. This suggests that P-CREB defects could be contributing to the neurodevelopmental aberrations in ASD, however, targeting this pathway via db-cAMP seems to increase neurites in the Sib too. Db-cAMP is a very powerful agonist of the PKA pathway and it floods the cells with an excess of cAMP-like 2nd messenger thereby revving up P-CREB levels. It is possible that a subtler agonist would allow us to tell if ASD NPCs could be specifically rescued by targeting this pathway. As a defect in PACAP response is what prompted my use of db-cAMP, I wanted to see if using a low-dose of the drug to "prime" cells would now allow ASD-1 NPCs to respond to PACAP and other EFs. This low dose of db-cAMP did not stimulate neurite outgrowth on its own. Interestingly, low dose of db-cAMP allowed for ASD cells to respond to both PACAP and 5-HT but not NGF. On the other, in Sib-1 NPCs, low db-cAMP did not change responses to any EFs. Interestingly, PACAP and 5-HT utilize G-protein coupled signaling which sometimes utilizes PKA (and cAMP) to signal. Thus, it seems that defects in cAMP levels or activity of this pathway are preventing ASD NPCs from responding to EFs that

utilize this pathway. While NGF has also been shown to activate CREB, it typically does not do so through cAMP/PKA signaling. This suggests that low P-CREB levels itself may not be contributing to the PACAP and 5-HT response. Rather, low P-CREB levels could reflect inadequate levels of cAMP which is essential for both PACAP and 5-HT but not for NGF signaling. Overall, targeting PKA reverses defects seen in I-ASD-1 but the agonist chosen also elicited responses in Sib. However, low doses of db-cAMP specifically rescue PACAP and 5-HT stimulated neurite outgrowth in just the ASD.

While experiments to elucidate the role of PKA signaling in Family-1 were being conducted, I began to probe the westerns I ran in Family-1 for mTOR and MAPK (ERK) pathway proteins. Quite a few studies looking at neurodevelopmental disorders have implicated the mTOR and ERK pathways in disease pathogenesis. Specifically, alterations in the members of the mTOR pathway are associated with several forms of monogenic ASDs. Analyses of the ERK pathway in Family-1 revealed an increase (50% higher) in P-ERK/T-ERK ratio in ASD-1 NPCs compared to Sib-1 NPCs. More strikingly, studies looking at P-AKT and P-S6 levels in Family-1 NPCs revealed 2 and 4-fold lower levels of these molecules respectively in ASD-1 NPCs. I hypothesized that these defects could be contributing to the neurite, migration, and EF response defects seen in the ASD-1 NPCs. In particular, studies have shown mTOR is important for regulating migration and neurites [500, 507, 523, 783-787]. Moreover, mTOR pathway members have also been shown to regulate receptor endocytosis and trafficking to the membrane [788, 789]. Without proper receptor distribution on the membrane cells cannot respond to EFs. Thus, it is possible that mTOR abnormalities are contributing to abnormal receptor distributions in our cells which could be preventing EF responses. Lastly, EFs like NGF are also known to signal through mTOR,

thus it seemed that alterations in this pathway could really be contributing to all the defects seen in our cell. Interestingly studies conducted by Dr. Williams in our lab showed that the signaling defects seen in these ASD NPCs were not present in the iPSCs! iPSCs from ASD NPCs had normal P-AKT, P-S6, and P-ERK, and P-CREB levels compared to Sib iPSCs. This suggests that defects seen in our cells occur upon differentiation to a neural lineage. Thus, neural development but not early blastocyst development is impaired in ASD. It would be interesting to see if differentiated neuronal lineages such as neurons, astrocytes, and oligodendrocytes or other non-neural cell types carry these signaling defects. This would help elucidate whether iPSC to NPCs transition or neural differentiation is specifically impacted or whether differentiation as a whole is dysregulated.

As ASD NPCs had lower mTOR pathway activity, I began to look for drugs that could increase mTOR levels in ASD NPCs. However, most drugs targeting the mTOR pathway are inhibitors used in the treatment of cancer which generally has elevated mTOR activity. One drug, SC-79, increases the level of P-AKT in cells by binding to AKT and opening up the phosphorylation sites such that there is an increase likelihood that PDK1 can phosphorylate the opened pocket [790]. SC-79 increased neurite outgrowth and migration in ASD such that they were equivalent to Sib levels. Unlike db-cAMP SC-79 had no effects on the Sib NPCs showing a targeted and specific correction of ASD defects. Of course, I wanted to test if treating cells with sub-threshold doses of SC-79 (similar to db-cAMP experiments) would allow ASD-1 NPCs to now respond to the EFs. Excitingly, treatment with low- and ineffective dose of SC-79 allows ASD-NPCs to respond to all 3 EFs: PACAP, NGF, 5-HT, indicating that mTOR pathway dysregulation is contributing to all defects seen in our ASD-NPCs. To further cement the role of mTOR in the

developmental pathology of our NPCs, I utilized an mTOR pathway antagonist MK-2206 to reduce mTOR signaling in the Sib-1 NPCs. MK-2206, is an AKT inhibitor which works in the same exact manner as SC-79, had the opposite effect. Application of this drug on Sib-1 NPCs led to reduction of neurite and migration to the level of ASD NPCs. Moreover, combining low doses of MK-2206 with EFs in these Sib NPCs abolished the normal EF responses to PACAP, NGF, and 5-HT! Thus, normal mTOR function is necessary for neurite outgrowth, migration, and responses to important developmental regulators!

Lastly, in addition to the in-depth studies conducted on signaling pathways, Family-1 NPCs were also sent out for metabolomic analyses. The metabolome reflects the interaction between an organism or a cell's genome, proteome, and environment. Thus, the metabolome is an excellent way to see how altered signaling and the culture environment differentially affect Sibling and ASD cells. Analysis of over 243 metabolites in these cells showed that there were 130 that were differentially expressed between Sib-1 and ASD-1 NPCs indicating that the genome/proteome environment interaction leads to widely disparate metabolites in ASD and Sib. When the metabolites were arranged by enzymatic pathways, we found the largest changes were seen in purine/pyrimidine metabolism, lipid metabolism, and amino acid metabolism. Proper metabolism of these macronutrients is essential for everything from the integrity of the cell membrane to normal DNA synthesis. The wide dysregulation in the ASD cells show us it is not merely one process or one small thing that is altered in our ASD NPCs. Rather, a cell wide dysregulation of numerous factors is present. This altered metabolomic profile can be due to changes in important enzymes that conduct metabolism or in the molecules that regulate these enzymes. In I-ASD-1 cells, levels of nucleobases and nucleosides such as adenosine, adenine, guanosine,

uridine were all elevated while di-and-tri nucleotides were lower. This suggested that either di-and-tri nucleotides were being synthesized slower or they were being degraded faster. Analysis of metabolism maps provided by Metabolon showed that there were 2 enzymes-ribonucleotide reductase (RNR) and purine nucleotide phosphorylase (PNP) which were involved in the degradation process of all purines and pyrimidines. On the other had there were a multitude of nucleobase specific enzymes involved in the synthesis of the di-and-tri nucleotides with no common points. It seemed unlikely that all of these enzymes were somehow dysregulated and thus, I postulated that dysfunction in RNR or PNP led to altered nucleotide metabolism leading to elevated nucleobases and reductions in tri and di nucleotides. As there was an excess of degradation products, I hypothesized that RNR and PNP were overactive in ASD cells. I postulated that this dearth of di-and-tri nucleotides along with buildup of their metabolites potentially impacted neurite and migration in ASD-1 NPCs. By antagonizing these enzymes, I showed that both neurites and migration could be restored in I-ASD NPCs! However, blocking RNR, which is an important enzyme for making dNTPs for DNA synthesis, did decrease cell numbers and proliferation in our ASD NPCs. I-ASD-1 NPCs already had lower proliferation, thus RNR was not rescuing this defect in the NPCs. However, by altering enzymes that degrade these nucleotides, neurites and migration were rescued in cells. Of course, I did not conduct studies to see whether targeting RNR or PNP reversed the metabolic abnormalities seen in our cells. Thus, without further experiments, it is unclear whether a reversal of metabolic abnormalities is really leading to the phenotypic rescue seen in our cells.

After this in-depth analysis of Family-1, I decided to characterize NPCs from 2 more Sibling pairs. Armed with the knowledge gained from Family-1, I narrowed my

studies to assessing neurite outgrowth, migration, and neurite responses to PACAP, 5-HT, and NGF. I also focused my western studies to analysis of PKA, mTOR, and ERK. Beginning with Family-2, my analyses showed that ASD-2 NPCs also had reduced neurites, reduced migration, and no response to EFs, much like ASD-1 NPCs! Based on these results, I anticipated that the signaling defects seen in ASD-1 NPCs would also be found in ASD-2 NPCs. Surprisingly, however, despite common neurobiological alterations, the signaling "profile" of ASD-2 NPCs was different. Despite lack of response to PACAP, Family-2 ASD NPCs had no difference in P-CREB stimulation under PACAP, suggesting an alternate mechanism for the lack of PACAP response in this individual. Moreover, P-AKT levels were not different between the Sib and ASD in this family. However, ASD-2 NPCs had almost a 4-fold higher level of P-S6 than Sib-2 NPCs. Thus, unlike ASD-1 NPCs, ASD-2 NPCs had higher mTOR pathway activity. This suggests that over- or underactivity of the mTOR pathway leads to common phenotypes. In the 3rd family, once again, ASD NPCs had reduced neurite outgrowth, reduced migration, and lack of response to EFs! In terms of signaling, preliminary studies suggest that ASD-3 NPCs, much like ASD-1 NPCs, have blunted PACAP stimulated P-CREB levels, decreased P-AKT and decreased P-S6 levels (though the magnitude of difference does vary from Family-1). Thus 2 out of 3 patients in our idiopathic cohort had identical neurobiological and signaling phenotypes while the other individual had similar neurobiological phenotypes but different signaling profile. Interestingly, Family-1 and 3 ASD NPCs proliferated slower than their sibling counterparts, while the ASD NPCs from Family-2, with the higher P-S6 levels, had faster proliferation rates than Sib-2. Thus, signaling profile, particularly mTOR activity, may correlate with proliferation. This is not

particularly surprising, as studies in cancer have consistently shown that higher mTOR pathway activity is associated with increased proliferation.

As Family-2 ASD NPCs had higher mTOR activity, I wanted to see if the drugs related to those used with Family-1 (except reversed) would help ameliorate the neurite defects seen in Family-2. First, I tested multiple doses of MK-2206 on both the Sib-2 and ASD-2 in this family. As discussed in Sib-1, MK-2206 inhibited AKT levels and thereby reduced neurite outgrowth. In ASD-2, where mTOR was hyperactive, MK-2206 reduces both P-AKT and P-S6 levels. This reduction in P-S6 is accompanied by an increase in neurite outgrowth and migration. While Sib-1 NPCs began to show reductions in neurite at 10 nM MK-2206, Sib-2 only showed a decrease at the 100 nM dose, suggesting there are differential sensitivities to MK-2206 amongst "typical individuals". However, in both Sibs we see that reduced mTOR levels do lead to neurite impairments. Moreover, in the ASD-2, targeting overactive mTOR also fixes the reduced neurites and migration, suggesting that a fine balance of this pathway is necessary for normal development. Likewise, SC-79, the AKT activator, had no effects on neurite outgrowth in ASD-2 but at high doses it inhibited neurite outgrowth in Sib-2. Thus, even in Family-2 we see that manipulation of the mTOR pathway can ameliorate or mimic developmental defects. Due to limitations in time, I was not able to test if sub-threshold doses of MK-2206 could rescue EF defects in I-ASD-2. However, it would be interesting to see if both lower and higher mTOR were contributing to EF response defects.

Remarkably despite the heterogeneity of ASD, 3 randomly selected individuals from our cohort had common neurobiological defects. Of course, these patients do share some common traits which could account for why such similar phenotypes were observed.

For one, all 3 patients with ASD were selected from families where there is one first-degree relative with a language disorder known as specific language impairment (SLI). It is possible that families where both ASD and SLI are present have some common genetic or etiological factors thereby reducing heterogeneity of our sample. Indeed, this use of "endophenotypes" to narrow heterogeneity has been adopted by other groups who generally use macrocephaly to narrow heterogeneity in I-ASD studies. Secondly, all of the patients studied are severely affected and often non-verbal as indicated by ADOS modules of 1 or 2 in all of our patients. The similar severity of the disorders also further reduces heterogeneity in our cohort which could lead to these similar outcomes. All individuals from this study are from the same geographic area (NJ) and speak English as their primary language at home, which could potentially limit ethnic variability in the cohort. Thus, while it is incredibly exciting that 3 randomly selected patients from our cohort all exhibited similar phenotypes, this does not necessarily mean such differences will be found in other cohorts of individuals with idiopathic ASD or other subtypes of ASD. Review of the literature, however, shows the changes in neurite outgrowth (at least in post-mitotic neurons seems to be a common feature in ASD. An unpublished study by Dykxhoorn et al with 7 patients who have idiopathic ASD also found neurite defects. Defects in migration have largely remained unexplored. Thus, while not all every patient with ASD will have neurite defects, this aberration seems to occur in many individuals with ASD. It would be interesting to screen other cohorts of individuals with I-ASD or monogenic ASDs at the NPCs stage to see if neurite defects are present in this stage in other groups of individuals with ASD too.

Shortly after I began characterizing Family-2 NPCs, we received fibroblasts and

lymphocytes from 3 patients with 16p11.2 deletion and ASD from the Simon's Foundation VIP cohort. This put our lab in the unique position of being able to study early neurodevelopmental phenotypes in 2 different sets of individuals with ASD using our established methods. The 16p11.2 deletion accounts for about 1% of all cases of ASD, making it one of the largest genetic contributors to ASD. Around 30% of individuals with this deletion have ASD. The 16p11.2 area include 27-29 genes one of which codes for ERK1, an important member of the MAPK pathway. Of course, as reviewed in the introduction, the ERK genes are important regulators of neurodevelopment. Interestingly, humans with this deletion have higher incidence of macrocephaly suggesting alterations in development. Indeed, in our cohort, M-1 has macrocephaly (99th percentile), the female has above average head size (80th percentile) and the M-2 has a slightly larger than average head size (70th percentile). However, the mouse model of 16p11.2 del has microcephaly indicating species specific differences in either CNV/ERK1 effects or neurodevelopment in mice vs humans. Thus, modeling this deletion in human neural cells was of particular interest especially since no such data had been published at the time. However, at this point, the Gage lab at the Salk Institute and the Vaccarino lab had published data on multiple individuals with idiopathic ASD and concurrent macrocephaly. The proliferation of NPCs in both studies was increased, thus, we hypothesized that these NPCs would have a proliferation defect. However, in terms of neurite outgrowth and migration, the literature was unclear. Pucilowska et al who studied the mouse model of 16p11.2 deletion suggested that 16p cells had an accelerated/early differentiation [608]. Thus, it was possible that neurites were increased in the 16p patients or would have an accelerated increase with time in culture or an increase by passage. However, with differential brain sizes in the mouse

(reduced) and human (increased), it was unclear how translatable the mouse model defects would be to human NPCs. In zebrafish, deletion of orthologous genes to those found in the 16p11.2 locus in humans led to macrocephaly and increased proliferation of neural progenitors. Interestingly, in the zebrafish model, this phenotype was recapitulated by deletion of a single gene in the 16p11.2 locus, KCTD13, though another analysis failed to replicate this finding[791, 792]. KCTD13 is known to interact with PCNA, a cell cycle regulatory molecule[792]. In addition to roles in the cell cycle, studies have found the KCTD13 interacts with the RhoA family, whose members are important regulators of cytoskeleton dynamics [791]. Moreover, in the cortex of mice, KCTD13 was also found to be important for neuronal positioning and dendrite maturation. Thus, in humans with the 16p11.2 deletion, loss of KCTD13 could produce neurite and migration phenotypes.

Initial studies in the 16p NPCs derived from both males and the female showed that unlike the idiopathic ASD patients, 16p patients had typical increases in neurite outgrowth in response to EFs. In fact, dose response studies conducted with PACAP, NGF, 5-HT, FGF, and OXT showed that 16p patients responded with increased neurites at all the doses where Sib NPCs were responsive. Initially, we planned on comparing 16pdel NPCs to the Sibs in Family-2 and Family-3 (which have lower neurites than Family-1 Sib). Initial studies did not show significant differences in neurites between age-matched Sibs (Family-2 and 3) and the 16pDel patients. Thus, it seemed like in terms of EF response and neurite outgrowth, 16pDel patients looked "typical". At this time, concerns were raised that genetic load of the Sibs (who come from a family with both SLI and ASD) increased the risks of these Sibs being "less than typical". For example, it was possible that the Sibs themselves had a "sub-clinical" version of ASD or SLI that could influence their neurobiology.

Specifically, when comparing to unrelated 16pDel individuals this point became more salient. Thus, we acquired iPSCs from the NIH/NIMH Regenerative Medicine Tissue Bank. These iPSCs were derived from cord blood taken from newborns who were identified as having no known genetic diseases, but their clinical developmental status is unknown. Studies on 2 NIMH patients revealed that their neurite outgrowth was around 13%, a level that is fairly similar to the 11% seen in the average of all 3 Sibs. With further studies, it became clear that M-1 and F from the 16pDel cohort had lower neurites than the average of all 3 Sibs and the average of 2 NIMH controls. M-2 however had great variability between clones and inductions in neurite values. Based on studies from 2 clones, the 16pDel M-2 has around 8% neurites which is a little less than the unaffected individuals, but this data is not significant. Studies will need be conducted in a 3rd clone to better understand the neurite behavior in this individual. The 16pDel M-2 and F also had passage-based increases in neurites. That is, with each passage, there was about a 2-5% increase in the percentage of neurites. Thus, there were significant differences in results obtained between P3-5 and P6-P8. This increase was not observed in M-1, however, for the 16p, data for neurites was restricted between P3-P5. These discrepancies will be further discussed in a later section. Unlike neurite studies, migration studies in the 16pDel cohort were much more consistent and clear-cut. The average migration in all Sibs compared to the average migration in the NIMH controls were similar (1000 vs 1200) while all 3 16p patients had migration values less than 800! Thus, all 3 16p patients regardless of passage or clone showed reduced migration compared to Sibs and NIMH controls. Unlike I-ASD NPCs, however, 16pDel NPCs did have increased migration under PACAP stimulation, indicating subtle differences in neurobiology. Thus, it seemed that much like I-ASD NPCs,

16pDel NPCs also had reduced migration and neurite outgrowth. However, the 16pDel NPCs had no issue with EF responses. Thus, it was likely that the underlying mechanisms contributing to the defects seen in the 16p and I-ASD were different. Moreover, with our study design, we are able to subtype and uncover different "forms" of ASD.

Of course, as the 16pDel patients were missing 1 copy of the ERK gene, I expected this pathway would be altered in our patients. However, it was unclear whether ERK would be "under" or overactive (as determined by P-ERK/T-ERK ratio). In the mouse model of 16p11.2 deletion, compared to total-ERK levels, there was a relative increase in P-ERK compared to WT, indicating relative "overactivity" of ERK signaling. As expected, western studies in our NPCs showed Total ERK levels reduced by an average of 50% in the 16p patients (between 30-70% for each patient). P-ERK levels, on their own, were reduced by approximately 40%. However, when normalized to T-ERK (which is reduced), the P-ERK/T-ERK ratio was similar to Sibs/NIMH. There were also no differences in the PACAP stimulated P-CREB levels in 16p, which was expected as these cells were responsive to PACAP. Due to responsiveness to EFs, it was unclear whether mTOR defects would be observed in the 16pDel NPCs. While P-AKT levels were unchanged in 16pdDel NPCs, all patients with the 16p11.2 deletion showed higher levels of P-S6 indicating overactive mTOR! M-1 had a 90-140% increase in P-S6, while M-2 had a 60-90% increase while the female had the smallest difference ranging from a 30-70% increase. Moreover, this phenotype, (lower P-ERK, normal PKA, normal AKT, and increased P-S6) were similar to the I-ASD-2 patient from our idiopathic cohort. Interestingly, both the I-ASD-2 and the 16pDel NPCs had higher levels of proliferation. The relative increase in P-S6 also seemed to mirror the proliferative defect. That is, the higher the percent increase

of P-S6, the higher the proliferation. As such 16pDel M-1 and I-ASD NPCs had the fastest proliferation rates followed by the M-2, F. Of the 2 I-ASD patients with lower P-S6, I-ASD-1 proliferated the slowest (and had the largest decrease in P-S6 levels)! Thus, there seems to be an interesting correlation between mTOR and proliferation. Yet, higher and lower mTOR are correlated with reduced migration and reduced neurites!

At the inception of my studies, there were no publications of iPSC studies investigating idiopathic disorders due to concerns that no common phenotypes would emerge. Moreover, even now, very few iPSC papers have conducted the extensive in-depth mechanistic analyses that I have performed. By exploring cellular biology, metabolism, and signaling pathways whilst also manipulating these pathways, I have uncovered both common and interesting patient-specific defects in our cohorts. Overall, my studies analyzed iPSC-derived NPCs from a total of 11 individuals. 5 of these individuals were unaffected and 6 had ASD. Of the unaffected, 3 individuals were Siblings of 3 individuals with idiopathic ASD and 2 individuals were unrelated unaffected controls. Three patients from the ASD group had idiopathic ASD while the other 3 had a known genetic CNV disorder, 16p11.2 deletion. Surprisingly, all the ASD patients showed reduced neurite outgrowth and migration when compared to both groups of unaffected individuals. This suggests that dysregulations in early developmental processes are indeed present in ASD. However, the two "groups" of ASD could be distinguished by their response profiles to EF such as PACAP, 5-HT, and NGF suggesting different underlying mechanisms for neurobiological defects. Moreover, this shows that carefully designed studies and EFs could be used to "subtype" ASD which may help with molecular and clinical categorization of the disorder. Western blot studies revealed that I-ASD-1 and I-ASD-3 had very similar

signaling profiles: Low P-AKT, Low P-S6, and blunted PACAP stimulated P-CREB levels. On the other hand, I-ASD-2 and the 3 16pDel patients had similar but not identical signaling defects. Both I-ASD-2 and the three 16pdel patients had normal PACAP stimulated P-CREB levels, normal P-AKT levels, but higher P-S6 levels when compared to their respective controls. These similarities between I-ASD-2 and 16pdel NPCs may explain the common hyperproliferative phenotypes observed in these cells. However, while P-ERK levels alone were similar in 16pdel and I-ASD-2, when normalized to the T-ERK levels, the 16pDel patients had similar or slightly higher P-ERK/T-ERK ratios compared to the unaffected control individuals while I-ASD-2 had a lower P-ERK/T-ERK level. This indicates that despite missing 1 copy of the ERK1 gene, the "activity" of the ERK pathway is normal in 16pdel NPCs but reduced in I-ASD-2 NPCs. This subtle difference in the ERK pathways between the 16pdel NPCs and I-ASD-2 could explain why 16pdel NPCs respond to EFs while I-ASD-2 does not, despite similar mTOR and PKA profiles. Ultimately, despite the heterogeneity of ASD, we see that among 6 patients in our cohort there are commonalities in many phenotypes and 2 main "signaling" profiles that underlie these phenotypes. One interesting note is that 16p11.2 deletion patients often manifest language defects. In cases of this deletion, even without the presence of ASD, language disturbances are seen. Thus, it is possible that our 16pDel patients have commonalities with our I-ASD cohort because they both have genetic backgrounds that are associated with language and communication issues. Thus, it is conceivable that in individuals with ASD and language impairments, neurite, migration, and mTOR/ERK defects are common points of convergence. If this is indeed the case, this sub-group of individuals with ASD could be screened for mTOR/ERK defects and then given therapeutic treatment based on these

defects. However, to further understand how pervasive the phenotypes I uncovered are, studying another sub-group or another cohort of ASD using our studies would be highly useful and informative! Moreover, we have access to the SLI patient from our idiopathic cohort who can be studied to see what phenotypes are unique to ASD vs language impairments. Ultimately, with molecular studies, we can further sub-group ASD patients. This "profiling" could ultimately help develop therapeutics that are targeted to molecular defect rather than broad behavioral phenotypes!

Implications of iPSC studies: Does the dish reflect the Patient?

Since their introduction, iPSCs have been heralded for their ability to revolutionize biomedical research and medical therapies. Initially, iPSCs were promoted as a method of treating degenerative disorders as "stem cell therapy". Later, however, researchers realized that iPSCs provided the unique opportunity to model complex diseases in cells that were difficult to culture or access in the past. iPSCs also seemed to provide an excellent model to test pre-clinical drugs. In fact, when faced with the increased failure rate of drugs piloted and tested in mice, a human system for drug testing is really a boon. Lastly, as iPSCs retained the genetic signature and characteristics of the patient from who they're derived, iPSC technology opened the door for precision medicine and personalized medical approaches in fields such as neuropsychiatry where drug discovery has largely stalled since the 70s. Thus, iPSCs seem to represent the pinnacle of bench to bedside research. Now, more than a decade since their initial introduction, we can acknowledge that iPSCs have indeed changed the way we study disease, but has it changed medicine and patient outcomes as promised? Some of the enthusiasm around iPSCs have been dampened due to questions and concerns about the cells and their derivatives. For one, iPSCs are not

identical to embryonic stem cells and thus may not be as pluripotent as originally believed. This brings into question the cells derived from iPSCs and their similarity to tissue in human beings. The field is also grappling with the enormous variability that exists between iPSCs and differentiated cells derived from these iPSCs. Moreover, as reviewed, iPSC derived post-mitotic cells like neurons seem to be more similar to fetal cells than adult cells. Thus, their ability to reflect the current status or the progression of degenerative diseases is questionable. Lastly, safety concerns and potentially tumorigenicity have stalled studies of iPSC derived cells being used in clinical trials. Moreover, researchers are still grappling with what the results of these iPSC studies mean for the patients from whom they are derived. Do the aberrations in neurons seen in a dish have any bearing on the phenotypes seen in a living, breathing patient? Will drugs that rescue defects in a simplified culture system do anything for the individual with severe degenerative disorders like ALS? These questions remained largely unanswered and further studies and careful correlation of iPSC data with patient data will need to be done in the future.

My results show that all the patients that I have studied have defects in neurite outgrowth and in cell migration. In mouse models, migration defects have often led to altered cortical lamination or the presence of ectopic neuronal cells or dysplastic regions in brain. Such defects have also been observed in post-mortem analyses of humans with ASD. Thus, it is possible that these migration problems could indicate such aberrations are present in our patients too! Likewise, reductions in immature axons and dendrites (neurites) can change numerous parameters in the brain including axon tracts or white matter volume, brain volume, dendritic spine density, and brain connectivity, all of which have been shown to be altered in ASDs. Of course, these are just postulations of potential abnormalities in

the brains of our patients based on data seen in mouse and post-mortem studies. Some of these defects can only be confirmed by post-mortem analysis. Others can be studied by MRI and it would be valuable to conduct these studies on our patients to see if what is seen in the dish has any bearing on the patient phenotype. Interestingly, Marchetto et al 2016 studied 8 patients with various degrees of macrocephaly and found that NPCs derived from every affected individual proliferated faster than control NPCs [757]. Fascinatingly, the severity of macrocephaly, as assessed by brain MRI, was correlated to the NPC proliferation rate (R =0.4)! This suggests that the proliferative increases seen in the neural cells derived from these individuals could be contributing to the large brains seen in these individuals. On the other hand, in the study of ASD individuals with macrocephaly conducted by Mariani et al, organoids derived from these patients only showed a transient increase in size at 11 days [756]. However, by 31 days, ASD and control derived organoids had similar size suggesting that the correlation between iPSC models and patients may not hold at all developmental time points. Interestingly, for our patients, head circumference data, which is a proxy for brain size, is available for 5 out of 6 of our patients. Proliferation data acquired by Drs. Madeline Williams and Robert Connacher in our lab showed that I-ASD-2 and the 2 male 16pdel patients proliferated faster than Sib NPCs. On the other hand, I-ASD-1 and I-ASD-3 NPCs had either slower proliferation or proliferation rates similar to Sib. Interestingly, the fastest NPCs were derived from I-ASD-2 and 16pdel M-1 who also had the largest head circumferences in our cohort at the 97th and 99th percentile respectively (controlled for age). 16pDel M-2 proliferation was slower than M-1 and I-ASD-2 but faster than the average Sib and 16pdel F, ASD-1 and ASD-3 showed a larger than average head size (71st percentile) at 18 months of age but by age 15 however, this

individual did exhibit a head circumference in the 99th percentile. The 16p11.2del female also had a head size that was larger than average, in the 80th percentile (which stayed at this percentile from age 7-8) did not show increased NPCs proliferation relative to controls but were faster than the I-ASD-1 and I-ASD-3 NPCs. Interestingly, our slowest NPCs were derived from Family-1 and surprisingly this patient had the smallest relative head size (in the 20th percentile) which is below average! Thus, overall, much like the Marchetto study, there seemed to be correlation between brain size and proliferation. However, proliferation is not the only developmental process that contributes to the size of the brain (synapse formation and cell death are involved too!). Moreover, we do not have head size data from the Siblings or unaffected family members of these ASD individuals. Studies have found that macrocephalic phenotypes can be found unaffected relatives of patients with ASD and macrocephaly. If the unaffected, related individuals have larger heads but not greater proliferation phenotype, it would mean the correlation between NPC proliferation and head size are not correlated. However, it would mean that the interaction between ASD and macrocephaly may be reflected by NPCs proliferation. While proliferation data can be correlated with head size, for the results of my studies, this can only be verified by MRI as there is no real "proxy" for connectivity or cortical lamination.

Interestingly, there may also be a correlation between ASD severity and neurite defects in our cohort. Five out of 6 of our ASD patients showed statistically significant reductions in neurite outgrowth when compared to typical controls. The 16p11.2 del M-2 NPCs had lower neurites, but this was not statistically significant. Interestingly, while all the I-ASD patients had severe ASD and the other two 16p11.2 patients have "autism", M-2 has Asperger's and a high IQ which could indicate why this patient is the only one

without significant neurite reductions. Moreover, this patient's NPCs did show large increases in neurites with passage. More patients would need to be studied to determine whether high IQ contributes to different neurite outgrowth or whether low ASD severity translates into milder phenotypes! No current iPSC studies have really compared severe vs mild autism in such a manner. However, MRI studies have sometimes investigated brain differences between individuals who by the DSM-IV criteria had either "autistic disorder" (more severe) or Asperger's (mild). A meta-analysis of these MRI studies conducted in 2011 by Yu et al reported that grey matter alterations were less commonly reported in individuals with Asperger's compared to individuals with autistic disorder [793, 794]. Likewise, a study conducted by Lotspeich et al 2004 compared low functioning autistic individuals with high functioning autistic individuals using MRI [795]. Cerebellar gray matter volume was enlarged in both low-functioning and high functioning individuals. However, increases in cerebral gray matter were milder in individuals with high functioning autism when compared to individuals with low functioning autism. This suggests that "milder" forms of autism may have "milder" neurobiological alterations.

Of course, the correlations seen between our NPCs and our patients could be a lucky accident. It is important to remember that the NPCs we are studying are most likely mirroring cells that were present in the individual during fetal development. Moreover, our NPCs do not recapitulate the 3-D nature or complex interactions between cells that are occurring in the actual developing brain. Thus, what we are seeing in the dish may not necessarily have any bearing on what is currently happening in the patient's brain. As I will discuss in detail below, alterations in proliferation, migration, and neurite outgrowth could indicate a defect in the cytoskeleton of these NPCs. This cytoskeletal defect could

then translate to abnormalities in synapse formation and even electrophysiological functioning of neurons. Then, these aberrations could translate into the behavioral impairments seen in ASD. Thus, it is possible that the aberrations seen in the dish are reflective of a more global dysregulation in cell function. Thus, migration defects in the dish may not necessarily correlate with heterotopias but could reflect a cytoskeletal change that manifests itself as synaptic alterations in a patient.

In addition to the common cellular phenotypes, our patients also exhibited dysregulations in signaling pathways. These molecular dysregulations provide us with potential targets for therapeutics and represent an opportunity for precision medicine in ASD. In cancer, screening tumors for a genetic, signaling, or receptor dysregulation has allowed for targeted biologic or drug therapeutics to be developed for these aberrations. Not only has this precision medicine approach improved survival outcomes but it allowed for the development of new treatment for cancers that did not have good prognoses. Moreover, dividing patients by sub-groups has also increased the likelihood that a drug, which would only impact people with a particular defect, passes clinical trials. My studies have shown that there are two distinct "signaling profiles" in our patients. If similar molecular profiles are seen in other autism patients, iPSC-derived NPCs from this group of patients could be used to test drugs. Indeed, I have already tested mTOR pathway agonists and antagonists NPCs which rescued all phenotypes observed. Thus, it is possible that the drugs that successfully led to rescues in my cells could be beneficial to the patients we are studying. In fact, some of these drugs such as MK-2206 are already in Phase II clinical trials for cancer patients [796]. If these studies are successful, it makes it easier for the use of this drug in individuals with ASD. Indeed, even though mTOR pathway dysregulations are seen very

commonly in ASD, there aren't any drugs indicated for the treatment of ASD that target mTOR. Yet, unlike cancer cells, which can be directly derived from patient tumors, iPSC-derived NPCs are not developmentally identical to the postnatal brain cells found in our patients. Furthermore, it is unclear whether mTOR defects seen in the dish are present currently in the patient. Of course, it is also uncertain whether a drug that ameliorates neurodevelopmental phenotypes in culture would have any impact on the already-developed brain. However, if mTOR defects are still present in the brains of our patients, it is likely this defect is contributing to neuronal or glial pathology. Thus, while targeting mTOR may not "re-write" the aberrant developmental program that occurred in these individuals, it is possible that mTOR drugs could still aid in ameliorating symptoms in these individuals. In the iPSC field, numerous groups have shown that drugs such as calcium channel inhibitors, IGF-1, and lithium, and anti-psychotics can "rescue" defects seen in iPSC-derived cells. However, not all these studies have taken these drugs to clinical trials to see if the patients whose cells show responses benefit from the drug. One interesting study in bipolar disorder (BPD) found that neuronal hyperexcitability was seen in neurons derived from all 6 patients with BPD [797]. Clinically, 3 of these patients were responsive to lithium while the others were not. Surprisingly, the hyperactivity observed in iPSC-derived neurons was only ameliorated by lithium if the patient was responsive to the drug! This suggests that in-culture responses to drugs may parallel patient drug responsiveness. Thus, iPSC could be utilized to uncover out what drugs may be useful for treating what disorders and could even be used to tailor therapeutics to an individual in a personalized medicine approach. In the coming years, numerous drugs that have been tested on iPSCs will likely move into clinical trials. These studies will help determine

whether treatments discovered by studying cells in a dish can really impact the patient!

Protocols for the Generation and Study of NPCs: Is this optimal?

Historically, embryoid bodies (EB), which are floating 3D clustered of iPSCs, were used for the generation of NPCs from ESCs. The EB method requires weeks of time, manual manipulation and selection of EBs, and selection and dissociation of rosettes which is laborious, time consuming, and requires technical expertise. However, in 2009, Chambers et al published a method by which EBs could be bypassed and NPCs could be induced from a 2D monolayer of ESCs[726]. This monolayer method was faster and significantly less laborious as it did not require manual selection of EBs nor the formation and selection of rosettes. The original EB method had about a 95% success rate in generation of NPCs while the original monolayer method had a greater than 80% success rate of generating NPCs. These techniques pioneered on ESCs were later adopted by iPSC biologists. Our lab currently uses a commercially available monolayer induction kit from GIBCO ThermoFisher to generate NPCs. The GIBCO kit, like the Chambers et al 2009 method, likely uses dual-SMAD inhibitors for the induction of NPCs. However, the kit is proprietary, and we do not know the contents of the supplement used to generate the NPCs. In our hands, the GIBCO kit only generates high quality NPCs that exhibit robust PAX-6 staining in about half our inductions. The other half the time, cells acquired from the induction process do not retain neural morphology or have less than 10% PAX6+ cells! Interestingly, a study by Yan et al 2013 tested the GIBCO method of neural induction and were able to successfully induce SOX1, SOX2, NESTIN + NPCs that could give rise to multiple different subtypes of neurons. However, the percentage of PAX-6 expressing cells were only around 50% indicating that perhaps this kit had a lower efficiency at producing

forebrain NPCs or were producing cells earlier or later in the developmental trajectory such that PAX-6 expression has not yet begun or has already faded. Testing cells for TBR2 staining could indicate whether the cells have "moved on" from their radial glial lineage into a more mature "IPC" lineage which is characterized by expression of TBR2. Staining for mid and hindbrain NPC markers could also help ascertain whether NPCs generated are from a more caudal lineage. Yet, in some cases, in addition to the low PAX-6 expression, the cells generated from induction had very heterogeneous morphology- with very few being the typical neural stem cell fate morphology. Up to 4 different types of cells (based on morphology) have been observed in our NPC cultures. The reasons these alternative cells forming in our culture or the low success rate for NPCs generation is unclear as our collaborating lab (who use the same iPSCs) has a much a higher success rate. However, other labs such as the D'Arcangelo and Tischfield lab at Rutgers have encountered difficulties with the GIBCO Neural Induction kit. It is likely that small changes in lab microenvironment could be contributing to these drastic differences in induction efficiency between labs. Indeed, in iPSCs, lab microenvironment has been shown to influence gene expression and differentiation potential. A study by Newman et al 2010 found that around 20% of genes were differentially expressed between in iPSC derived from the same exact fibroblasts but cultured in different lab. The fibroblasts however, despite being cultured in different labs showed identical transcriptome profiles. This suggests that there is some unidentified feature in lab microenvironments that can impart distinct genetic signatures on iPSCs. In fact, some studies have found that there are even significant variations between iPSC lines generated at the same time from the same original somatic tissues in the same lab [798]. Some of these genes were associated with pluripotency of the iPSCs and

thus could affect the ability of the iPSC to form certain cell types or can bias iPSCs to become one cell type over the other. Newman et al suggested that oxygen tension, culture matrix, and media could contribute to these differences. Indeed, oxygen tension was suggested as a potential culprit of lower induction efficiency by members of the Tischfield lab. In addition, there are some papers which suggest that NPC in culture could have the ability to "transdifferentiate" into a range of non-neural lineages. In the developing brain, NPCs are largely constrained to a small niche where they are surrounded by signals that "force" them to go into a neural lineage. Thus in vivo, it is unlikely that NPCs develop into non-neural cell types. In culture, some of these neural signals are absent and furthermore, other external signals (that are not present in the developing brain) could also lead to NPCs turning into different cell types. Studies in adult NPC cultures have shown that, in culture, NPCs can "transdifferentiate" into hematopoetic, epithelial, and endothelial lineages [799-802]. Indeed, when co-cultured with ES cells, E12.5 neural tissue derived from mice were able to generate smooth muscle and keratinocyte cells, indicating that microenvironment could influence cell fate [803]. An interesting study by Rajan et al 2003 showed that NSCs plated at low density differentiated into smooth muscles when SMAD is activated [804]. On the other hand, NSCs plated at high density differentiated into glia (astrocytes) when SMAD is activated. As monolayer NPCs are generated by inhibiting SMAD, it is likely that if SMAD inhibition is not complete or strong enough, NPCs, especially early passage NPCs (which GIBCO indicates are "unstable") could differentiate into other cell types.

It is of utmost importance that high quality NPCs with PAX6+ staining are utilized. In the cases where poorly induced NPCs were used, neurite outgrowth, migration, and even EF responses were altered- which could greatly alter the fidelity of the data acquired. For

example, in Family-1 Sib, 5 clones from which multiple neural inductions were derived were utilized in my studies. In Clone-4, 2 inductions were done. One induction had proper neural morphology and bright, evident PAX-6 staining. In these cells, typical neurite percentage, migration, and EF responses were seen (when compared to C1, C2, C3 and the NPCs induced from these clones). On the other hand, the 2nd induction had almost no PAX6 staining and a preponderance of flat cells. Studies on these "poor quality" NPCs led to very low neurite percentages, strange neurosphere shapes, and a complete lack of EF response- an atypical profile for Sib NPCs. Thus, quality control and careful assessment of cell morphology and staining are necessary to ensure proper results. Moreover, for future studies, it may be worth changing or optimizing the GIBCO protocol to increase NPCs generation efficiency in our hands. First, we could try utilizing different culture materials (such as plates and coatings) to see if induction efficiency can be improved. Secondly, while this may be more difficult, experiments can be conducted on incubator settings such as temperature and CO2 levels to see if altering these can increase NPC generation success [805]. Oxygen tension and pressure could be changed though these metrics are harder to manipulate without specialized incubators [806, 807]. Thirdly, addition of growth factors to the current GIBCO kit could also optimize and increase NPCS generation. For example, Shi et al 2012 found that the addition of retinoids to the Chambers et al 2009 monolayer protocol increased the efficiency of NPCs generation and more importantly increased the percentage of forebrain PAX-6 positive cells acquired from the protocol [727]. EB protocols often utilize EGF or FGF to increase the efficiency of NPC generation. For example, Zhang et al found that addition of FGF increased NPC generation by almost 50% [678]! More recently, GIBCO updated their protocols to suggest that plating iPSCs in clumps (rather

than as dissociated cells) may be a better strategy. Thus, this approach could also be utilized to ensure better induction rates. Of course, we could also adopt other methods of neural induction such as opting for another commercially available kit. For example, Stem Cell technologies offers a neural induction kit of similar price that has yielded better results for multiple other labs (as per discussions with other labs at SFN). The Chambers protocol or modified version of that could also be used. This would allow for better control of added factors and help us better troubleshoot problems that may arise (than if working with a proprietary supplement). Changing protocols however, should be done with caution, because it is unclear if changing the way NPCs are generated would change the behavior of these cells and thereby alter results that we have consistently observed. Of course, high quality, undifferentiated iPSCs that are clump passaged are also necessary to get good neural inductions. iPSCs with karyotype abnormalities or differentiating colonies can vastly alter NPC generation efficiency [808]. Ultimately, generation, maintenance and induction of iPSCs and NPCs are still being optimized by the larger scientific community. Thus, careful observation and quality control are necessary to obtain high quality results.

Of course, in addition to optimizing NPC generation techniques, it may also be important in future studies to optimize the methods I developed to study migration and neurite outgrowth. The current methods utilized were developed within the first 6 months of my time in the lab. Although multiple careful experiments were conducted to select my experimental conditions (see results), our knowledge of NPC behavior and variability were highly limited at that time. While I was testing different conditions for my methods, we had a total of 4 NPC lines we could work with- 2 Sib and 2 ASD. We were blind to diagnosis for about the first 3 months and thus pilot studies for methods were done with

very little knowledge. Moreover, we could not anticipate the large variation in phenotypes such as percentage of cells with neurites from line to line. Now, with this knowledge, some changes could be made to our protocol to obtain better results. First, the conditions I selected for neurite outgrowth experiments have led to a state where the highest percentage of neurites we observe in culture is around 25%. Thus, a majority of the NPCs we are studying do not exhibit the behavior of interest. In some of the more impaired ASD lines, neurite percentages have been as low as 0.6%. Furthermore, changes seen with EFs were also rather minor- for example, PACAP treatment in Sib would sometimes change NPC neurite outgrowth from 8% to 12%. In absolute terms this is only a 4% increase in the total number of neurites (though a relative 50% change) which is a very small difference. This 4% change could reflect an increase in just 10-20 neurites per dish-which is a minor difference in the big picture. With such small numbers, experimental error is higher, and reproducibility is more difficult. Moreover, it is unclear how biologically relevant it is to observe small changes in a minority cell population. Thus, it may be more useful to study neurite outgrowth in conditions where a larger percentage of the NPC population had neurites. Based on my experiments, using higher fibronectin concentrations, switching to Matrigel coating, extending culture time from 48 to 96 hours, and increasing initial plating density from 50K to 150K could all increase the numbers of neurites we see in culture. For example, in one study, I plated Sib and ASD NPCs in control and PACAP conditions for 2, 4, and 6 days. The results were as follows: Sib- D2: Control: 13%, PACAP 22%; D4: Control: 27% PACAP 38%; D6: Control: 42% PACAP: 62%. For ASD, Control and PACAP values had no differences even after multiple days of culture D2: 5%; D4: 15%; D6: 30%. By D4, we find that, in Sib almost 1/3 of the NPCs have neurites. Moreover, the

absolute difference in neurites between PACAP and Con was about 40 neurites instead of 15 neurites at D2! Thus, minor counting differences from investigator to investigator are less likely to significantly alter data at D4. In addition, D4 culture still preserved the differences seen between ASD and Sib and the larger numbers hold more biological relevance. Similar results were observed for increased density. When conducting the neurite assay, there are some important factors that need to be taken into consideration such as distribution of cells and cell clumping. These factors have been extensively reviewed in our publication of our NPC methods in Williams and Prem et al 2018 [762]. To increase efficiency and reduce investigator bias, neurite assay analyses can also be automated. This would greatly speed up the data acquired from our lab.

In general, the neurosphere migration protocol does not seem to need much optimization. Results obtained are usually fairly consistent and the changes in migration are not minor. However, proliferation rate/neurosphere size have consistently been raised as a concern by other researchers when I've presented my work. However, interestingly, despite having ASD cells that proliferate faster (I-ASD-2, 16pDel NPCs) and ASD cells that proliferate slower (IASD-1 and I-ASD-3) both these groups of individuals have lower migration, showing no correlation between migration and proliferation rates. Moreover, when migration is normalized to total neurosphere size, the ratio obtained is still higher in unaffected lines compared to all affected lines. Moreover, when plotting migration as a function of inner cell mass area, there is little correlation between inner mass size and neurosphere expansion in both Sib ($R^2 = 0.25$) and for ASD ($R^2 = 0.05$). Proliferation and migration could not be completely separated using the neurosphere model, as NPCs in plated spheres will indeed continue to proliferate and this could of course influence the

sizes of both the migrating carpet and the inner cell mass. By dividing total migration by sphere size, concerns about sphere size contributing to migration changes would be partially ameliorated. Of course, migration could also be tested via other methods such as Boyden's Chamber Assays and scratch tests. Much like the neurite assay, when conducting neurosphere assay it is important to plate cell carefully and ensure no sphere-to-sphere contact is occurring. These important considerations are thoroughly reviewed in our JOVE methods paper. Lastly sphere measurement can also be automated to increase efficiency.

While room for optimization and improvement are always present, the results I've conducted based on current protocols shows, with experimental rigor and carful quality control, exciting reproducible results can be revealed by the study of human NPCs. Yet, improvement and optimization would better convey these results and thus, the suggestions made above should be considered for future studies.

Control Groups: What is Normal? The variations in Control Behavior

A majority of ASD studies have been conducted on mouse models largely due to the relative ease of manipulating genetic factors in mice. Many of the mice strains used in these experiments are heavily inbred such that individuals of a strain are nearly identical to each other in genotype genetic constitution and variability between individuals is minimal [809]. In general, a strain is considered to be inbred when it has undergone at least 20 generation of brother- sister or offspring- parent mating such that the at least 98.6% of the loci of an individual strain is homozygous. Thus, each individual mouse is almost a clone of another mouse and differences between individuals are largely determined by environmental factors. This inbred homozygosity is highly useful for experimental studies as it leads to increased reproducibility in both control and in mutant lines. Essentially, WT mice are

"isogenic" to mutant mice and differ only at the genetic locus altered for the experiment. This allows us to study the exact function of a gene without worrying about genetic background influencing phenotype changes. Unlike these model mice, humans, even from the same family, are highly variable and diverse in their genotypes and phenotypes. Thus, when studying human populations, questions of "what is normal" and "what makes a good control group?" are particularly important. Indeed, fields that often study human behavior and disease such as medicine, sociology, and psychology have grappled with this variability for decades. These fields have coped by ensuring studies are adequately powered and by using sample sizes in the hundreds or even thousands. Moreover, thorough and careful screening of patient behavior and disease state is also often necessary to ensure acquisition of adequate control and disease groups. However, with the human iPSC field, with current technology obtaining sample sizes in the range of the hundreds of individuals would be nearly impossible. Even with the onset of automation, these studies would take a very long period to complete. Thus, careful selection of controls and ensuring rigor and reproducibility are of utmost importance.

In human behavior and disease, there is quite a large "range" of phenotypes that are considered "normal". For example, IQ scores between 70 and 130 are considered "normal" and in fact 95% of humans fall into this range. However, there are vast differences in cognitive ability and intelligence between someone who scores a 70 on an IQ test and someone who scores a 130! Yet, when looking for "normal" human controls, both these individuals could be considered. At the same time, an individual who scores a 65 on an IQ test would be considered mildly "intellectually impaired" but would not be too different than a "normal" individual with an IQ of 70. Thus, for studies of IQ impairment, either

controls would have to be selected from the narrow median range of 90-110 or many individuals representing all the IQ brackets would need to be selected for comparison to impaired groups. Likewise, medical studies of diseases ranging from blood pressure to Alzheimer's have shown that in general, phenotypes between normal and disease patients are best represented by two overlapping bell curves (Fig 102). Thus, there are groups of people who despite being "normal" display disease phenotypes or individuals with disease that display "normal" phenotypes which greatly complicates human studies. In iPSC studies, this variability is further enhanced by the fact that reprogramming and induction processes to generate neural cells themselves can alter cell behavior and phenotypes.
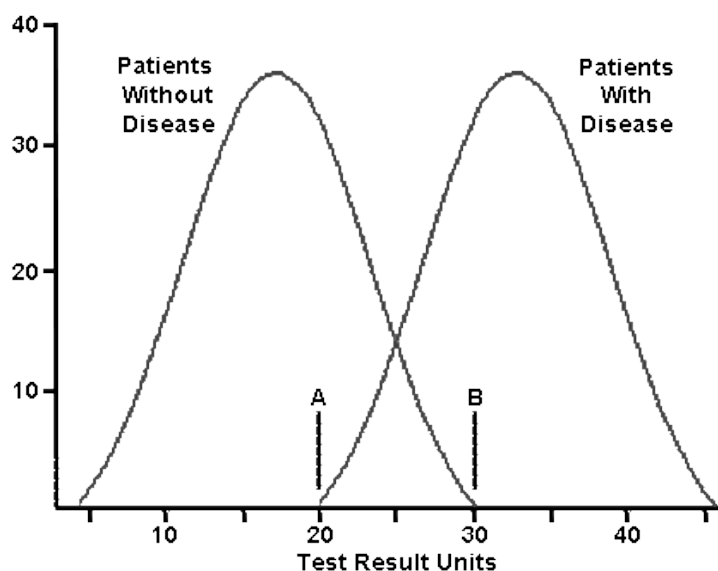
Figure 101: Overlapping bell curves which show that data acquired from patients without disease and patients with disease can sometimes intersect at the "extremes". Thus in some cases, unaffected patients can "appear" to have disease when in reality they don't. Likewise affected patients can appear "unaffected" when they are not.

So, what are the ideal controls for iPSCs studies [810, 811]? For studies of single-gene ASDs, researchers believe the best controls are isogenic lines generated by either "fixing" the genetic mutation in a diseased individual or by "causing" the genetic aberration in a typical individual and comparing the results. Much like mouse model studies, this

comparison controls for the effects of varied genetic backgrounds and potential differences in controls. Moreover, by "making" and "fixing" these mutations in multiple genetic backgrounds, we could get a better idea of why certain disease associated genes cause disease in some individuals but not others. Indeed, such studies could also be conducted in mouse models by looking at the consequence of a genetic mutation in different mice strains. However, for multigenic or complex idiopathic disorder such an approach is not possible. While CRISPR and other technologies have been used to create multi-site mutations, these technologies have not yet evolved to cause mutations in low-penetrance genes at multiple loci or loci as large as those seen in CNV disorders associated with ASD [812]. Thus, for idiopathic or polygenic disorders, the only choice is to derive iPSCs from a "healthy" control. In the best case-scenario, for disorders such as ASD, the best "control" individual would be an identical twin. However, world-wide, only about 0.3% of the population are identical twins. From this already small sample, there would need to be a case where one twin has autism while the other does not. With the high heritability of ASD, these cases are exceedingly rare and thus, such a study would be very difficult to conduct. Another option is to use Sibling or parental controls, who share approximately 50% of their genome with the proband. However, familial controls themselves are not without problems. More recently, several studies have suggested that less pronounced social, cognitive, and personality characteristics that are associated with ASD may be present in the first-degree relatives of individuals with ASD [813, 814]. This "broader autism phenotype" of course could mean that neurobiologically, familial controls and the ASD proband could have similar phenotypes. Thus, in iPSC studies where only a Sibling control is utilized, it is possible that phenotype uncovered may appear "minor" or phenotypes can be lost because the Sib

also has the defect. Of course, this could be both a blessing and a curse, as comparisons to Sib would allow us to see what specifically in a given genetic background caused one individual to have ASD and the other to not. Thorough screening of Sibs and related controls would also help reduce this issue. Comparing Sibs of the same sex also help reduce heterogeneity and allow for more similar control groups.

Our cohort of idiopathic ASD patients came from a larger study conducted by the Brzustowicz lab where families with at least 5 individuals with 1 unaffected Sibling, 1 Sibling with SLI, and 1 Sibling with ASD were recruited. Unlike many other iPSC studies, all family members and patients in our study were thoroughly screened on multiple metrics including IQ, social interaction, communication, and language parameters by the same exact group of people. From this larger group of Families, we selected 8 families ensuring that families with severely affected proband and sex-matched SLI and Sib were selected. Due to the thorough behavioral testing our Sibling controls went through, we know that these patients do not have SLI or autism, thus worries about the Sib's being partially affected were minimized. Yet, concerns raised about the "genetic loading" of these Sibs making them less than ideal controls. Moreover, for comparison to the 16p11.2 del patients we acquired another set of control iPSC lines from the NIMH Center for Regenerative Medicine. Yet, unlike our Sib controls, these NIMH control lines were acquired from newborn umbilical cord blood from infants who had no known genetic mutations. However, no behavioral testing or follow-up studies for disease were conducted on these patients. Thus, it is possible that some of the NIMH controls could be derived from people who have idiopathic disorders including neuropsychiatric diseases like autism, bipolar disorder, or schizophrenia! Of course, with such issue, the data from the NIMH controls

need to be evaluated carefully. Similar concerns should extend to studies by other groups where controls are not well characterized. A look at my data shows the importance of having two control groups and the amount of variability that is seen just between the "typical unaffected individuals". In terms of neurite outgrowth, Family-1 Sib NPCs have an average of 12% neurite outgrowth. On the other hand, Sib NPCs from Family-2 and Family-3 had around 8 and 9% neurites respectively. This represents 25-35% reduction between these NPCs- which in some Families is the same difference seen between Sib and ASD. Does this imply that compared to Sib-1, Sib-2 and Sib-3 NPCs are impaired in some way? Or does the fact that they still have normal responses to EFs and have higher neurite than their proband brothers make then "okay"? When compiling the data from all the idiopathic Families by clone, we see that some Sib clones have the same exact neurite outgrowth percentage as some ASD clones -even within the same Family! In fact, on average Sib NPC neurite outgrowth varied from 6% to 30% (a huge range!). Neurite outgrowth in ASD was tighter but did indeed overlap with Sib values ranging from 1%-13% neurites. Thus, we see the "overlapping bell-curve" phenomenon of disease in our data. This variability sometimes meant multiple clones and neural derivations needed to be studied to detect phenotypes or to see "separation" in the data. Yet, in the end, we see that on average Sib NPCs from each family had higher neurite outgrowth when compared to the ASD NPCs. There is also wide variation in migration between "normal individuals" too. Moreover, responses to drugs and EFs were also slight different between controls. Luckily, our study design is such that comparisons were made within Family, which helped control for some of this variability. However, for the 16p11.2 comparisons were made to the average Sib and average NIMH data. On average, Sib NPCs have about 10.5% neurite

outgrowth and NIMH NPCs had about a 12% neurite outgrowth, which is a minor difference. There were rather big differences in migration, yet again on average the "unaffected" individuals tended to have higher values despite overlap with 16p11.2 del NPCs. Due to this variability, we see that in the 16p11.2 data, M-2 did not have significant differences in neurite outgrowth.

In addition to variations in neurites and migration, study of signaling is an area where the Sib-to-Sib comparison really helped reduce heterogeneity. However, this poses a problem for the 16p11.2 del studies. When placed on the same blot, Sib-1 NPCs have a statistically higher level of P-S6 than any other Sib or NIMH line. In fact, when compared to Sib-2, Sib-3, and both NIMH individuals, the P-S6 levels in this individual are almost 1.5-2. fold higher! This high P-S6 level in Sib-1 NPCs exaggerates the deficit of phosphorylated S-6 seen in ASD-1 NPCs. In fact, compared to other Sibs and the NIMH, ASD-1 NPCs have about a 50-80% reduction in P-S6 (significant) yet when compared to his Sib, the reduction is almost 4-fold different. Likewise, ASD-2 NPCs when compared to Sib-2 has a 4-fold higher P-S6 whereas compared to Sib-1, this difference is now between 30-70% higher (significant). Thus, comparing to non-sib controls does not alter the significance or direction of changes in our ASD NPCs but it does change the magnitude. When averaging together P-S6 densitometry between Sibs for 16pDel studies, we end up with large error bars in the data due to the variability between Sibs. NIMH control data is however tighter and seems to resemble Sib-2 and 3. Thus, when compared to Sib, studies of 16pdel NPC signaling requires either averaging all controls together, normalizing to NIMH, or conducting more experiments to see differences. Indeed, the data I have presented shows the raw data for 16p, NIMH, and Sib for Westerns. This is because we are

still figuring out which is the best way to compare data as is evident from the results section where data is represented in multiple ways including as averages of clones, broken down by clone, average of individuals and comparisons within and between families.

In conclusion, with our ability to now study human neurobiology in a dish, is incredibly important for us to cognizant of the variability in the human population such that selection of controls and proper study design are done properly and carefully.

Language Impairment, Autism, and Neurobiology: The common arena?

Despite the vast heterogeneity of ASD, my results show strikingly common defects in multiple patients with ASD from different cohorts. Similarities in our I-ASD cohort while surprising, could be due to the similar backgrounds these patients share. Our I-ASD patients come from similar families in the sense that they all are from NJ, speak English at home, and in addition to having autism in the family, there is also one member who has specific language impairment. Moreover, two out of 3 of the idiopathic patients studied are non-verbal while the other has severe verbal impairments and language impairments as assessed by multiple language assessments. Language impairment is not uncommon within ASD as one of the core symptoms of ASD is a communication defect. It is unclear how many children with ASD have some kind of language impairment, however, it is estimated that approximately 25% of children with ASD are non or very minimally verbal [815]. It is likely then, that the children with severe verbal problems represents an "endophenotype" of ASD and may be a distinct group than children with ASD who do not have verbal problems. Interesting, much like our I-ASD cohort, individuals with 16p11.2 deletion have high prevalence of language disorders. One study by Mei et al conducted on 55 individuals with 16p11.2 deletion found that 77% of the children met criteria for a speech disorder

known as childhood apraxia of speech (CAS) [816]. Likewise, 70% of the individuals had receptive and expressive language impairments. Co-occuring neurodevelo-pmental conditions and non-verbal IQ did not correlate with the verbal disorders. This suggests that 16p11.2 deletion locus is highly penetrant for language disorders. Indeed, all three of our 16p11.2 deletion patients, despite variable severities in ASD, have language impairments. M1 who has autism, has articulation disorder along with receptive and expressive language disorder, M-2 has Asperger's and expressive language disorder and the female has mild autism and an articulation and communication disorder. Thus, language impairments are a common feature in our cohort and could explain the homogeneity of our results. Perhaps, having language impairment itself is correlated with certain neurobiological abnormalities.

The acquisition of language is a ubiquitous and essential process for humans. Yet, the neurobiological substrates of language and language disorders are not well characterized [817]. As humans are the only species currently thought to possess sophisticated language, language impairments in general are difficult to model in animals. Studies suggest that up to 7.4% of children have specific language impairment (SLI). Despite the name, SLI is not "specific" any one feature, rather, it is associated with any alterations or delay in production or understanding of speech which is not caused by autism, low IQ, or physical impairments. Much like ASD, SLI is thought to have strong genetic underpinnings though, there is no "single" gene that accounts for all cases of SLI. Studies have found that in some cases, children with SLI often have "subclinical" ASD-like symptoms suggesting that language disorders and ASD may have commonalities. Like individuals with ASD, a proportion of individuals with SLI also show brain abnormalities like atypical asymmetry in the language cortex, white matter changes, and cortical

dysplasia. This similarity of neuropathology between ASD and SLI can suggest similar developmental alterations. Genetic studies of SLI hare fairly limited. One study by Chen et al 2017 used next-generation sequencing to identify gene variants associated with SLI [818]. Surprisingly, this study found alterations in genes such as GRIN2A AUTS2, CNTNAP2, and FOXP1 which are all genes implicated in the pathology of ASD. Non-ASD genes such OXR1, KMT2D, ERC1, and SRPX2 were also observed. Interestingly, the genes such as CNTNAP2 and AUTS2 are associated with defects in migration in mouse models. Moreover, 1 iPSC studies of CNTNAP2 heterozygous deletion found defective migration in patient derived NPCs [819, 820]. Interestingly, migration defects were common in all our ASD patients- who do also have language impairments.

Due to the phenotypic similarities in our ASD NPCs and the common language impairments seen in our ASD patients, it would be very informative to compare our patients to individuals with language disorders. Our lab and collaborators are incredibly fortunate to have access to lymphocytes derived from the SLI family member of each of our idiopathic families! These blood cells are being made into iPSCs and NPC so that we can now explore the neurobiological and developmental similarities between ASD and SLI. These studies will give great insight into language disorders and will help us better understand the influence of language disorder on ASD phenotypes.

Migration and Neurite outgrowth: An Issue of the Cytoskeleton?

During neurodevelopment, the cytoskeleton of neural precursor cells and newborn neurons undergo extensive, dynamic remodeling to facilitate proliferation, migration, differentiation, and synapse formation. Indeed, alterations in the cytoskeleton or cytoskeleton associated proteins often lead to drastic alterations in neurodevelopment and

brain structure [821-824]. Recently, genetic studies of neurodevelopmental disorders have analyzed the thousands of genes associated with these disorders and looked for points of convergence. These studies have found changes in the cytoskeleton and its regulators as a common point of dysregulation in ASD and other NDDs [108] . Moreover, "synaptic" and "activity dependent" molecules such as the neuroligins and SHANK-3 which are associated with NDDs are also often cytoskeletal proteins or proteins that interact with the cytoskeleton. This suggest that dysregulation in the cytoskeleton may be a key pathological feature of neuropsychiatric disorders. In disorders such as autism, there are often aberrations in numerous steps of neurodevelopment including proliferation, migration, neurite outgrowth, and synapse formation. The successful execution of these steps is dependent on extracellular and intracellular cues which I reviewed in great detail. However, all of these signals ultimately converge on the neural cytoskeleton which undergoes constant dynamic changes to facilitate the proper execution of these developmental processes. Results my work and work from others in our lab have shown that ASD NPCs have alterations in all early developmental processes including proliferation, migration, and neurite outgrowth. The proper function of the cytoskeleton is essential for the regulation of these processes and thus, alterations in the cytoskeleton could be the reason why our cells are showing defects in multiple neurodevelopmental domains. This potential cytoskeletal defect would also likely manifest in mature neurons as alterations in dendritic spines, axon/dendrite formation, and synapse formation.

The neuronal cytoskeleton includes actin microfilaments, intermediate filaments, and microtubules which are all essential players in the entire neurodevelopmental program. In migration, for example, microtubular dynamics are indispensable for normal movement

and position of developing neurons [825]. Indeed, alteration in microtubule associated proteins such as Lissencephaly 1 (Lis1) and Doublecortin (DCX) lead to drastically altered cortical layering and changes in migration. Both these proteins are involved in the stabilization and destabilization of microtubules which is necessary for cellular movement. Likewise, actin and actin associated proteins are incredibly important in early neurite outgrowth and polarization of neurites [826, 827]. In particular F-actin dynamics and the regulation of actin by small GTPAses of the Rho family are pivotal for the establishment of neuronal polarization [828-830]. Indeed, the RhoA signaling pathway include members such as ROCK and cofilins that are well known to regulate actin dynamics. Dynamics of these cytoskeletal elements are also essential for regulating differentiation programs, synapse formation, and even neuronal electrical activity. Some regulators such as doublecortin are specific to early development while members of the RhoA pathway for example, are pervasive and important throughout development. Thus, which cytoskeletal elements are dysregulated will largely influence the type of defects seen in neural cells and the brains of diseased individuals. In our cells, the ROCK inhibitor, which blocks the RhoA pathway, thereby increasing actin dynamics, increases both migration and neurite outgrowth in our NPC. However, the magnitude of change caused by the ROCK inhibitor is not different between Sib and ASD. This suggests that the Rho pathway is not completely dysfunctional in our I-ASD cells. However, baseline function of this pathway was not assessed. Cytoskeletal dynamics are also influenced by signaling pathway such as mTOR and many extracellular factors such as NGF. Indeed, mTOR activation can lead to the phosphorylation of RhoA signaling pathway which regulates cytoskeletal dynamics [831].

In our NPCs, aberrant mTOR pathways and altered development are reasons to suspect altered cytoskeletal dynamics. In 16p11.2, studies by other groups have suggested alterations in cytoskeletal regulators. One transcriptomic study of individuals with 16p11.2 deletion and duplications noted differential expression of RhoGTPase signaling and cellular adhesion molecules [832]. Moreover, work by Lin et al 2015 found that KCTD13, which is located within the 16p11.2 CNV influences the level of RhoA [833]. Initial actin stain of 1 Sib, ASD, and 16p11.2 del NPCs showed no gross morphological changes in the actin cytoskeleton. Furthermore, immuno-staining was conducted for P-cofilin, an actin binding protein that regulates actin dynamics. Phosphorylated cofilin is thought to stabilize F-actin which reduces polymerization[834-836]. Reduced polymerizat-ion should lead to reductions in cell motility and neurite outgrowth. Thus, I expected that our ASD NPCs, which had lower neurite outgrowth and migration, would have elevated levels of P-cofilin. Surprisingly, all but 1 ASD NPC had identical levels of P-cofilin to Sib and NIMH controls. Family-1 I-ASD NPCs did show distinctly higher P-cofilin levels, which paralleled the neurite defect seen in these lines. Thus, coilin did not seem to be a "point of convergence" for the neurite defects in all our cells. However, there are numerous other cytoskeletal molecules that could be assessed for abnormalities. Moreover, it is possible that different cytoskeletal aberrations are leading to the same ultimate phenotype in our cells.

When numerous developmental processes are aberrant, it is likely that some convergent mechanism is contributing to these defects. Indeed, in our cells, mTOR signaling represents one of these convergence points. However, mTOR pathway regulates the cytoskeleton in order to achieve the dynamics needed to allow for cell morphology and

movement. Thus, in our cells, cytoskeletal abnormalities could be another point of convergence for developmental abnormalities.

EF responses and ASD: Underpinnings and Implications

The biggest difference between the 16pdel NPCs and the I-ASD NPCs are in their response to the EFs. Specifically, while I-ASD NPCs failed to respond to numerous EFs, the 16p11.2 responds typically to all EFs tested. The underpinning reason for this response difference has not been elucidated. Furthermore, the developmental consequences of this difference and the implications are currently unclear, particularly since my study is one of the first to extensively investigate the response of ASD NPCs to EFs. However, a review of patient phenotypes and an in-depth analysis of receptor distribution and signaling pathway differences in our cells could help us understand the differences in these cells.

In our I-ASD cohort, both higher mTOR and lower mTOR signaling are associated with lack of EF response. However, there are other differences in signaling between I-ASD-1 & 3 which have lower mTOR activity and I-ASD-2 which has higher mTOR activity. Unlike I-ASD-1 and 3 NPCs, I-ASD-2 NPCs have a normal increase in P-CREB stimulation upon treatment with PACAP. Based on this, we would expect PACAP to stimulate neurite outgrowth in ASD-2 NPC. However, the I-ASD NPCs do not respond to PACAP. This suggests that effectors downstream of CREB, such as cytoskeletal regulators, may be contributing to the impaired PACAP responses in ASD-2. Additionally, interaction with other dysfunctional signaling pathways could also lead to lack of PACAP responses in I-ASD-2. Unlike I-ASD-1 and I-ASD-3, I-ASD-2 NPCs show lower levels of ERK-1 phosphorylation. This ERK-1 defect could interact with P-CREB or downstream molecules to prevent the P-CREB based transcription of genes needed for neurite outgrowth. Indeed,

in adipocytes, cardiac neurons, and SH-SY5Y cells, PACAP has been shown to activate ERK1/2 signaling [837] (Monagahn et al 2007). Specifically, in SH-SY5Y human neuroblastoma cell lines, PACAP mediated ERK1/2 activation was essential for neurite outgrowth. Blocking ERK1/2 in these cells abolished the ability for PACAP to induce neurites. Thus, it is possible that response to PACAP and other EFs are not simply based on canonical activation of a single pathway, rather EF binding is likely to activate numerous signaling pathways and defects in any node can translate to alterations in response. Indeed, more and more studies are finding that "canonical" models of signaling activation are rather simplified and binding of an agonist to a receptor can activate numerous second messenger systems and lead to activation of other pathways which are not independent and isolated as originally believed [838, 839]. However, the 16p11.2 del NPCs have similar signaling profiles to I-ASD-2 but can respond to EFs. Yet, a close look at the signaling defects again reveal that there is a difference in the ERK signaling defects in I-ASD-2 vs 16pDel NPCs. While I-ASD-2 NPCs have lower P-ERK levels and lower relative ERK activity (the P-ERK/T-ERK ratio), 16pdel NPCs have lower P-ERK levels but a normal relative ERK activity. This relatively "normal" signaling of the ERK pathway could contribute to the "typical" EF responses seen in 16p11.2 del patients. This shows how multiple factors and minor differences could contribute to differences in phenotypes.

EFs are important regulators of brain development as they signal cells to take certain "steps" in a timely coordinated fashion. Altered or lack of response to even a single EF can alter the course of brain development. For example, the Vaccarino lab created animals that were compound knockout for 3 FGF receptors in the telencephalon using EMX driven CRE lox system [840]. Due to lack of FGFR these animals are unable to respond

to FGF. Due to this, the mutant animals had severe deficits in cortical surface area growth at E12.5. Likewise, overexpression of transgenic PAC1 receptor lead to altered cortical size and increased apoptosis in mice [268] . Thus, in our I-ASD NPCs, lack of response to EFs could lead to developmental abnormalities. In the context of brain development, it is possible that I-ASD individuals, whose cells do not respond to EFs could have more severe brain or behavioral abnormalities than the 16pDel individuals whose cells do respond to EFs. Essentially, while at baseline, 16pdel NPCs exhibit lower neurites and migration than unaffected NPCs under EF stimulation both migration and neurites can be increased (though levels are still lower than unaffected individuals under EFs). In I-ASD, lack of EF responses and already low neurite outgrowth could lead to a more severe phenotype. Indeed, a look at our patients show that 2 of our 3 idiopathic patients are non-verbal and the other has very severe language defects. On the other hand, while having language issues, the 16p11.2 del patients are all verbal and 2/3 have Asperger's or milder forms of ASD. Of course, this correlation of EF response to more severe phenotype could just be a coincidence as, in vivo, there are more than 3 or 4 factors that influence development and ultimately behavior. Further studies on ASD patients of different severity would help elucidate if such a correlation exists.

In addition to implicating differences in developmental severity, lack of response or ability to respond to EFs could have major implications in treatment of individuals with ASD. In piloting therapeutics for ASD, heterogeneity of patients makes it difficult to conduct clinical successful clinical trials. For a drug to get FDA approval, it has to be able to impact a majority of study participants such that a significant drug efficacy is demonstrated. In ASD, drugs that may work wonderfully in one sub-population of

individuals may not work in another. This variability may explain why therapeutics targeted towards ASD often fail in clinical trials. Clinical trials are expensive and often take years of time. Moreover, when a drug fails, even if it works in a small percentage of people, it will not be marketable or available to that group unless a separate trial is done. In idiopathic ASD, our current knowledge does not allow for this "subtyping" of individuals. However, with iPSC technology, it is possible to use patient derived cells to test drug efficacy. For example, oxytocin has been studied in ASD before and has often led to mixed results [841] . My data shows that our I-ASD patients do not respond to OXT but 16pDel patients do. Thus, it is possible that 16pDel patients are more likely to respond to oxytocin as a therapeutic while I-ASD patients are not. Similarly, antidepressants are often given for the management of ASD. However, in I-ASD patients who don't' respond to 5-HT at typical doses, it is possible that higher doses of antidepressants may be needed for efficacy. Of course, it is possible that the responses and alterations we see in culture do not reflect patient biology. Specifically, since iPSC derived cells are fetal in nature, it is possible they are representing biology that is no longer relevant to the patient. Thus, it is important to test whether drug or EF responses in iPSCs really indicate or reflect responses in patients. Currently, such studies are limited, however drug testing and discovery is a promising avenue of iPSC research. In fact, there are numerous studies in multiple fields utilizing iPSCs to screen and test drugs. One example of drugs that have shown efficacy in iPSC-derived neurons and in human clinical trials is IGF-1. In 2010, Marchetto et al found that IGF-1 application rescued abnormal neuronal phenotypes seen in Rett-patient derived neurons [742]. Now, IGF-1 has passed Phase-2 clinical trials and has shown efficacy in improving social and other defects in Rett patients [539, 842]. Thus, EF and drug response

profile in our patient may be beneficial to uncover potential therapeutics for our patients.

Gene Dosage, mTOR, and Development

Despite common phenotypes, the underpinning signaling defects in our patients are not identical. This suggests that the same neurobiological alterations can occur due to different etiologies. Interestingly, this could mean that the numerous etiological factors associated with ASD could converge onto a few different cellular or molecular defects! Altered mTOR is common amongst all our patients. However, while 2 patients show underactive mTOR pathway the other 4 have overactive mTOR. Yet, neurite and migration defects we see are the same. However, the proliferation abnormalities observed in our cells seem to correlate with mTOR activity. Slow-proliferating NPCs have low mTOR activity while fast-proliferators have high mTOR. This suggests that mTOR signaling balance (not too low, not too high) are necessary for normal cellular function. Interestingly, most studies of ASD have generally found higher mTOR signaling activity and higher proliferation. This is generally due to focus on syndromic disorders such as tuberous sclerosis where gene aberrations lead to higher activity or focusing on macrocephalic models which are more likely to have higher proliferation rates/higher mTOR activity. Thus, our study is one of the few to observe lower mTOR signaling in ASD. So, it is difficult to know how "over and underactive" mTOR are related to neurobiological defects. However, this concept of "gene dosage" has been noted in several disorders including CNV duplications and deletion along with Rett syndrome vs MECP2 duplication Syndrome [843]. Though our mTOR overactivity is not caused by any known deletion or duplication in genes associated with the mTOR pathway the idea of too much or too little leading to similar problems is illustrated through deletion and duplication studies.

A recent study by Deshpande et al investigated iPSC-derived neurons from 16p11.2 deletion and duplication patients in parallel [752]. Neurons derived from deletion patients had longer dendrites and larger soma while duplication patient neurons had smaller soma size. Yet, both duplication and deletion patients shared common increases in mESPC amplitude, decreases in synaptic density, and reduction in VGLUT2 puncta. Why do both over and under-expression of a gene or gene product lead to similar phenotypes? The product of a gene may be important for reducing proliferation. In the absence of the gene, proliferation would be increased. However, overexpression could lead to a dominant negative effect which would also lead to increased proliferation. For example, too much of a repressor protein could lead to complexing, which means this protein is not functional anymore. In our cells, it is possible that too little mTOR activity leads to reduced transcription of genes associated with cytoskeletal dynamics. On the other hand, too much mTOR means excess of these genes/gene products which could alter the cytoskeleton and prevent migration and neurites. Of course, further studies would need to be conducted to parse out the exact mechanism of this phenomenon.

mTOR, Metabolism, and Interactions with other Signaling Pathways

In science, a reductionist approach is often taken to identify the effects or contribution of a single variable on a system. While this approach is useful to gain knowledge the variable of interest, it does grossly simplify the complicated interactive processes that occur in an organism or cell. For example, my results show that manipulating mTOR signaling can rescue all of the defects seen in our ASD cells. However, mTOR alterations were not the only signaling pathway abnormality in our cells. Moreover, signaling and mTOR are not the only factors that regulate neurite outgrowth and migration

in a cell! In reality, there are numerous interactions between different signaling pathways, metabolism, and the cytoskeleton that lead to the ultimately phenotypes seen in our cells. These interactions are important to consider as they could help explain some of the phenotypes seen in our cells or could help us understand how to better study and treat ASD.

The mTOR pathway is a major eukaryotic signaling network that coordinates cellular functions and development with environmental conditions to play an important role in cellular physiology. As such, it is no surprise that mTOR has cross-talk with metabolism and other signaling pathways. In our cells, we find that I-ASD-1 and 3 which have low mTOR show reduced PACAP stimulated P-CREB and higher ERK pathway activity. Interestingly, in the high mTOR cells, there are no alterations in P-CREB and ERK activity is lower. This suggest cross-talk between the mTOR, PKA, and ERK pathways. In particular there seems to be a negative correlation between ERK and mTOR activity. Studies have indeed found that the Ras-ERK and P13K- AKT pathways can negatively regulate each other's activities [581, 844, 845]. AKT for example, can negatively regulate ERK activation by phosphorylating inhibitory sites on RAF. In cancer, mutations that activate the PI3K-AKT pathway dampen RAS-ERK signaling levels, almost as a compensatory mechanism to reduce cell proliferation. Thus, it is likely that such interactions are occurring our cells. In subtypes of ASD where either ERK is altered it may be useful to look at mTOR activity and vice versa. Moreover, if the alteration in one pathway is a compensation for upregulation or downregulation in the other, therapeutics need to be designed carefully. That is, if a compensatory downregulation in a pathway is targeted for therapeutic intervention, it could exacerbate a phenotype instead of ameliorate it! Moreover, in cancer, often normalization of both ERK and mTOR pathways are needed

to treat a tumor [846]. Thus, it is important to remember pathway interactions when designing therapeutics for ASD. Interesting, in Family-1 ASD NPCs, treatment of the underactive mTOR pathway has shown to normalize the "overactive" ERK seen in these cells further supporting cross-talk between these pathways.

The mTOR pathway is an important regulator of nucleotide, lipid, and amino acid metabolism. mTOR activity is also influenced by feedback from metabolites in the cell. Interesting, our metabolomics studies showed that ASD-1 NPCs had reduced levels of ATP/ADP which are the main source of cellular energy. ATP is also important for the production of important second messengers like cAMP. In cells, there is a heterotrimeric complex called AMPK that monitors the ATP/AMP ratio [847]. When ATP drops and AMP increases, AMPK turns on catabolic pathways to increase ATP production while turning off pathways that consume ATP. One way in which AMPK turns off ATP consumption is by inhibiting the mTOR pathway which is a major regulator of anabolic pathways (which consume ATP). Moreover, mTOR itself is responsive to ATP. Studies have found high levels of ATP increase mTOR signaling [848]. In addition to this cross-talk between metabolites and mTOR, the mTOR pathway itself is involved in the synthesis of purines and pyrimidines which are essential components of ATP and other trinucleotides [849, 850]. Specifically, mTOR activation induces the synthesis of both purines and pyrimidines thereby expanding the pool of these molecules for RNA synthesis, DNA synthesis, and energy. Interestingly, in ASD-1 NPCs we see low levels of mTOR coupled with low ATP and high AMP/nucleosides/nucleobases. It is possible that either the low ATP status is contributing to mTOR defects or low mTOR is contributing to the altered purines and pyrimidines [851]. Interestingly, Ben-Sahra et al 2016 studied the purine metabolism of

normal and TSC2 knockout fibroblasts [849]. TSC-2 knockout cells, which have higher mTOR activity, interestingly showed increases in IMP, AMP, and GMP which is similar to our cells. However, unlike our cells, the TSC2 knockout fibroblasts show increased DNA and RNA synthesis and increased levels of ATP. On the other hand, our ASD-1 NPCs have lower DNA synthesis and decreased ATP. This suggests that in TSC2, the upregulation of purine intermediates leads to increased ATP and increased trinucleotides needed for RNA and DNA synthesis. As I mentioned earlier, high ATP itself can further increase mTOR which then creates a feed-forward mechanism where mTOR increases purine synthesis which goes onto increase ATP which then increases mTOR! This cycle could worsen the defects seen in the cell. On the other hand, in our ASD-1 NPCs, the increase mononucleotides is correlated with reduced ATP and reduced DNA synthesis. Moreover, our cells show a huge accumulation of nucleobases and nucleotides suggesting that there may be excess degradation of ATP and other energy molecules. This suggests that mTOR regulates the balance of synthesis vs degradation in purine metabolism. This regulation can then alter the energy status of the cell which can then itself feedback onto the mTOR pathway. Interestingly, in ASD-2 NPCs, we see a higher mTOR and higher DNA synthesis. Thus, much like the TSC-2 cells it is possible that metabolomic analyses will show elevation of catabolic pathways in these NPCs. On the other hand, I-ASD-3 NPCs look similar to I-ASD-1 NPCs signaling-wise, thus it would be interesting to see how similar the metabolic profiles of these cells are to Family-1 NPCs.

In addition to these low levels of DNA synthesis and ATP seen in our I-ASD-1 NPCs, these cells show blunted P-CREB levels when stimulated with PACAP. For PACAP to signal it requires the presence of cAMP which is made from ATP. It is possible that the

lower mTOR, which contributes to low ATP leading to impaired cAMP generation. This low cAMP could, in turn, prevent signaling through the Gs pathway and reduce P-CREB levels. Indeed, when I-ASD cells are treated with low levels of db-cAMP, an analogue of cAMP, they can now respond to PACAP and 5-HT (Figure 78). Likewise, PACAP and 5-HT response are also restored by SC-79 (Figure 84) signaling. Western blot analysis shows that when I-ASD NPCs are treated with low dose SC-79, PACAP now is able to stimulate P-CREB levels in ASD to mirror the Sib (Figure 85). This suggests that manipulating mTOR can indeed fix the PKA signaling defects. However, metabolomic assays will need to be done to see whether SC-79 increases ATP and thereby cAMP in our cells. Again, I-ASD-2 NPCs which do not have low mTOR and therefore could have higher ATP production, do not show blunted PACAP stimulated P-CREB suggesting no alterations in cAMP! These interesting interactions between metabolism, energy balance, and signaling have important repercussions for ASD treatment. Due to the responsiveness of signaling pathways to environment, things like dietary alteration or vitamin supplementation could even be considered for helping manage ASD. Furthermore, these results are amongst the first to ever show an association between metabolism and mTOR in human neural cells.

Chapter 11: Ongoing Studies, Future Directions, and

Conclusion

Research never really has a conclusion. While certain well-constrained questions can be thoroughly explored and answered, these answers often prompt multiple new questions each needing years of study to get to an answer. Indeed, many seasoned researchers joke that their work has led to more questions than answers. In science, when the belief that "we know everything to know" arises, it is often proven to be utterly untrue by a new study or a paradigm shifting observation. Likewise, new technology and techniques further our ability to continue asking questions, often in systems we know well and have explored thoroughly. While my thesis work has shown exciting common neurodevelopmental and signaling phenotypes in autism patients, this work is just a foundation for future studies. Indeed, there are many small experiments that I have conducted that have shown promising and exciting results begging for follow-up! Moreover, as the iPSC field is in it's infancy, there are so many more avenues that can be explored in the future. Some of these studies are currently ongoing while others could be interesting roads to pursue in future by new members of our lab.

Ongoing studies:

A significant proportion of my years in the lab were focused on characterization and in-depth analysis of Family-1 from I-ASD cohort. As iPSCs were still being generated and expanded, NPCs generation was still being optimized, study of the other Families was not a possibility in the initial years. Due to this, there are still experiments that need to be finished in Family-2 & 3 and the newer 16p11.2 deletion cells which we only received in 2016. In Family-2, characterization of neurites, migration, EF responses, and signaling

pathways is complete. Rescue studies were also conducted on neurite outgrowth and migration. However, studies determining whether manipulation of mTOR in this patient could facilitate EF responses were not completed. These experiments are currently underway and should be completed soon. In Family-3, characterization of neurites and migration is complete. However, western experiments for signaling have only been done once on a single clone. Protein has been collected in control and PACAP conditions from 2 inductions from the same clones and 2 inductions from the 2nd clone. Westerns need to conducted on this protein and then analyzed to confirm signaling dysregulation in Family-3. In addition to western studies, immunocytochemistry (ICC) studies of P-S6 need to be completed for Family-2 and 3. ICC studies will help us ensure that the phenotypes observed through western, which are run on protein collected from high density cell cultures mirror the P-S6 phenotypes seen in our low-density cultures. In Family-1 P-S6 was shown to be lower by both western and ICC. It is important to confirm that the other two families also have parallel defects using both these methods. Since I-ASD-3 mirrors the I-ASD-1 phenotype, I was not planning on conducting extensive rescue studies in these patients. However, testing SC-79 and MK-2206 in these patients would further strengthen the mTOR pathway data I have acquired so far. For the 16p11.2 patients, neurobiological analyses and signaling studies are largely complete. However, for M-2, a 3rd clone needs to be analyzed to determine neurite defects and for the F, an unaffected female NIMH control needs to be characterized. Currently, iPSCs from the female NIMH control have been induced and characterization is underway. Rescue studies could be conducted for the mTOR pathway in 16p11.2, though preliminary data suggests that M-1, like I-ASD-2, has increases in neurite outgrowth when treated with an mTOR antagonist (Figure 95).

Analysis of EF Receptors:

Due to concerns that receptor distribution may be contributing to the lack of EF response in ASD cells, qPCR experiments are being conducted on all NPCs for the following receptors: PAC1, TRK1,2&3 (receptors for NGF, BDNF, and NT3), FGFR2, 5HT2A, 5HT1A ,5HT1C, and 5HT7. Primers have already been designed and received for all these genes. Currently, cells have been collected in trizol and RNA has been extracted from 2 clones of Sib and ASD from Family-1. RNA was converted into cDNA and these samples were used to run qRT-PCR studies. The results are presented in the appendix and show that most receptors tested are present in the cells however there are statistical differences in receptor mRNA expression between Sib and ASD. For example, both TRKA and PAC1 receptor expression is significantly lower in the ASD cells while TRK2, FGFR2, 5HT2A and 5HT7 receptors were normal. 5HT1C and 1A expression was not detected in any clone including Sib and interestingly the TRK3 receptor expression was 4-fold higher in the I-ASD-1 NPCs. The lower expression of PAC and NGF receptors could be contributing to the lack of EF responses seen in our patients, however, my studies showed that targeting the mTOR pathway in ASD-1 NPCs allowed for EF responses to occur. This suggests that either sufficient receptors are present on the ASD cells to signal through these EFs and signaling defects are the primary cause of EF response defect or that correcting the mTOR defects corrects the receptor level defects thereby allow for cells to respond. To tease these two possibilities apart, qPCR could be conducted on RNA derived from ASD cells treated with SC-79 to see if receptor expression is normalized in these cells compared to vehicle treated cells. For the NPCs, RNA has been collected for 2 clones each of Sib and ASD from Family-2, cells are in Trizol for the 2 male 16pdel patients and Family-3 NPCs.

Proteome and RNA-Sequencing

In addition to these studies, I have been collecting protein from Family-1, 2 & 3, along with 16pDel M-1 & M-2 for proteomic analyses. Thus far, protein has been collected in control conditions for all patients except for Sib-3 and ASD-3. For Family-1, PACAP stimulated cells have also been collected for protein. We have already discussed study design and methods of analysis with the Lobel lab who will be conducting mass spectrometry on these cells to analyze the overall proteome and phospo-proteome of these cells. This will allow to see the global signaling dysregulations that may be occurring in our cell and represents an unbiased complementary approach to the western studies I have already conducted. In addition, the RNA samples collected for q-RT-PCR were collected in parallel to the proteome samples for all patients. Thus, RNA-sequencing analyses can be conducted and compared to the proteome. Discussions have already been started between our lab, the Millonig and Rasin labs for conducting this RNA-Seq analysis.

Analysis and Identification of Aberrant Genes in I-ASD

Genome sequencing of our patients has already been completed by the Brzustowicz lab as part of their larger study of ASD and SLI. Recently, the data for our specific patients were pulled and it is currently being analyzed for SNV, CNVs, and SNPs. Based on this data, studies could be conducted to assay whether these genetic changes are reflected in our NPCs by q-RTPCR. Then, aberrant genes can be manipulated to assess whether any of them individually are contributing to NPC phenotypes. Of course, while highly penetrant de novo mutations in genes can be causative in I-ASD, in some cases, low penetrance polygenic factors are what contribute to disease. Thus, in the polygenic cases, genetic alteration may not necessarily be the best mechanism to rescue or study I-ASD phenotypes.

Studying SLI NPCs:

Recently, we acquired NPCs from 1 clone of the SLI patient from Family-1. NPCs were expanded, and studies were set up to assess neurite outgrowth and migration in these lines. Preliminary neurite studies have suggested normal EF response and neurite outgrowth in these cells! However, further studies need to be conducted to see if these results replicate. These studies will help us determine the developmental similarities and differences between language disorders and ASDs which often have a language component.

Future Directions:

Studying other I-ASD patients:

As mentioned, there are 8 total families in our idiopathic cohort. Of these 8, I characterized 3 randomly selected patients for my studies. The remaining 5 patients are uncharacterized and of these five, 1 is a female-female pair! It would be interesting to screen these patients to see if neurite and migration defects are common in our cohort. Moreover, it would be interesting to see if similar signaling profiles are also found in these cells. In addition, each family also has 1 SLI individual from whom we have acquired blood samples. These SLI iPSCs are being generated and, as mentioned, the SLI individual from Family-1 already has NPCs generated. In each Family, it would be interesting to see whether SLI individuals look like Sib, ASD, or an in-between. This would give better insight into how language disorders and ASD are similar or different and whether similar neurobiological processes are contributing to these disorders. Lastly, if more patient's samples could be acquired from the Simons VIP cohort, these individuals could be studied too.

The effect of other EFs on NPCs:

One avenue of exploration, which could be a good project for future undergraduates, is

testing additional EFs on our NPC lines. This includes factors such as vasopressin, NT3 and IL-17a. While oxytocin studies were conducted, due to time constraints, vasopressin which was purchased and aliquoted were not tested on NPCs. Unlike OXT, vasopressin and the receptor for vasopressin appear in the developing brain around mid-neurogenesis in the mouse and rat brain .[852] Thus, it is likely, that vasopressin plays a more important role in development than oxytocin. Moreover, clinical studies are being piloted for vasopressin treatment of ASD and thus, these studies could be potentially insightful. NT3 is of interest because the TRKC receptor expression level is 4-fold higher in our ASD-1 NPCs indicating that perhaps, ASD NPCs may be compensating for lack of response to other EFs by increasing TRKC receptors and NT3 response. In Sib NPCs NT3 did not elicit any responses, however NT3 was not tested in ASD NPCs. Furthermore, BDNF, did elicit a neurite response in ASD-1 NPCs but not in Sib suggesting differential neurotrophin responses in Sib vs ASD. The reason for the differential response is currently undefined, however, relative differentiation of the cells or cell fate could be playing a role. Likewise, which signaling pathways are active and the level of receptors could of course also play a role. Il-17a, is a cytokine that has been implicating in contributing to fetal brain defects and autism like symptoms in pups in models of maternal immune activation [853, 854]. One experiment I conducted on Il-17a effects on neurite outgrowth showed that Sib NPCs had a decrease in neurite outgrowth with Il-17a exposure suggesting that this cytokine leads to ASD-like neurite outgrowth in the Sib. Interestingly, in I-ASD and 16pDel NPCs, Il-17a led to increases in neurite outgrowth. Cell numbers were unaffected in I-ASD and Sib but reduced in the 16pdel NPCs. Thus, IL-17a seems to have interesting effects on our cell and has differential effects in each patient types. Lastly, other EFs which I have not yet piloted

could be tested on our cells including other neurotransmitters like dopamine and norepinephrine or neuropeptides like Osteocrin and VIP. These studies could help unveil the potential developmental relevance of these molecules in a human system and help understand whether ASD patients have differential responses to these regulators.

Other Metabolomic Analyses and Experiments

As mentioned metabolomic studies were only conducted in 1 Family in our idiopathic cohort. Thus far, almost no iPSC studies have analyzed the metabolism of neural cells derived from individuals with neuropsychiatric diseases. In general, metabolite studies in these disorders have been conducted on urine or blood. Thus, our metabolome studies place us in a unique uncharted territory in the iPSC field. Moreover, even in Family-1 the breadth of metabolic data we acquired has not been used to it's potential. While I have taken a small look into the purine/pyrimidine metabolic abnormalities in Family-1 NPCs, there are many other pathways such as lipid and amino acid metabolism that can be targeted for further analysis. Moreover, it would be useful to re-run metabolomic or a select metabolic profiling in additional clones in this family. With regards to purine/pyrimidine metabolism, the more specific PNP inhibitor drug studies were only conducted once and could be replicated in more ASD clones and done in a Sib clone to see if reducing degradation of purines and pyrimidines helps ameliorate neurite and migration defects. Moreover, enzymatic ELISA tests could be run to check the level and activity of the enzymes which are suspected to be dysregulated based on the metabolite maps. Interestingly, as discussed, there is interplay between the mTOR pathway and many metabolic pathways, including purine/pyrimidine metabolism, lipid metabolism, and amino acid metabolism. In particular, in our low mTOR cells (Family-1 &3 ASD NPCs) we see lower levels of

ATP/ADP which could be contributing to a cAMP deficit in these cells. This cAMP deficit then prevents the cells from being able to respond to cAMP based EFs such as 5-HT and PACAP as suggested by my db-cAMP rescue studies. In these cells, targeting and fixing mTOR abnormalities allows for PACAP to stimulate P-CREB levels suggesting potential "normalization" of the PKA pathway. Thus, it would be interesting to see if treating ASD-1 & 3NPCs with SC-79, the AKT activator drug, reverses the metabolic defects seen in our cells. Of course, it is difficult to tell if ATP dysregulation feeds into the mTOR phenotype or if it is the mTOR defect that leads to metabolism issues. Thus, it would also be interesting to see if providing cells with permeable trinucleotides would help ameliorate mTOR and neurobiological defects. These studies would not only provide an interesting perspective on the interaction between metabolism and signaling but would also illustrate how these processes are contributing to developmental dysregulation.

Studying Post-Mitotic Neurons and NPC Differentiation:

Of course, our studies have been conducted exclusively on NPCs, it is logical to propose studies on post-mitotic neurons derived from our patients! These studies would allow us to see if, for example, neurite outgrowth defects seen in NPCs translate into defects in neurons. We would also be able to assess whether signaling defects noted in NPCs are also found in the differentiated neurons. Moreover, neurons allow us to assess other developmental parameters such as synapse formation, neuronal polarization, connectivity and electrophysiological parameters. Generation and early characterization of neurons is currently underway in the Millonig lab.

In addition to studying terminal differentiation, it would also be valuable to study the differentiation of NPCs over time utilizing the neurite paradigm. As discussed, 2 out of

3 16p11.2 del patients showed a curious increase in neurites with passage. Specifically, M-2 had about a 2-8% increase in neurites with every passage such that P3 NPCs were around 3-5% neurites but by P7 neurites ranged from 20-305%! The female also showed similar increases. This steady increase in neurites over time was not observed in any other line as reproducibly or clearly. Likewise, all three 16p lines also exhibit decreases in proliferation over passage. This indicates that there is some change in the 16p NPCs from proliferative progenitor to non-proliferative neuron/glia over time. As other lines do not have this issue, it is possible that 16p11.del NPCs are differentiating faster or prematurely. Indeed, an accelerated differentiation and early exit from the cell cycle were observed in the mouse model of 16p11.2 deletion [608]. Recently, I piloted a study where Sib and I-ASD NPCs were plated at low density (neurite conditions) in dishes for either 2,4 or 6 days. This allowed me to monitor neurite outgrowth and differentiation of the cells over time. In this preliminary study, Sib NPC at days 2,4 and 6 had the following neurites: 15%, 27, % 42%, while ASD NPCs: 5%, 15%, and 30%. By day 4, in Sib NPCs, neurites had greatly elongated, and the presence of flat astrocyte-like cells became apparent. By D6, in Sib culture, there were hardly any small compact NPCs and all cells were either flat "astrocytes" or neurons/immature neurons with long and sometimes branched processes. In I-ASD, glia-like cells and long neurites did not appear until D6, suggesting slower differentiation. Interestingly, comparison of these experiments to 16pDel M-2 NPCs at P6 cultured for 2 days showed interesting phenotypes. By 2 days in culture, high passage 16p NPCs already showed 25% neurites (mirroring D4 Sibs) and showed some "glial cells". Low passage from the same patient and induction, however, only had 5% neurites and no glial cells. This suggests that 16pDel NPCs may have an accelerated differentiation

phenotype. In the future, these 2,4,6-day assays can be set up with the 16p lines both at low and high passage to see if they have an "accelerated" differentiation over time in culture and by passage. Moreover, immunocytochemistry will need to be conducted to determine the identity of these cells over time. For example, stains will need to be done for NPC markers likes Nestin and PAX-6, immature neuronal markers like TUJ1, mature neuronal markers such as Tau, and astrocyte markers such as S100β or Aldh1a1 to determine the proportion of these cells over time. Moreover, it would help ascertain of the "glial" cells that appear are indeed astrocytes. Ultimately, these studies would help us gain a more dynamic view of the neurite and differentiation defects seen in our cells.

Genetic Manipulation of the 16p11.2 Locus:

With the 16pdel NPCs, there are 27-29 genes known to be deleted in the locus. According to Monal Mehta's gene expression work from the Millonig lab, 11 of these genes are expressed in NPCs. Moreover these 11 genes are expressed at 50% levels when compared to NIMH controls and Sibs. Thus, it is possible to increase expression of the genes in 16pDel NPCs, one at a time, with different viral vectors to see if defects such as neurites or migration can be manipulated by changing one gene. Moreover, in Sib and NIMH, gene expression can be reduced with siRNA or other methods to see if neurite or migration defects can be recapitulated by altering a single gene. In one study conducted in a zebrafish model, altering the gene KCTD13 alone led to the phenotypes associated with 16p11.2 deletion and duplication [792]. No other genes in the locus faithfully recapitulated the macrocephalic and cortical phenotypes observed in 16p11.2 deletion. Thus, similar findings may be observed in our human cells. These studies would help elucidate the developmental roles of the genes in the 16p11.2 locus.

Bench to Bedside?

iPSCs are a wonderful way to model human development in a simplified system. However, as I discussed in the previous chapter, how results gained from iPSCs translate to the clinic is still largely unclear. Moreover, since human neurons could not really be studied directly from the brain in live culture, there is also no way to confirm that iPSC-derived cells are faithfully recapitulating human neurobiology. In some sense, the data we have acquired from our patients could reflect the status, severity, or phenotypes of their disease as discussed in the previous chapter. Likewise, EF studies in our patients could help uncover potential "personalized" medical therapeutics for our patients. Indeed, some studies have begun to correlate phenotypes or responses of cells in the dishes to patient outcome. For example, proliferation rate of patient-derived NPCs has been correlated to brain size. Likewise, rescue of neuronal defects by IGF-1 in Rett syndrome iPSC models have been associated with positive outcomes for IGF-1 in clinical trials with Rett syndrome. In our patients, we find that EF responses are different between 16pdel NPCS and I-ASD NPCs. It would be interesting to see if, for example, reduced sensitivity to 5-HT in I-ASD leads to a reduced response to 5-HT base drugs like antidepressants in our patients. Neurite defects are common in our patients, these defects could indicate altered white matter tracts or changes in "connectivity" in the brain. It would be interesting, thus, to conduct MRI and fMRI studies on our patients to see what their brains look like. In the case of our 16p11.2 del patients, MRI scans are already available and thus data could potentially be accessed through the Simon's Foundation. Furthermore, as mTOR defects are seen in our patients, it would be interesting if we could test already available mTOR drugs on our patients (and other patients like them) in a clinical trial. Additionally, testing of drugs like caffeine which increases cAMP, could also be interesting and valuable. Of course, these experiments are

not feasible to do in our lab alone. First, the patient cohort we have studied was not recruited by us and thus, the status of these patients is currently unclear and many could be lost to follow-up. Secondly, our lab is not suited to conducting clinical trials or MRI studies. But in the future, through collaboration such studies may be feasible. These studies would be incredibly valuable as they would allow us to see if what we learn in iPSCs really reflects patient biology and whether it can influence the treatment and well-being of the patient in true "bench to bedside" fashion.

Last year, I submitted a grant to ASF based on the oxytocin studies I conducted. In this grant, I proposed that NPC response to oxytocin made a patient more likely to respond to OXT as a therapeutic treatment. Currently, Karen Parker's lab at Stanford has been conducting trials on ASD patients using both oxytocin and vasopressin. Of course, with the heterogeneity of ASD, it is likely difficult to get clear cut results on these drug trials. One potential avenue of collaboration would be to get blood cells from children enrolled in the vasopressin/oxytocin clinical trials. Then, these cells could be reprogrammed into NPCs and then tested on their ability to respond to these neuropeptides. Then, we could determine whether NPC response does correlate to patient response in a trial! This would help us determine if iPSCs are a good way to screen drugs for potential human use.

In addition to drug screening, iPSCs can be used to screen high-risk individuals for the potential onset of disease. For example, studies have shown that younger siblings of a child with ASD have almost a10-20X higher risk of developing ASD. Thus, in our cohort, if any Families chose to have a new child, cord blood could be banked from the newborn sibling and reprogrammed into iPSCs. We could then compare these iPSCs to their already affected Sibling to determine if the child will go onto develop ASD. Thus, iPSCs could be

an early screening and diagnosis tool for ASD. This approach could be used in other cohorts or by other groups too, however, the appropriate controls would need to be utilized.

Conclusion:

Ultimately, my studies are the initial foray of the DiCicco-Bloom lab into the world of human iPSC derived neural cells. Based on currently literature, my studies are one of the first to deeply investigate the neurobiology of NPCs in ASD iPSC-derived cells. My results have shown that early neurodevelopmental processes are indeed dysregulated in ASD, indicating the importance of studying NPCs. Moreover, despite disease heterogeneity, our six patients, who have different genetic backgrounds and severities of ASD, showed common aberrations in neurite outgrowth and migration. By designing studies that use EFs, I showed that we could distinguish between ASD subtypes by EF responses. In addition to these cellular alterations, I studied signaling pathways in our NPCs to find two distinct "signaling profiles" in our cells. This molecular characterization divided our patients into two subgroups. ASD-1 and ASD-3 NPCs had decreased P-AKT and P-S6, higher levels of P-ERK, and blunted PACAP stimulated P-CREB. I-ASD-2 NPCs and 16pdel NPCs had normal P-AKT, normal PACAP stimulated P-CREB, lower P-ERK and higher P-S6. These alterations then helped me select agonist and antagonist drugs to manipulate signaling systems to see if neurobiological defects could be ameliorated. Indeed, by agonizing mTOR pathway in ASD-1 NPCs, neurites, migration, and EF defects were fixed. Likewise, antagonizing mTOR activity in I-ASD-2 NPCs successfully rescued neurite and migration defects. In the end, these studies show us that autism NPCs have changes in numerous different cellular processes which seem to converge onto alterations in mTOR signaling. Moreover, these dysregulations can be reversed by a precision medicine approach. My

studies have laid the foundation for numerous avenues of further inquiry, some of which I have presented above. In the future, we will hopefully know whether the defects we see in the dish pertain to brain pathology and function in our patients. Moreover, we will learn whether molecular abnormalities seen in NPCs reflect patient biology and whether targeting these abnormal pathways in people with ASD will lead to better management and treatment. In the end, iPSC technology has opened up a wonderful way to study and characterize human neuropsychiatric disorders. Moreover, it has given me the opportunity to deeply understand and characterize human neurobiology in ASD.

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