IPSC-DERIVED NEURONS AS A MODEL FOR STUDYING THE ROLE OF RELN IN AUTISM

Author: Ryan Mokhtari

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Approved __________________________

(Sponsor’s signature)

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Abbreviations

ADHD: Attention Deficit Hyperactivity Disorder
ANOVA: Analysis of Variance
APA: American Psychiatric Association
ApoER2: Apolipoprotein E Receptor 2
ASD: Autism Spectrum Disorder
CCM: Control Conditioned Media
CDC: Centers for Disease Control and Prevention
CNV: Copy Number Variation
CP: Cortical Plate
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CRMP2: Collapsin Response Mediator Protein 2
Ct: Threshold Cycle
DEG: Differentially Expressed Gene
DMSO: Dimethyl Sulfoxide
DNM: De Novo Mutation
DSM: Diagnostic and Statistical Manual of Mental Disorders
EAGLE: Evaluation of Autism Gene Link Evidence
EEG: Electroencephalography
ER: Endoplasmic Reticulum
FDR: False Discovery Rate
GABA: Gamma Amino Butyric Acid
GAD: Glutamic Acid Decarboxylase
GO: Gene Ontology
GSK3β: Glycogen Synthase Kinase 3β
GWAS: Genome Wide Association Study
ID: Intellectual Disability
IF: Immunofluorescence
iPSC: Induced Pluripotent Stem Cell
KO: Knock Out
LCH: Lissencephaly with Cerebellar Hypoplasia
LGD: Likely Gene Disrupting
MAP2: Microtubule Associated Protein 2
mGluR5: Metabotropic Glutamate Receptor 5
MRI: Magnetic Resonance Imaging
MRS: Magnetic Resonance Spectroscopy
mTOR: Mammalian Target of Rapamycin
Mtr: Maternal
MZ: Marginal Zone
NGN2: Neurogenin 2
NPC: Neural Progenitor Cell
NPY: Neuropeptide Y
NT3: Neurotrophin 3
Pbd: Proband
PBMC: Peripheral Blood Mononuclear Cell
PBS: Phosphate Buffered Saline
PCA: Principal Component Analysis

PI3K: Phosphatidyl Inositol 3 Kinase

Ptr: Paternal

qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction

RCM: REELIN Conditioned Media

RGC: Radial Glial Cell

RXR: Arginine Amino acid Arginine

SBM: Surface Based Morphometry

SFARI: Simons Foundation Autism Research Initiative

SNP: Single Nucleotide Polymorphism

SNV: Single Nucleotide Variant

UTR: Untranslated Region

VGLUT1: Vesicular Glutamate Transporter 1

VLDLR: Very Low Density Lipoprotein Receptor

VZ: Ventricular Zone

WB: Western Blotting
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Abstract

iPSC-derived neurons as a model for studying the role of \textit{RELN} in autism

Author: Ryan Mokhtari
Sponsor: Dr. Brian Howell

\textit{RELN} is strongly associated with Autism Spectrum Disorder (ASD). Homozygous loss of the encoded protein REELIN is associated with severe neurodevelopmental phenotypes characterized by lissencephaly and cerebellar hypoplasia, yet the ASD-linked variants are typically heterozygous and appear to require additional genetic risk to cause ASD. To functionally characterize a \textit{RELN} variant in a patient with ASD, we used induced pluripotent stem cells (iPSCs) from a family of non-autistic parents and their son who had ASD (the proband). The proband has a maternally-inherited missense variant (R2457C) in the RXR motif of the REELIN protein. We differentiated the iPSCs into two types of neurons, inhibitory neurons which model the inhibitory forebrain neurons that secrete REELIN, and excitatory neurons which model the cortical pyramidal neurons that respond to REELIN. Immunoblotting revealed that the proband inhibitory neurons had a lower ratio of extracellular/intracellular REELIN compared to that of the parental neurons, suggesting a decreased REELIN secretion. Sholl analysis on the proband excitatory neurons showed reduced dendritic complexity and reduced total length compared to the parental neurons. REELIN treatment increased the dendritic length and complexity in proband neurons up to
the level of parental neurons. CRISPR/Cas9-mediated RELN KO did not change the dendritic phenotype in the excitatory neurons, ruling out a cell autonomous role for REELIN in these neurons. The proband excitatory neurons also had lower mRNA expression of WNT target genes in response to WNT3a, suggesting an underactive WNT signaling, as well as higher total GSK3β protein and lower phosphorylation at the inhibitory S9 site, indicating an overactive GSK3β signaling. Inhibition of GSK3β improved the proband neurons dendritic complexity in the proximal parts of the dendritic arbor. However, inhibition of mTOR signaling, which has shown to regulate REELIN signaling, did not change the dendritic morphology. In conclusion, the pathophysiology of ASD in the proband likely consists of a reduced REELIN secretion from the inhibitory neurons and an additional vulnerability in the REELIN-responding excitatory neurons, the latter likely being an overactive GSK3β and an underactive WNT signaling, all of which result in reduced dendritic complexity.
Chapter 1

Introduction

Contribution:

Ryan Mokhtari did all the experiments and wrote this chapter. Brian Howell generated the RELN KO cell lines, supervised the experiments, and edited the text.
Autism is a psychiatric disorder with a strong genetic component. The genetic underpinning of autism is complex, however, which means a single gene variant is not usually sufficient to cause the clinical phenotype. In order to understand how genes contribute to autism pathophysiology, it is useful to study gene variants associated with autism in the context of other abnormalities at the genetic and cell biological levels and discover the ways in which different risk factors interact with each other.

### 1.1. Definition and clinical manifestations of autism

According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, Text Revision (DSM-5-TR) (APA, 2022), **Autism Spectrum Disorder (ASD)** is defined by the following two criteria. First, having persistent deficits in social communication and social interaction across multiple contexts, including deficits in social-emotional reciprocity, nonverbal communicative behaviors, and developing, maintaining, and understanding relationships. Second, having restricted, repetitive patterns of behavior, interests, or activities, including stereotyped motor movements or speech, highly restricted, fixated interests that are abnormal in intensity or focus, insistence on sameness, inflexible adherence to routines or rituals, and hyper- or hypo-reactivity to sensory input or unusual interest in sensory aspects of the environment.

Symptoms are usually present in the early developmental period, but may not become fully manifest until social demands exceed limited capacities, or may be masked by learned strategies in later life. Language impairment or intellectual disability do not have to be present, but both frequently co-occur with ASD (APA, 2022).
1.1.1. The “spectrum” nature of autism

The word “spectrum” was added to autism diagnosis in the DSM-5 to recognize previously separate diagnoses such as Asperger’s disorder as different variations of the same condition (APA, 2013). However, studying autistic traits in the general population has revealed that the spectrum nature of autism extends beyond the clinical definitions, since subthreshold autistic traits are reported in a significant proportion of the population (Lundström et al., 2011; Robinson et al., 2011), sometimes called the Broader Autism Phenotype (Sucksmith et al., 2011). There is some evidence that this population-level continuity has parallels at the genomic level as well (Bralten et al., 2018).

1.2. Epidemiology

1.2.1. Prevalence

The Centers for Disease Control and Prevention (CDC) estimates that one in 36 children in the United States have ASD (CDC, 2023). ASD is reported to occur in all racial, ethnic, and socioeconomic groups (Maenner et al., 2021). A recent systematic review estimates that globally, one in 100 children are diagnosed with ASD (Zeidan et al., 2022). The reported prevalence of autism was much lower in the 20th century, as low as 5 in 10,000 in the 1980s (Gillberg et al., 1991).

There has been much debate about the reason behind this dramatic rise in the reported prevalence of ASD. Many experts believe that the apparent increased prevalence does not reflect a real increase in the incidence. Instead, it reflects a heightened awareness among clinicians and families as well as expanding the diagnostic criteria which would qualify more patients for receiving healthcare (Newschaffer et al., 2007; Blumberg et al., 2013).
However, some experts have pointed out that the increased detection and expanding criteria might not explain the entire trend. For example, Hansen et al. (2015) who followed up a population cohort of Danish children for more than two decades, concluded that changes in reporting and diagnosis accounted for only 60% of the increased prevalence of ASD (Hansen et al., 2015).

1.2.2. Environment is unlikely to account for ASD rise

The steep rise of ASD in a short period of time has made some researchers think environmental factors must be responsible (Windham et al., 2006). However, evidence for such causal effect is scant (Newschaffer et al., 2007; Modabbernia et al., 2017). Moreover, the potential environmental candidates would likely harm the developing brain in a rather indiscriminate way, causing a broad range of neurodevelopmental disorders, including intellectual disability (ID) (Lanphear 2015). Yet, the prevalence of ID has declined over the same period of time in which ASD rate has risen (Polyak et al., 2015), which suggests that some children that would have been diagnosed with ID in the past are now more likely to be diagnosed with ASD.

1.2.3. Sex distribution

ASD is more common among boys, with a male-to-female ratio of 4 to 1 (Maenner et al., 2021; Zeidan et al., 2022). However, this ratio is as high as 8 to 1 when individuals with Asperger syndrome or high-functioning autism are included (Brugha et al., 2011), and even higher (11 to 1) if this latter group is studied exclusively (Gillberg et al., 2006). With similar risk profiles, males have lower thresholds than females for meeting diagnostic criteria, which is known as the “Female Protective Effect” hypothesis (Jacquemont et al., 2014).
Many of the psychological and neuroanatomical phenotypes of ASD are those traits that are considered sexually dimorphic (Baron-Cohen et al., 2005), and ASD appears as an exaggerated form of the male brain, which is the basis for the “Extreme Male Brain” theory of autism (Baron-Cohen et al., 2010). Differential effects of sex hormones on the prenatal brain development are thought to underly this dimorphism (Nguyen et al., 2017). For example, there is evidence that fetal testosterone levels predict local gray matter volume of specific brain regions (Lombardo et al., 2012), and that fetal testosterone levels positively correlate with autistic traits later in life (Auyeung et al., 2009).

However, recent findings portray a more complex picture, since fetal levels of other sex steroids, including estrogen and progesterone, also positively correlate with the risk of autism (Baron-Cohen et al., 2015; Baron-Cohen et al., 2020). Moreover, autism was reported to be higher among people who identify as transgender or gender-diverse (Warrier et al., 2020), a finding that is not easy to interpret, given our limited knowledge of the neurobiological basis for gender identity (Hines, 2011).

1.3. Etiology and pathophysiology

1.3.1. Heterogeneity

ASD is a heterogeneous disorder, and different risk factors have been proposed for ASD (Buxbaum & Hof, 2013). The heterogeneity of ASD is manyfold, i.e., different types of risk factors (e.g., genetic, environmental, prenatal) may play causal roles, yet each category contains a multitude of potential risk factors.

Dividing ASD risk factors into categories may be an oversimplification, not only due to the presence of complex gene-environment interactions, but also due to the fact that some
risk factors that are considered environmental may act through a genetic mechanism. For example, advanced age of fathers at conception is thought to increase the risk of ASD through higher rates of de novo mutations (Kong et al., 2012) (see below).

Autism, either as ASD or as subclinical autistic symptoms, can be a manifestation of another neurodevelopmental disorder, often a syndrome with a known genetic mutation (e.g., fragile X syndrome) (Fernandez & Scherer, 2017). Syndromic forms of autism typically include other physical and neuropsychiatric symptoms as well, e.g., intellectual disability (ID) (Thurm et al., 2019) and epilepsy (Besag, 2018). However, the latter two conditions are also common comorbidities in idiopathic autism, cases with no identifiable cause (Buxbaum & Hof, 2013).

1.3.2. Prenatal factors

1.3.2.1. Advanced age of parents

Advanced parental age at conception is associated with increased risk of several medical and psychiatric conditions, including ASD (Bergh et al., 2019). However, the uniquely steep rise in ASD prevalence in recent decades, which coincides with significant increase in average age of parents, has raised the possibility of a causal effect. Although there has been some evidence for the importance of maternal age in ASD risk (Larsson et al., 2005), most studies have focused on the age of father as the more relevant factor, arguing for the role of de novo mutations (DNMs) and their higher rate in older fathers (Kong et al., 2012). However, quantitative analyses have shown that the association of ASD with delayed fatherhood cannot be accounted for solely by DNMs. Instead, older fathers themselves might carry some genetic predisposition for ASD and possibly have autistic traits as well (Gratten et al., 2016).
1.3.2.2. Maternal immune activation

Infections during pregnancy, particularly in the first and second trimesters, increase the risk of autism later in life (Jiang et al., 2016). However, this correlation is non-specific on both directions, i.e., a wide range of viral or bacterial agents have been associated with the ASD risk, and ASD is not the only neurodevelopmental disorder that is associated with prenatal infections (Knuesel et al., 2014). These observations as well as findings from animal models (Smith et al., 2007) revealed that the infectious agents do not have a direct causal role. Rather, the activation of maternal immune system and increased levels of pro-inflammatory cytokines are the direct risk factors (Boulanger-Bertolus, et al., 2018). Other sources of prenatal stress, including psychosocial stress during pregnancy, are also associated with higher risk of ASD, likely through maternal immune activation (Beversdorf et al., 2018).

1.4. Structural and functional phenotypes

1.4.1. Brain size

There have been many studies on the relationship between brain size and ASD, using methods such as head circumference, postmortem brain weight, and magnetic resonance imaging (MRI). Some studies found that ASD is associated with increased head size (macrocephaly) (Sacco et al., 2015) as a result of brain overgrowth (Hazlett et al., 2011). Other studies, however, suggested that there is little difference between brain sizes of autistic individuals and healthy controls (Redcay & Courchesne, 2005). On closer examination, two factors may explain this discrepancy. First, the brain overgrowth in autistic children is limited to the first 2 – 4 years of life, after which the growth reaches a
plateau (or slightly declines in some cases), and then the brain growth curve closely resembles that of the general population (Courchesne et al., 2007; Courchesne et al., 2011a). Second, macrocephaly is observed only in a subset of individuals with ASD (around 20%), reflecting its heterogeneity, and there is a correlation between larger brain size and lower function in patients with ASD (Nordahl et al., 2011; Sacco et al., 2015). Although the mechanism behind the brain overgrowth is unknown, evidence suggests that it is partly due to an increased number of neurons (Courchesne et al., 2011b).

1.4.2. Neuronal morphology

Morphological abnormalities in both dendrites and axons have been observed in brains of autistic individuals (Fetit et al., 2021). The total number of dendrites was lower in dorsolateral prefrontal cortex (Mukaetova-Ladinska et al., 2004), and the dendritic branching was reduced in CA1 and CA4 areas of hippocampus in autistic individuals (Raymond et al., 1996). Also, anterior cingulate cortex had fewer long-range axons, more short-range axons, reduced axon thickness, and increased axonal branching in ASD (Zikopoulos & Barbas, 2010). Abnormalities in neurite morphology, outgrowth, and migration have also been observed in animal models of autism (Martínez-Cerdeño, 2017; Prem et al., 2020). For example, an animal model of Rett syndrome showed reduced complexity of dendritic arborization in hippocampal neurons (Nerli et al., 2020), and animal models of fragile X syndrome have shown reduced dendritic branching and abnormal morphology of dendritic spines in the cortex (Berman et al., 2012) and in spinal cord motor neurons (Thomas et al., 2008).

Studies on iPSC-derived neurons from patients with both syndromic autism (Fink & Levine
and idiopathic autism (Griesi-Oliveira et al., 2015) have shown abnormalities in the morphology of axons and dendrites. For instance, different copy number variations (CNVs) at the locus 16p11.2 are associated with ASD, and the iPSC-derived forebrain cortical neurons from those patients revealed abnormalities in dendritic length and soma size (Deshpande et al., 2017).

Abnormalities in dendritic spine morphology and density have also been associated with ASD, although the specific findings can be different depending on the types of studies (Martínez-Cerdeño, 2017). For example, postmortem studies on brains of autistic individuals have shown an increased density of dendritic spines in pyramidal neurons, particularly in layers II and V (Hutsler & Zhang, 2010; Weir et al., 2018), which was associated with lower cognitive functioning (Hutsler & Zhang, 2010).

Some animal models suggest that the density of immature dendritic spines increase, whereas the density of mature spines decrease in autism (Martínez-Cerdeño, 2017). Other animal models have found that spine density can be increased or decreased depending on the brain region (Bringas et al., 2013). There is also evidence from animal studies for insufficient pruning of excitatory synapses in layer V pyramidal neurons, which is due to impaired autophagy and overactive mTOR signaling (Tang et al., 2014; Pagani et al., 2021). However, a study on induced pluripotent stem cells (iPSCs)-derived neurons from idiopathic ASD patients showed lower synaptic density compared to control neurons (Marchetto et al., 2017). This finding is not surprising since iPSC-derived neurons are less mature than postnatal neurons (Prem et al., 2020), and there is no mechanism for synaptic pruning in vitro.
1.4.3. Neuronal Function

Various research findings converge on the *excitation-inhibition imbalance* of neurons as an underlying pathophysiology of ASD (Nelson & Valakh, 2015; Bozzi et al., 2018). For example, epilepsy is more common in people with ASD than in general population (Besag, 2018), and it is associated with more severe symptoms (Capal et al., 2018). Even in seizure-free autistic patients, EEG phenotypes such as epileptiform activity and increased gamma oscillations have been reported (van Diessen et al., 2015; Capal et al., 2018). The excitation-inhibition imbalance can also explain some sensory phenotypes of ASD, such as slower rate of binocular rivalry (Spiegel et al., 2019).

Dysfunction of both excitatory and inhibitory neurotransmission have been proposed as the primary mechanism for the excitation-inhibition imbalance (Nelson & Valakh, 2015). Evidence for the inhibitory origin of this imbalance in ASD are mostly based on human studies. For example, postmortem brain studies showed that parvalbumin interneurons were reduced in the prefrontal cortex (Hashemi et al., 2017), and that Purkinje cells had lower density in the cerebellum of individuals with ASD (Whitney et al., 2009). Magnetic resonance spectroscopy (MRS) imaging also revealed lower GABA levels in patients with ASD (Schür et al., 2016).

Evidence for the excitatory origin of the imbalance is mostly limited to the animal models of autism (Lee et al., 2017). Metabotropic glutamate receptor 5 (mGluR5) is thought to play a pivotal regulatory role in this context, being proposed as a unifying explanation for synaptic abnormalities in ASD (Zantomio et al., 2015). Different ASD-linked genes have converging effects on the activity of mGluR5, and interestingly, modulation of mGluR5 in
opposite directions are both associated with autistic phenotypes (Auerbach et al., 2011).

The excitation-inhibition imbalance was also manifested in a study by Mariani et al. (2015), in which iPSCs from patients with idiopathic autism were differentiated to neurons to make 3D cultures (organoids). The inhibitory neurons were overproduced in the patient-derived organoids compared to that of the controls (Mariani et al., 2015).

1.4.4. Macroscale connectivity

There is evidence for disrupted connectivity between different areas of the brain in ASD, both the structural connectivity, measured by diffusion MRI and white matter tractography (Ameis & Catani, 2015), and also functional connectivity, measured primarily by functional MRI (fMRI) (Müller et al., 2011). Specifically, local and short-range connections tend to be more dense in ASD, whereas long-range and interhemispheric connections tend to be more sparse; hence there is a local overconnectivity and a global underconnectivity (Kana et al., 2011; Schipul et al., 2011). Overconnectivity is more prominent in the frontal lobe (Courchesne et al., 2005), while underconnectivity is often between the frontal and parietal lobes (Just et al., 2007). Also, neuroimaging studies on autistic children have revealed functional connectivity abnormalities in the cerebellum (Khan et al., 2015). Moreover, there is correlation between the connectivity phenotypes and the cognitive and behavioral phenotypes in ASD (Ameis & Catani, 2015).

1.5. Genetics

1.5.1. Heritability based on pedigrees and twin studies

Twin studies have consistently shown a high degree of heritability in ASD. A meta-analysis of 13 twin studies calculated a 98% correlation between monozygotic twins and a 53-67%
correlation between dizygotic twins, with a 64-91% heritability estimate (Tick et al., 2016). Another study on a 16-year follow-up cohort in 5 countries estimated a heritability of 80% for autism (Bai et al., 2019). Heritability is defined by the proportion of phenotypic variability that is not explained by the environment (Knopik et al., 2017), the latter being divided into shared environment (same in a household) or non-shared environment (unique to each individual). In most behavioral traits, the non-shared environment (individual’s unique life trajectory) has much more impact than the shared environment (parenting style, socioeconomic status) (Plomin, 2011).

The most critical part of the non-shared environment in relation to ASD and other neurodevelopmental disorders is the prenatal conditions (Knuesel et al., 2014). The critical role of the prenatal period is reflected by the finding that the concordance rate of autism in dizygotic twins is twice that of non-twin siblings (Bohm et al., 2013). There are multiple mechanisms by which prenatal conditions contribute to the risk of ASD, including epigenetic changes due to stressful pregnancy (Beversdorf et al., 2018; Waye & Cheng, 2018) and also the environmental factors that increase the risk of de novo mutations (Pugsley et al., 2022).

1.5.2. De novo mutations (DNMs)

Mutations that occur in the germline are called de novo, since they do not exist in either parent’s genome. Every human has approximately 60 DNMs (also called spontaneous mutations) (Shendure & Akey, 2015), most of which (around 85%) originate in the paternal germ cells (Campbell et al., 2012; Francioli et al., 2015), and as the father’s age advances, the risk of DNMs increases (about two mutations per year) (Kong et al., 2012). This is due
to the different timing of paternal vs. maternal germ cell division, the former continually happening throughout adulthood, but the latter being near-complete at birth. The increased risk of DNMs in older fathers also accompanies the additional risk that they are more likely to fall on the protein-coding regions (Francioli et al., 2015). Whole-genome sequencing studies on families with one autistic child (*proband*, i.e., the subject of genetic study) and one unaffected child have shown that proband DNMs are more likely to be in the coding regions (Michaelson et al., 2012). Moreover, *likely gene-disrupting (LGD)* DNMs (nonsense, frameshift, or splice site altering mutations) are more common in probands than in non-autistic children (Turner et al., 2017).

The non-coding DNMs in ASD probands are enriched in the 5’ and 3’ untranslated regions (Yuen et al., 2016), and also enriched in the promoters and enhancers specific to prenatal neurodevelopment (An et al., 2018; Turner et al., 2017). Exome-sequencing studies have also shown that DNMs are more likely to be gene-disrupting in probands than in unaffected siblings (O’Roak et al., 2011; Iossifov et al., 2012). ASD-linked DNMs (both coding and non-coding) are often enriched in chromatin remodeling pathway (Yuen et al., 2016; Kim et al., 2022), which is among the most important pathways in ASD genetics. Overall, DNMs contribute to a significant proportion (at least 30%) of ASD risk (Yoon et al., 2021).

**1.5.3. Inherited variants: common vs. rare**

Defined as having population frequency of greater or less than 1% respectively, common and rare variants contribute differently to the risk of a complex genetic disorder such as ASD. Each common variant has only a small effect, thus many such variants need to be present in a genome to increase the risk, whereas rare variants have strong effects individ-
ually, so a few of them can be sufficient to cause the disorder (Schork et al., 2009).

This is explained by the degrees of negative selection pressure on common vs. rare variants, the latter being tolerated less and removed from the gene pool more quickly. However, rare variants cannot be completely eliminated, partly due to the presence of DNMs which are immune from the negative selection.

1.5.4. Rare variants: CNVs and SNVs

Rare variants can be inherited or de novo, and both categories are usually studied together and contrasted with common variants. In the context of ASD, many of the characterized rare variants have been copy number variants (CNVs), deletions or duplications larger than 1 kb (aka structural variations) (Vicari et al., 2019). Rare CNVs tend to be more common (Sebat et al., 2007) and larger (Pinto et al., 2014) in ASD, and one copy of a CNV is often sufficient to confer the risk (dominant effect) (Manoli & State, 2021). However, it is challenging to understand the mechanism of ASD-linked CNVs, since most of them are also associated with other psychiatric disorders such as schizophrenia and ADHD (Gudmundsson et al., 2019). Moreover, opposite structural variations at the same locus may present with the same phenotype. For example, both deletions and duplications at the locus 16p11.2 are associated with ASD (Deshpande et al., 2017; Niarchou et al., 2019).

Rare variants can also be single nucleotide variants (SNVs), and children with ASD have an excess of inherited truncating SNVs in the conserved genes (Krumm et al., 2015). Interestingly, the inheritance of these variants shows a mother-to-son bias (Iossifov et al., 2015; Krumm et al., 2015). This finding is in line with the Female Protective Effect hypothesis, because an unaffected mother with a risk variant could transmit the variant to
a son who would be more vulnerable to it, and to a daughter who would be protected from it, like her mother.

The largest ASD whole-exome sequencing study so far (Satterstrom et al., 2020) analyzed the likely disruptive rare variants (de novo and inherited) and identified 102 risk genes with statistically significant effects. Functionally, the genes were involved in either gene expression regulation (including chromatin modification and transcription factors) or neuronal communication (including synaptic function). However, the former group does not regulate the latter, nor do they align in their spatiotemporal expression profile. The regulatory genes had a prenatal bias and weak enrichment in the cortex, whereas the synaptic genes had a postnatal bias and strong enrichment in the cortex (Satterstrom et al., 2020). These two categories, i.e., transcription regulation and synaptic function, have consistently appeared as the top enrichment pathways in ASD studies (De Rubeis et al., 2014; Krumm et al., 2014).

1.5.5. Common variants

Despite the significant role of rare variants, the scientific consensus is that common variants contribute to a much larger proportion of the ASD risk (Gaugler et al., 2014; Manoli & State, 2021). However, finding SNPs (single nucleotide polymorphisms) that pass the genome-wide significance threshold was a longtime challenge due to underpowered GWAS (genome-wide association studies) (Anney et al., 2012). That was until 2019 when for the first time, 12 genome-wide significant loci were identified, using a national database of Danish population (Grove et al., 2019). Five loci were specific to ASD and seven loci were added using a method (Turley et al., 2018) that leverages data
from traits with overlapping genetic architectures, in this case, schizophrenia, major depression, and educational attainment. Functional annotation revealed that the ASD-associated common variants are enriched in regulatory elements predicted to be involved in fetal corticogenesis (Grove et al., 2019).

1.5.6. Monogenic and syndromic forms of autism

As a complex genetic disorder, ASD-linked variants follow a frequency-strength-number continuum, such that the risk of ASD is conferred by either “many common variants with weak effects” or “a few rare variants with strong effects.” At the common end of the continuum, the presence of many variants makes it difficult to pinpoint to a cause for ASD, hence these cases are called idiopathic autism. At the rare end of the continuum, a single variant with very strong effect is sufficient to cause ASD, in which case it can be called monogenic autism, assuming the gene or the genomic locus in known (Manoli & State, 2021). However, even the strongest ASD-linked gene variants do not have 100% penetrance (de la Torre-Ubieta et al., 2016), which means other genes or environmental factors may be necessary for the emergence of the clinical phenotype.

Syndromic autism is a subtype of monogenic autism, in which a well-defined set of clinical manifestations are present, often including somatic symptoms, reflecting the impact of the mutations on other organs. Autistic symptoms are only a part of the whole phenotype in syndromic autism. Among the best known examples are Rett syndrome, fragile X syndrome, and tuberous sclerosis (Szteinberg & Zoghbi, 2016).
1.6. The role of RELN gene in ASD

1.6.1. REELIN protein functions

RELN gene encodes a large secreted extracellular matrix protein (REELIN), which has several important functions in the developing and adult brain (D'Arcangelo et al., 1995; Hirotsune et al., 1995). The name is after the Reeler mouse, which has a ‘reeling’ gait due to a mutation in Reln, the mouse ortholog of human RELN. All vertebrates express Reln, but the expression is amplified in mammals, giving rise to more elaborate layering of the neocortex (Bar et al., 2000).

The distinct organization of neocortical layers depends on the secretion of REELIN by Cajal-Retzius cells in the marginal zone (MZ) (Ogawa et al., 1995). The early projection (pyramidal) neurons produced in the ventricular zone (VZ) migrate into and split the preplate (PP), forming the cortical plate (CP), which will eventually form the 6 layers of the neocortex. In the Reeler mouse, however, the pyramidal neurons cannot migrate and split the PP, and will be displaced outward, making a disorganized “superplate” instead (Sheppard & Pearlman, 1997). Injection of REELIN into the cortical explants from Reeler mouse partially rescued the PP splitting phenotype, suggesting a necessary but not sufficient role for REELIN (Nichols & Olson, 2010). Neocortex is developed in an inside-out fashion, i.e., each round of migrating neurons are positioned on top of the deeper layers that migrated earlier. This pattern is disrupted in the Reeler mouse (Caviness, 1982).

A similar but more elaborate process of neuronal migration and cortical lamination occurs in the human brain, which ultimately leads to extensive cortical folding and appearance of gyri and sulci (Llinares-Benadero & Borrell, 2019). Absence of cortical folding in humans
is a condition called lissencephaly (lit ‘smooth brain’), a subtype of which (lissencephaly with cerebellar hypoplasia, LCH) is associated with homozygous loss of RELN (Chang et al., 2007, Hong et al., 2000).

REELIN is also necessary for the growth and organization of cerebellum (Goffinet, 1983). Radial migration of Purkinje cells from the ventricular zone to the cerebellar cortex requires REELIN production from granule cells in the external granular layer (D'Arcangelo, 2014; Miyata et al., 2010). Migration defects and mispositioning of neurons also occur in the olfactory bulb (Hellwig et al., 2012) and the hippocampus (Zhao et al., 2004) in the Reeler mouse. Similarly, REELIN deficiency in a mouse model for temporal lobe epilepsy leads to dispersion of hippocampal granule cells (Heinrich et al., 2006).

Several models for the role of REELIN in neuronal migration have been proposed, such as being an attractant signal, a detach-and-stop signal, and a detach-and-go signal (D'Arcangelo, 2014; Jossin, 2020). However, REELIN functions appear to be more complex than once thought, and our understanding of its roles continues to evolve.

REELIN also promotes the neurite growth and branching in radial glial cells (RGCs) and pyramidal neurons during neocortical development (Figure 1.1). The Reeler mouse RGCs have severely disrupted basal processes, and exposure to REELIN increases the RGC branching as well as the dendritic complexity of migrating cortical neurons (Chai et al., 2015; O’Dell et al., 2015). Dendritic development in the hippocampal neurons also requires an intact REELIN signaling (Matsuki et al., 2008; Niu et al., 2004).

Postnatally, however, REELIN has inhibitory effects on dendritic growth and complexity in cortical pyramidal neurons (Chameau et al., 2009) and cortical interneurons (Hamad et
al., 2021). However, in the postnatal and adult brain most of REELIN production occurs only in a subset of interneurons (Pesold et al., 1998), and the main role of REELIN is the regulation of synaptogenesis (Bosch et al., 2016), synaptic plasticity (Pujadas et al., 2010), and the excitation-inhibition (E-I) balance (Bouamrane et al., 2016).

At the molecular level, most REELIN functions are mediated through the canonical REELIN signaling, which consists of REELIN binding to the membrane receptors VLDLR (very-low-density lipoprotein receptor) and ApoER2 (apolipoprotein-E receptor-2) (D'Arcangelo et al., 1999) and the resulting dimerization and phosphorylation of the intracellular receptor-associated adaptor DAB1 (Disabled-1) (Howell et al., 1999). DAB1 phosphorylation in turn activates multiple downstream pathways, including its own degradation which is necessary for the function of REELIN on cortical layering (Feng et al., 2007). One of the key REELIN-DAB1-activated pathways is the Crk-CrkL/C3G/Rap1 pathway (Matsuki et al., 2008; Park & Curran, 2008), which regulates the orientation of migrating multipolar neurons towards the pial surface by stabilizing N-cadherin on the neuron membrane (Jossin & Cooper, 2011).

REELIN-induced DAB1 phosphorylation also activates PI3K (phosphatidylinositol-3-kinase), which is required for neuronal migration, since inhibition of PI3K in vitro disrupts the cortical plate (Bock et al., 2003). Activated PI3K triggers the phosphorylation and activation of AKT (Protein kinase B), which is also necessary for the organization of the cortical plate (Jossin & Goffinet, 2007).

The PI3K/AKT pathway subsequently activates mTOR (mammalian target of rapamycin), which is required for the growth and branching of dendrites of hippocampal neurons (Jossin
The mTOR signaling itself regulates the REELIN signaling by changing the levels of phosphorylated DAB1 through the ubiquitin ligase CUL5 (Moon et al., 2015). The mTOR signaling is an important regulator of protein synthesis (translation) (Lipton & Sahin, 2014), and protein synthesis is among the pathways that are frequently enriched in ASD genetic analyses (Santini & Borgkvist, 2020).

GSK3β (Glycogen Synthase Kinase-3β) is another protein regulated by the REELIN/DAB1/PI3K/AKT pathway (Ohkubo et al., 2003). GSK3β is an important signaling molecule at the intersection of several pathways such as canonical WNT signaling, which is one of the pathways most frequently associated with ASD in gene enrichment analyses (Krumm et al., 2014; Packer, 2018). GSK3β also regulates neurodevelopmental processes implicated in ASD, e.g., neuronal migration and synaptic function (Hur & Zhou, 2010). Although no GSK3β mutations have been reported in ASD patients, GSK3β overactivation has been associated with autistic-like behaviors in animal models (Mines et al., 2010; Zhang et al., 2012), and with ASD-linked cellular phenotypes in vitro (Kim et al., 2006; Rui et al., 2013).

### 1.6.2. RELN mutations in ASD

According to the Simons Foundation Autism Research Initiative (SFARI), RELN is a high-confidence ASD gene (category 1), which means it is “clearly implicated in ASD” with at least three reported de novo likely-gene-disrupting mutations (SFARI Gene, 2023). RELN is among the top 100 ASD genes based on the EAGLE (Evaluation of Autism Gene Link Evidence) scoring system (Schaaf et al., 2020). The EAGLE score reflects the strength of evidence for involvement of a gene in the pathophysiology of ASD as well as
the specificity of a gene to ASD exclusively. A gene with strong ASD association which is also implicated in other neurodevelopmental disorders receives a moderate (rather than high) EAGLE score. This is the case with RELN, since it is also associated with disorders such as schizophrenia and epilepsy (Chen et al., 2017).

This should not come as a surprise, given the crucial roles of RELN in neurodevelopment and synaptic function. In fact, homozygous loss of RELN is associated with lissencephaly with cerebellar hypoplasia (LCH), a severe disorder in which neuronal migration defects prevents the formation of cortical gyri and sulci, and in which cerebellum and several other brain structures are significantly underdeveloped (Chang et al., 2007, Hong et al., 2000).

Milder forms of lissencephaly have been reported in a few patients with heterozygous RELN mutations, some of whom had autistic symptoms (Di Donato et al., 2022). Also, more subtle forms of cortical folding abnormalities which are detected by surface-based morphometry (SBM) have been found in individuals with ASD (Nordahl et al., 2007; Pappaianni et al., 2018).

Cerebellar hypoplasia in LCH indicates that REELIN is necessary for cerebellar growth. This finding may be relevant to ASD pathology as well, since there is evidence from postmortem studies that REELIN expression is lower in the cerebellum in autistic individuals (Fatemi et al, 2001). The dependence of cerebellar growth on REELIN is also reflected by the finding that Purkinje cells are lost even in the heterozygous Reeler mouse, which does not have most phenotypes of the homozygous Reeler (Hadj-Sahraoui et al., 1996). However, this effect was observed only in the male mice, which can be relevant to the Female Protective Effect in ASD (Jacquemont et al., 2014).
Most *RELN* variants that are associated with ASD are heterozygous rare variants, and 207 unique rare variants (including 26 de novo) have been reported as of 2023 (SFARI Gene, 2023). De Rubeis et al. (2014) analyzed rare coding variants from nearly 4000 ASD cases and found *RELN* to be among the 22 genes with FDR (false discovery rate) of less than 0.05. In a targeted sequencing study on nearly 12000 patients with neurodevelopmental disorders, *RELN* was among the 91 genes with an excess of severe de novo mutations and among the 8 genes that had bias for ASD (as opposed to ID or developmental delay) (Stessman et al., 2017).

A few common variants of *RELN* have been associated with ASD, including coding and non-coding SNPs and GCC repeats in the 5'UTR (Scala et al., 2022; SFARI Gene, 2023). However, two meta-analyses on the GWAS found little evidence that common *RELN* variants are associated with ASD, probably with the exception of the rs362691 (p.Leu997Val) variant (Hernández-García et al., 2020; Wang et al., 2014). Also, *RELN* was not among the candidate genes in the only GWAS that so far has found genome-wide significant loci associated with ASD (Grove et al., 2019).

Our lab has previously shown that ASD-linked variants of *RELN* are enriched in a highly conserved RXR (arginine-amino acid-arginine) motif (Lammert & Howell, 2016). This is an evolutionary conserved region which implies a functionally important role. We previously characterized an ASD-linked RXR variant and showed that it reduces REELIN secretion and increased its intracellular levels, leading to the activation of ER (endoplasmic reticulum) stress response (Lammert et al., 2017).

Other groups have also shown that ASD-linked *RELN* variants result in decreased REELIN
secretion, causing migration defects (Riva et al., 2021) and mTOR overactivation (Sánchez-Sánchez et al., 2018). The latter group used iPSC-derived neural progenitor cells (NPCs), which is a useful model to study certain cellular phenotypes associated with ASD, e.g., cell proliferation (Marchetto et al., 2017). However, studying certain phenotypes such as dendritic complexity require mature neurons. A few other ASD-linked gene variants have been characterized in mature neurons derived from iPSCs (Deshpande et al., 2017; Griesi-Oliveira et al., 2015). However, RELN variants have not been characterized for cellular phenotypes in human iPSC-derived mature neurons.

Despite an extensive literature on REELIN signaling, two aspects of this field are understudied. First, most of our knowledge is based on animal models, and human studies have been historically limited to clinical and neuroimaging characterization of REELIN loss in patients with LCH. As such, our understanding of human-specific REELIN signaling is limited. Second, there have been few functional studies on the ASD-linked variants of RELN, which are typically heterozygous and do not appear to cause as severe cellular phenotypes as those of homozygous REELIN loss, hence making them more difficult to study in mouse models. The latter point is a reflection of the fact that ASD is rarely caused by a single genetic mutation. This underscores a need for a more realistic model system to account for the heterogeneity of ASD.

1.7. Induced Pluripotent Stem Cells (iPSCs)

The induced Pluripotent Stem Cell (iPSC) technology was invented by Shinya Yamanaka in 2006. Somatic cells are re-programmed by the introduction of four transcription factor genes (Myc, Oct3/4, Sox2, Klf4) (Takahashi & Yamanaka, 2006). This technology allows
us to use adult human cells (e.g., skin fibroblasts or blood cells) without having to use embryonic stem cells. iPSCs can be differentiated to many cell types, including different types of neurons, and can be used for regenerative medicine or disease modelling (Russo et al., 2019).

The iPSC technology is particularly a promising approach for modelling complex genetic disorders such as ASD, and it offers several major advantages. First, iPSCs almost eliminate the need for using human embryonic stem cells, assuaging some ethical concerns regarding the use of those cells. Second, the iPSC technology gives us an opportunity to use donated cells from patients who already have a clinical diagnosis, which can be either idiopathic autism or a monogenic/syndromic autism (Russo et al., 2019). Studying patient-derived iPSCs takes into account the heterogeneity of ASD, and it also allows us to explore the genetic interactions that have contributed to ASD in the patient’s cells. Patient-derived iPSCs are especially valuable for studying complex psychiatric disorders in humans, since there is no need for behavioral phenotyping that animal studies require, and there is evidence that the available animal behavioral assays have limited validity for human psychiatric disorders (Nestler & Hyman, 2010).

Besides behavioral complexity, there are important differences at the molecular and cellular levels that makes human neurons indispensable for studying ASD. For example, a direct comparison of human and mouse excitatory synapses revealed significant inter-species differences in protein components, including a set of post-synaptic density molecules involved in dendritic structural plasticity (Bayés et al., 2012). There is also a growing body of literature that focuses on the biophysical differences between human and
non-human neurons, with a particular focus on the role of human dendrites and their unique computational capacity (Beaulieu-Laroche et al., 2021; Kalmbach et al., 2018).

There are also limitation in using iPSCs for disease modelling, such as lack of cytoarchitecture and vascularization, limited cell type variety in a single culture, and heterogeneity among different cell lines (Hong et al., 2023).

### 1.8. Objective

We sought to leverage the human iPSC technology to explore some of the molecular and cellular underpinnings of ASD, and circumvent limitations of animal models.

Many animal models have been used to study autism, ranging from fruit fly (Tian et al., 2017) to nonhuman primates (Zhao et al., 2018), and dozens of mouse models with genetic mutations (Hulbert & Jiang, 2016) and environmental factors (Iwata et al., 2010). There are certain limitations to animal models in general, such as lack of a one-to-one correspondence between behavioral phenotypes in humans and animals, but there are also specific limitations to each animal model (Nestler & Hyman, 2010).

Animal models with Reln mutations are particularly limited for studying ASD. The animal behavioral phenotypes resulting from homozygous or heterozygous Reln loss are not specific to autism, and are seen in models of other disorders such as schizophrenia as well (Lossi et al., 2019). Also, there is no animal model with a specific Reln mutation that recapitulates a known RELN variant associated with ASD in humans.

Therefore, we sought to take advantage of the iPSC technology to characterize a rare RELN variant in a patient with a clinical diagnosis of ASD. This patient (henceforth called the
proband) is a male individual with an RXR *RELN* variant. For control cell lines, we used iPSCs from the proband’s parents, neither of whom had an ASD diagnosis (Figure 1.2a). Whole exome sequencing revealed that the proband’s *RELN* variant (R2457C) was maternally inherited. According to the PolyPhen-2 annotation tool (Adzhubei et al., 2010), this variant is *probably damaging* (Figure 1.2b).

The fact that the proband’s mother carried R2457C but did not have ASD had two implications. First, the variant is not sufficient by itself to cause ASD, since the proband’s mother has the same variant but does not have ASD. This suggests that the proband has additional genetic predispositions for ASD. Second, having two control (parental) lines with different *RELN* genotypes allows us to distinguish between the variant-specific findings and the proband-specific findings.

Therefore, we asked how the R2457C variant may have contributed to the ASD pathophysiology in the proband, and whether other ASD-related predisposing factors at the molecular level have added to the risk. To this end, we decided to differentiate iPSCs to a type of inhibitory neurons that model a population of REELIN-expressing cortical inhibitory neurons. Given that R2457C is an RXR *RELN* variant and our lab previously showed that RXR variants may reduce REELIN secretion (Lammert et al., 2017), we hypothesized that the iPSC-derived inhibitory neurons carrying this variant, i.e., the proband and the maternal lines, would secrete REELIN at lower levels compared to the paternal inhibitory neurons.

We also asked whether the proband has other genetic risk factors and whether those factors have converged on a REELIN-dependent cellular phenotype which is also implicated in
ASD, namely, dendritic complexity. We hypothesized that dendritic growth and complexity is compromised in the proband iPSC-derived excitatory neurons. We decided to choose a differentiation protocol that models cortical pyramidal neurons because these neurons are the main target of REELIN in early development and they require REELIN for dendritic growth.

We also hypothesized that the proband excitatory neurons had abnormalities in the signaling pathways that are downstream to REELIN signaling, particularly those pathways that are necessary for normal dendritic growth and are also implicated in ASD, such as WNT signaling and mTOR signaling.
**Figure 1.1.** A representation of the neocortical layers and the three types of neurons important in REELIN signaling. Cajal-Retzius cells and a subset of inhibitory cortical neurons secrete REELIN, which binds to receptors on the excitatory pyramidal neurons. This initiates the REELIN signaling, which is necessary for the migration and dendritic growth in the pyramidal neurons.
Figure 1.2. a. A pedigree representing the RELN genotype and the ASD phenotype of the family under the study. The proband and his mother carry the R2457 variant, but only the proband has an ASD diagnosis. b. The RELN variant R2457 is predicted to be probably damaging, according to the PolyPhen-2 annotation tool (Adzhubei et al., 2010). The inset shows the 3D structure of REELIN and the site of the missense mutation in red.
a

No ASD -> R2457C variant
ASD -> R2457C variant

b
http://genetics.bwh.harvard.edu/pph2/index.shtml

PolyPhen-2 prediction of functional effects of human nsSNPs

PolyPhen-2 report for P78509 R2457C

Query
Protein Acc Position AA1 AA2 Description
P78509 2457 R C Canonical; Recluster: Full=Reelin; EC=3.4.21.; Flag

Results
 darken Prediction/Confidence
HumDiv
This mutation is predicted to be PROBABLY DAMAGING with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)

HumVar
This mutation is predicted to be PROBABLY DAMAGING with a score of 0.997 (sensitivity: 0.27; specificity: 0.98)
1.9. References


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Chapter 2

REELIN rescues dendritic complexity in iPSC-derived neurons from an ASD patient with a RELN R2457C variant

Contribution:

Ryan Mokhtari did all the experiments and wrote this chapter. Brian Howell generated the RELN KO cell lines, supervised the experiments, and edited the text.
2.1. Introduction

REELIN is a secreted glycoprotein with critical neurodevelopmental roles such as facilitating the migration of cortical pyramidal neurons which is required for an organized cortical layering, normal dendritic growth in cortical pyramidal neurons, and regulating synaptic plasticity (Jossin, 2020). REELIN is encoded by the RELN gene, and variants of this gene have been frequently associated with autism spectrum disorder (ASD) (Lammert & Howell, 2016). RELN is considered a high-confidence ASD gene, according to the Simons Foundation Autism Research Initiative (SFARI) (SFARI Gene, 2023). However, with a few exceptions (Lammert et al., 2017; Riva et al., 2021; Sánchez-Sánchez et al., 2018), most ASD-linked variants of RELN have not been functionally characterized.

The induced pluripotent stem cell (iPSC) technology allows us to study a rare variant that is carried by a patient with ASD in neurons that are derived from the patient’s own cells (Kathuria et al., 2017). We used de-identified iPSCs from a family with non-autistic parents and their son (the proband) who had ASD diagnosis and had inherited a rare RELN variant from his mother. This variant was a missense mutation (R2457C) in a highly conserved region of the protein that is enriched in an RXR (arginine-amino acid-arginine) motif. We previously showed that the RELN mutations that are associated with ASD commonly occur in this region (Lammert & Howell, 2016), and that RXR variants of RELN may reduce the secretion of REELIN (Lammert et al., 2017).

We differentiated the iPSCs into inhibitory neurons that mimic the inhibitory forebrain neurons that secrete REELIN (Reed et al., 2021) and excitatory neurons that model the cortical pyramidal neurons responding to REELIN (Wang C. et al., 2017). REELIN binds
to cortical pyramidal neurons activating REELIN signaling, which is necessary for the growth and branching of dendrites (Chai et al., 2015).

Reduced dendritic complexity has been associated with autism, based on animal studies (Martínez-Cerdeño, 2017), studies on postmortem brain (Fetit et al., 2021) and iPSC-derived neurons (Fink & Levine, 2018). Thus, we used the iPSC-derived excitatory neurons to study dendritic complexity in the proband and compare it to the parental neurons. This phenotype is particularly important in this context, since it is REELIN-dependent in cortical pyramidal neurons. And if REELIN secretion is reduced in a brain, dendritic growth and complexity in pyramidal neurons will also be decreased.

Despite responding to REELIN, excitatory cortical neurons themselves are not among the cell types known to express REELIN. However, low levels of RELN mRNA have been detected in these neurons in human fetal brain samples (Polioudakis et al., 2019), and we found RELN mRNA in the iPSC-derived neurons as well. Nonetheless, consistent with the published protein databases (Sjöstedt et al., 2020; Uhlén et al., 2015), we did not find REELIN expression at the protein level in the excitatory neurons. We decided to knock out RELN in these neurons, however, to ensure that any observed phenotypes in the iPSC-derived excitatory neurons were independent of endogenous REELIN.

As mentioned earlier, a single ASD-linked variant is unlikely to be sufficient to cause a clinical phenotype. Thus, we hypothesized that the proband has additional genetic risk for ASD that has converged with that of the RELN variant. To investigate the additional risk, we focused on the signaling molecules and pathways that are downstream to the REELIN signaling or interacting with it.
GSK3β (Glycogen Synthase Kinase-3β) is an important protein downstream of the REELIN signaling (Beffert et al., 2002; Ohkubo et al., 2003), which also regulates neurodevelopmental processes implicated in ASD, including neuronal migration and synaptic function (Caracci et al., 2016; Hur & Zhou, 2010) and the growth and complexity of dendrites (Llorens-Martín et al., 2013). GSK3β overactivation has been associated with autistic-like behaviors in animal models (Mines et al., 2010; Zhang et al., 2012), and with ASD-linked cellular phenotypes in vitro (Kim et al., 2006; Rui et al., 2013). GSK3β is also an important regulator of the canonical WNT signaling (Wu & Pan, 2010), which is a crucial pathway for brain development, and there is strong evidence that WNT signaling is dysregulated in ASD (Kalkman, 2012; Zhang et al., 2014).

The mTOR (mammalian target of rapamycin) is another important pathway that is both regulated by (Jossin & Goffinet, 2007) and regulates REELIN signaling (Moon et al., 2015). The mTOR signaling is implicated in ASD as an important regulator of protein synthesis (Lipton & Sahin, 2014; Santini & Borgkvist, 2020), and it is required for dendritic growth (Jossin & Goffinet, 2007).

We studied the activity of GSK3β, the canonical WNT signaling, and the mTOR pathway, as they were related to the dendritic morphology in the iPSC-derived excitatory neurons. We hypothesized that these would be the underlying molecular and cellular processes that are dysregulated in the proband excitatory neurons. We also hypothesized that REELIN treatment would rescue some of these phenotypes.
2.2. Materials and Methods

2.2.1. Cell culture

All cell cultures were maintained at 37°C at 5% CO₂. HEK293 cells were grown in DMEM medium (Gibco) supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin. Stable HEK293 WT REELIN-expressing cells were a gift from Dr. Eckart Förster (Zelluläre Neurobiologie, Germany) (Förster et al., 2002). REELIN-conditioned media (RCM) and control conditioned media (CCM) were collected in serum free DMEM and concentrated in Centricon units (100 kDa cutoff, Millipore) as described previously (Howell et al., 1999). The presence of REELIN in RCM was validated with Western blotting (data not shown). Transfections on HEK293 cells were performed using X-tremeGENE HP (Roche Diagnostics) and Opti-MEM medium (Gibco).

iPS cells were de-identified cell lines and were a gift from Dr. Mark Cookson (NIA, NIH). The cells were reprogrammed from PBMCs (peripheral blood mononuclear cells) by transient expression of OCT4, SOX2, KLF4 and cMYC with a Sendai viral system at the NHLBI iPSC core (Churko et al., 2013). iPS cells were karyotyped for chromosome abnormalities (Karyostat Karyotyping Service, ThermoFisher) and found to be normal. iPS cells were plated on Matrigel matrix (Corning) and fed every other day with StemFlex medium (Gibco). iPSCs were passaged at near-confluency, using dissociation reagent ReLeSR (STEMCELL Technologies) and ROCK-inhibitor (RevitaCell, Gibco).

For differentiating inhibitory forebrain neurons (Reed et al., 2021), iPSCs were fed everyday with E8 medium (Gibco) and plated at 95% confluency (day 1) on Matrigel-coated 6-well plates with 5 ml of N3 medium [1/2 DMEM/F12 (Gibco), 1/2 Neurobasal medium].
(Gibco), 1X GlutaMAX (Gibco), 1X Non-Essential Amino Acids (Gibco), 1% Penicillin-Streptomycin, 0.25 μM 2-Mercaptoethanol], supplemented with 1X B27 supplement without RA (ThermoFisher), 1X N2 supplement (Gibco), and 10 μg/ml Insulin (Invitrogen). Neuronal induction was initiated by dual SMAD inhibition, adding 1.5 μM dorsomorphin (STEMCELL Technologies) and 10 μM SB431542 (STEMCELL Technologies) everyday to the media for 11 days, followed by 4 days of N3 + B27 + N2 + Insulin, and then 4 days of N3 + B27 + N2 + Insulin + 0.05 μM retinoic acid (RA) (Sigma-Aldrich). On day 19, cells were dissociated using Accutase (Gibco) and were passaged 1:2 on plates pre-coated first with PLO (poly-L-ornithine) (Sigma-Aldrich) for 24 hours and next with fibronectin (2 μg/ml) (STEMCELL Technologies) and laminin (0.2 μg/ml) (STEMCELL Technologies) for 24 hours. On days 20 – 28, cells were fed everyday with N3 + B27 + N2 + Insulin + RA, supplemented with BDNF (0.2 μg/ml) (STEMCELL Technologies) and GDNF (0.2 μg/ml) (STEMCELL Technologies). Cells were frozen on day 24 or continued to grow up to day 50, with less frequent media change (Figure 2.1a).

For differentiating excitatory neurons, the protocol published by Fernandopulle et al. (2018) was followed (i3N neurons) with minor modifications. Briefly, iPSCs were grown in mTeSR (STEMCELL Technologies), dissociated with Accutase, 2 x 10⁶ cells plated on Matrigel-coated 10 cm dishes, and after 2 hours, transfected with a Tet-On vector expressing hNGN2 inducibly and BFP constitutively in a PiggyBac transposon vector (K4, a gift from Dr. Michael Ward, NIH) and a PiggyBac recombinase expressing vector (K13, a gift from Dr. Michael Ward, NIH) using Lipofectamine Stem (Invitrogen). Selection started 48 hours later, using titrating doses of puromycin daily until all cells were fluorescent. 1 x 10⁶ cells were passaged onto a Matrigel-coated 6-well plate and were
grown for 3 days in Induction Media [DMEM/F12 (Gibco), 1X N2 supplement (Gibco), 1X GlutaMAX (Gibco), 1X Non-Essential Amino Acids (Gibco)] with daily supplement of doxycycline (2 μg/ml). Next, cells were dissociated with Accutase and plated on 12-well plates (for biochemistry) or glass coverslips (for immunocytochemistry) pre-coated with PLO and fibronectin/laminin (as above), and were grown in Cortical Media [BrainPhys medium (STEMCELL Technologies), 10 ng/ml BDNF (STEMCELL Technologies), 10 ng/ml NT-3 (STEMCELL Technologies), 1 μg/ml laminin], with feeding every 3 days for a week and then weekly half-volume replacements for 3-4 weeks (Figure 2.1b).

2.2.2. Constructs

The Reln R2457C variant (human numbering) construct was generated by Genscript from pCRL containing WT mouse Reln (Gift from Dr.s Tom Curran, Gabriilla D'Arcangelo) (D'Arcangelo et al., 1997). DNA constructs for PiggyBac recombinase insertion of the Tet-On hNGN2 expression system were developed and provided by Dr. Michael Ward (NINDS, NIH). The K4-PB-TO-hNGN2 vector contains a third generation Tet-responsive promoter that is activated by Tet-On(R) followed by a cDNA for human Neurogenin2. The Tet-On transcriptional factor (rtTA3G) that only binds the operator when bound to tetracycline is downstream of a constitutive CAG promoter. Puromycin N-acetyltransferase and BFP are separated by a 2A peptide from Thosea asigna leading to the production of 2 polypeptides since eukaryotic ribosomes do not insert a peptide bond between two resides of the peptide (Gly and Pro). This cassette is driven by the EF-1-alpha promoter and is floxed so it can be excised by Cremediate recombination. This system is integrated between 5' and 3' terminal repeats that facilitate integration of the genomic
elements into active regions of the chromatin by PiggyBac transposase. The K13 vector encodes the PiggyBac transposase.

2.2.3. Gene editing

The RELN KO alleles were generated by CRISPR/Cas9 mediated formation of indels in the indicated iPSC lines. The guide RNA target was CCCCCAGCAACAGCGCTAGG, which is followed by the PAM sequence AGG. The full-length gRNA (0.8 μl of 200 pmol/μl, Synthego) was incubated with Cas9 v3 protein (0.4 μl of 10 ug/μl, IDT) at 37°C for 15 min to generate a ribonucleoprotein particle which was electroporated into the iPSCs (1 x 10^6 cells). Cells were resuspended in P3 buffer (Lonza) and electroporated with the CA137 program on the Nucleofector (Lonza), quickly resuspended in StemFlex media with a ROCK inhibitor (RevitaCell). After 3 days growth the cells were plated at single cell density for approximately 10 days at which point colonies were picked into 96 well dishes for clonal analysis. DNA purified from each clone was amplified using primers RELN For-N2 (GAGCGAGCGGCGCGTAG) and RELN Rev-N2 (CAAGGCCCCTTGGAAGAAGG) using AccuPrime GC-rich DNA polymerase (ThermoFisher) and then analyzed by the Surveyor assay (IDT) to detect mismatch between heteroduplexes formed by heat-denaturing the DNA followed by slow cooling. Clones that had mismatches between alleles or had obvious deletions were sequenced. Sequence results were analyzed using the Synthego ICE program to disambiguate the sequencing results. Indels that would cause a frameshift on both alleles were chosen for further study. The following clones were used for our study: HT563b (F24, -10bp and >25bp deletion), HT564a (S14 -14 bp and +1 bp), HT565a (M31, -2, +1).
2.2.4. Drug treatments

The following drugs were used in this study. Recombinant Human WNT-3a (R&D Systems, 5036-WN), MG132 (UBPbio, F1100), IM-12 (Selleck Chemicals, S7566), Rapamycin (Sigma-Aldrich, R8781). WNT3a was dissolved in PBS and other drugs were dissolved in DMSO (Sigma-Aldrich).

2.2.5. qRT-PCR

RNA was extracted from neurons two weeks after plating, using RNeasy Mini kit (Qiagen). cDNA was made using iScript kit (BIO-RAD). The following primers were used:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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</thead>
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<td>TBXT</td>
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<td>CATGCACAGGAGGGCATATCA</td>
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<tr>
<td>VLDLR</td>
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<td>GGTGAAGTCTCGGGGACTAC</td>
</tr>
</tbody>
</table>

SYBR Green (Roche Diagnostics) was used to detect the amplicons in the CFX384 system (BIO-RAD), and the data were analyzed with CFX Manager (BIO-RAD). Fold changes were calculated with the $2^{-\Delta\Delta CT}$ formula.

2.2.6. RNA sequencing

RNAseq was performed on iPSCs and iPSC-derived inhibitory neurons to identify
differentially expressed genes (DEGs) in the proband cells compared to the parental cells. Three independently-generated clones from each of the three iPS cell lines (total n = 9 samples) were differentiated to inhibitory neurons as described earlier. RNA was extracted using RNeasy Mini kit (Qiagen) three weeks after plating neurons, and cDNA libraries were prepared using QuantSeq 3’ mRNA-Seq Library Prep Kit (Lexogen). Illumina NextSeq 2000 was used for sequencing, and the data were analyzed with Partek Flow. Data were normalized using TPM method, and filtered for transcripts with minimum of 10 copies. Differential analysis was performed using DESeq2 method, comparing the parental cell lines with the proband cell line. DEGs with p < 0.05 and fold change > ±2 were selected for pathway enrichment analysis. The selected DEGs were analyzed for protein-protein interactions using the STRING database (Szklarczyk et al., 2023). The edges (lines connecting proteins) were set to represent both functional and physical protein associations with medium and high confidence.

2.2.7. Antibodies

The following antibodies used in this study. REELIN (G10, Millipore, MAB5364), DAB1 (rabbit polyclonal, B3) (Howell et al., 1997), GSK3β (BD Biosciences, 610201), pS9-GSK3β (R&D Systems, MAB8934), GSK3α (Cell Signaling, 9338), S6 (Santa Cruz, 74459), pSer235/236-S6 (Cell Signaling, 81736), GAPDH (UBP Bio, Y1040), MAP2 (Chemicon, AB5622), βIII-Tubulin (Cell Signaling, D65A4), SMI312 (Biolegend, 837904), NPY (NeuroMab, 75456), GAD67 (Millipore, MAB5406).

2.2.8. Western blotting

Cells were lysed with RIPA buffer, adding Phosphatase Inhibitor Cocktail-1 (Sigma-
Aldrich) (1:100), Protease Inhibitor Cocktail (Roche Diagnostics) (one tablet per 10 ml), and phenyl-arsine oxide (Sigma-Aldrich) (1:1000), and sonicated to disrupt cell membrane. Lysates were mixed with 2X Sample buffer (1:1) and DTT (final concentration 100 mM) and heated at 95°C for 5 minutes. Samples were resolved by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. Blots were blocked with either 5% non-fat milk, or 5% BSA (bovine serum albumin) for phospho-proteins, in TBS with 0.1% Tween. After applying antibodies, blots were developed with Enhanced Chemiluminescence substrate (ThermoFisher) and visualized on the Chemidoc (BIO-RAD). ImageJ (Fiji) was used to quantify the protein bands (Schindelin et al., 2012), normalizing their signal intensity against the GAPDH bands as the control.

2.2.9. Immunocytochemistry

Coverslips were washed with PBS once, fixed with 4% paraformaldehyde in PBS, and blocked with 1% BSA in PBS with 0.1% Tween. After applying antibodies, coverslips were mounted using DAPI (Sigma-Aldrich) in Vectashield mounting media (Vector Laboratories). Images captured with Leica SP8 confocal microscope.

2.2.10. Morphological analysis

Images were analyzed using ImageJ (Fiji) (Schindelin et al., 2012) and the ImageJ plugin, SNT (Arshadi et al., 2021) for Sholl analysis on the excitatory neurons. Dendrites were defined as the neurites that were positive for MAP2 (dendritic marker) and negative for SMI312 (axonal marker). Twenty five concentric circles around the soma were drawn with 10 μm intervals, and the number of dendritic branches intersecting with the circles were recorded as the measure of dendritic complexity. The total dendritic length for each neuron
was also calculated. The following criteria were used for excluding neurons from the analysis: cells with non-neuronal morphology, cells with all projections shorter than 100 μm, cells with somas closer to image edges than 250 μm.

2.2.11. Statistical analysis

Statistical analyses were performed using Microsoft Excel. One-way ANOVA was used to compare the values among the 3 cell lines, and Tukey post hoc test was used for pairwise comparisons. Independent samples t-test was used for comparing each RELN-KO cell line with its unedited isogenic cell line. In all cases, the significance level was set at 0.05.
Figure 2.1. Overview of the differentiation processes for the iPSC-derived inhibitory neurons (a) and excitatory neurons (b).
a) Dual SMAD inhibition of iPSCs (11 days) → N3 media (4 days) → N3 + retinoic acid (5 days) → N3 + retinoic acid + BDNF + GDNF (4 weeks)

b) Transfection of iPSCs with inducible hNGN2 (2 days) → Puromycin selection (2-3 weeks) → Doxycycline induction (3 days) → Maturation of excitatory neurons (3-4 weeks)
2.3. Results

2.3.1. Expression of neuronal markers and REELIN signaling markers

Both types of iPSC-derived neurons expressed neuronal markers MAP2, βIII-Tubulin, and SMI312. The excitatory marker VGLUT1 and the inhibitory markers GAD67 and NPY were expressed in the respective neurons as well (Figure 2.2). A more comprehensive characterization of the excitatory neurons, including their electrophysiological properties, have been previously published (Wang C. et al., 2017). Both types of neurons across the three cell lines had similar density, purity, and maturity. And whenever a set of cultures was unequal in any of these measures, it was discarded.

The inhibitory neurons expressed REELIN approximately three weeks after plating, and the excitatory neurons expressed DAB1 approximately two weeks after plating (Figure 2.2). RELN mRNA was expressed in both inhibitory and excitatory neurons. However, there was no significant difference in the RELN expression level among the cell lines in either type of neurons. Similarly, the genes involved in REELIN signaling, DAB1, LRP8 (the gene for APOER2 protein), and VLDLR, were not differentially expressed at the RNA level in the excitatory neurons (Figure 2.2).

2.3.2. RXR variants reduce REELIN secretion

To examine the biosynthesis and proteostasis of the ASD-linked R2457C variant, we used HEK293 cells to heterologously express this variant, and compare it with both the wildtype (WT) RELN as well as with another ASD-linked RXR variant (R2290C), which our lab previously showed to reduce the REELIN secretion (Lammert et al., 2017). After 48 hours from a successful transfection (80% or higher efficiency), we collected the conditioned
media to probe for the extracellular REELIN and lysed the cells to probe for the intracellular REELIN. Compared to cells transfected with the WT *RELN*, cells transfected with either RXR variant had decreased extracellular and increased intracellular REELIN, suggesting a reduced REELIN secretion. The effect was slightly (but not significantly) greater in the R2457C than in the R2290C variant (Figure 2.3a).

To study the REELIN expression at endogenous levels, we generated inhibitory neurons (Reed et al., 2021) that mimic the REELIN-expressing inhibitory forebrain neurons (Pesold et al., 1998), using iPSCs from the proband and the parental cell lines. The R2457C variant is present in both the proband and the maternal lines, but only the proband is diagnosed with ASD. Therefore, this system allowed us to characterize the R2457C variant in two different genomic contexts with two different phenotypes.

The inhibitory neurons expressed REELIN approximately 3 weeks after initiation of differentiation protocol, by which time they already expressed the neuronal markers MAP2 and βIII-Tubulin, and the inhibitory markers GAD67 and NPY (Figure 2.2). At that point, we collected the conditioned media daily for one week, and then lysed the cells to compare both the extracellular and the intracellular REELIN levels by Western blotting. Compared to the paternal cells, the proband and the maternal cells, both of which carry R2457C, had increased intracellular and decreased extracellular REELIN levels. However, the extracellular/intracellular REELIN ratio was significantly lower only in the proband neurons compared to the parental neurons, and not the maternal versus the paternal neurons (Figure 2.3b). This finding has two implications. First, it provides further evidence for a possible role of RXR variants in reducing REELIN secretion. Second, it suggests that the
genomic/epigenomic background in the proband cells has an additional negative effect on the REELIN secretion, compared to the maternal cells, which may partly contribute to the ASD pathophysiology in the proband.

Given that the neurons are heterozygous for the RXR variant, we asked whether the reduced secretion is exacerbated by a dominant negative effect of the RXR allele on the WT allele. To address this question, we co-transfected HEK293 cells with a WT construct and an RXR variant (R2290C, R2290H, or R2457C). A dominant negative effect would reduce the level of extracellular Reelin below what was observed for the WT alone transfection. However, while co-transfection with the RXR variants increased the level of intracellular REELIN, the level of extracellular REELIN remained relatively unchanged (Figure 2.3c), suggesting that the RXR variants do not have a dominant negative effect on REELIN secretion.

2.3.3. Dendritic complexity is reduced in proband neurons

We differentiated the proband and the parental iPSCs into excitatory cortical neurons to study some of the molecular and cellular phenotypes that are relevant to ASD pathophysiology. We used a protocol involving inducible overexpression of the neuronal transcription factor neurogenin-2 (NGN2) at PiggyBac recombination sites, differentiating iPSCs to functionally mature glutamatergic cortical neurons, with high efficiency and purity (Fernandopulle et al., 2018). The iPSC-derived excitatory neurons expressed neuronal markers MAP2, βIII-Tubulin, SMI312, and the excitatory marker VGLUT1 (Figure 2.2). A more comprehensive characterization of these excitatory neurons, including their electrophysiological properties, have been previously published (Wang C. et al., 2017).
Abnormal dendritic morphology is a common cellular phenotype in autism (Fetit et al., 2021). To measure the complexity of dendritic arbor and the total dendritic length, we sparsely cultured the excitatory neurons and immunostained them with the dendritic marker MAP2 two weeks after plating. At this stage neurons had reached morphological maturity and had expressed other neuronal markers as well as MAP2. We used three independently-generated clones from each cell line (total of 9 clones) in all experiments, with the exception of the RELN KO lines each being generated from one clone (Table 2.1). All clones were grown and treated simultaneously and under identical conditions, such as cell density, purity, and maturity. We asked a colleague who was not involved in the experiments to blind the specimens before this and subsequent analyses. We then performed Sholl analysis using the ImageJ plugin SNT (Arshadi et al., 2021). We set the following criteria for excluding neurons from the analysis: cells with non-neuronal morphology, cells with all projections shorter than 100 μm, cells with somas closer to image edges than 250 μm (Figure 2.4a).

We found a significant reduction in the dendritic complexity in the proband neurons, compared to the parental neurons (Figure 2.4b). The total dendritic length was also significantly lower in the proband neurons (Figure 2.4c).

2.3.4. RELN gene disruption does not alter dendritic morphology in excitatory neurons

The iPSC-derived excitatory neurons model the neocortical pyramidal neurons, which are not known to express REELIN (Sjöstedt et al., 2020; Uhlén et al., 2015). However, low levels of RELN mRNA have been detected in these neurons in human fetal brain samples
(Polioudakis et al., 2019), and we found RELN mRNA in the iPSC-derived neurons as well (Figure 2.2). Nonetheless, consistent with the published protein databases (Sjöstedt et al., 2020; Uhlén et al., 2015), we did not find any detectable level of REELIN protein in the excitatory neurons by either Western blotting or immunofluorescence staining (data not shown).

Therefore, we decided to rule out any possibility of an endogenous REELIN expression or function in these cells by generating RELN KO neurons and analyzing their dendritic complexity. We used CRISPR/Cas9 in the iPSCs to generate indels in the RELN coding region proximal to the start codon, and for each cell line selected clones with frameshift variants on both alleles. We then differentiated the mutant and the unedited controls into excitatory neurons in parallel. Repeating the Sholl analysis, we did not find any pairwise differences between the RELN KO neurons and the isogenic unedited neurons in terms of dendritic complexity (Figure 2.5). This indicates that even if these excitatory neurons make an undetectable amount of REELIN protein, it is not consequential in terms of dendritogenesis.

2.3.5. REELIN treatment normalizes the dendritic phenotype in proband neurons

The cortical pyramidal neurons do not express REELIN but respond to the binding of REELIN to their membrane receptors, activating the intracellular adaptor DAB1 (Howell et al., 1999), which is necessary for the downstream effects of REELIN (Niu et al., 2004; Olson et al., 2006). We showed that the iPSC-derived excitatory neurons also express DAB1 (Figure 2.2), suggesting that they also respond to REELIN.
Given that REELIN promotes dendritic growth in pyramidal neurons, we hypothesized that the reduced dendritic complexity in the proband neurons can be normalized by REELIN treatment. To test this hypothesis, we produced REELIN conditioned media (RCM) and control conditioned media (CCM), using stably transfected HEK293 cells, as described previously (Lammert et al., 2017). The latter cells produce supra-physiological levels of REELIN, hence we added RCM at a final concentration of half the original media (25 μl of 10X RCM in 500 μl media, approximately 360 ng/μl). RCM or CCM was added on post-plating days 2, 4, 6, and 8. Neurons media were changed as usual, with half-volume replacements on days 4 and 11, and the neurons were fixed on day 15, the timepoint at which dendrites were analyzed in all experiments in this study. REELIN treatment increased both the branching complexity and the total length of dendrites in the proband neurons, making them statistically indistinguishable from the parental neurons with regards to the dendritic morphology (Figure 2.6).

The implication of this finding is that the reduced dendritic complexity in the proband’s pyramidal neurons is due to an underlying genetic risk unrelated to RELN, but one that can be rescued by exogenous REELIN. However, his RELN variant reduces the secretion of REELIN from inhibitory and Cajal-Retzius neurons, which maybe insufficient to rescue dendritic growth in the pyramidal neurons.

2.3.6. GSK3β is increased and overactive in proband neurons

If REELIN deficiency had an additive effect on the underlying risk factor in the proband excitatory neurons, the latter factor would likely be downstream to REELIN signaling and would likely have a role in dendritic growth and ASD pathophysiology. We hypothesized
that GSK3β is a candidate that matches that profile. GSK3β regulates dendritic growth and complexity (Llorens-Martín et al., 2013), and its overactivation is associated with autistic-like behaviors in animal models (Mines et al., 2010; Zhang et al., 2012).

Therefore, we investigated whether GSK3β is dysregulated in the proband neurons. GSK3β is a constitutively active kinase, and its signaling is typically through inactivation by phosphorylation at the serine-9 (S9) residue (Beurel et al., 2015). We probed the excitatory neurons for total GSK3β and the phosphorylated isoform pS9-GSK3β. The proband neurons had significantly higher levels of total GSK3β compared to the parental neurons, demonstrated by Western blotting and immunofluorescence staining (Figure 2.7a-d). The pS9-GSK3β was lower in the proband neurons, indicating increased activity (Figure 2.7b).

To explore the possible mechanisms of the increased total GSK3β, we treated the cells with the proteasome inhibitor MG132 to find out whether GSK3β is degraded differently among the cell lines. After treating neurons with MG132 (for 24 hours at final concentration of 100 nM), GSK3β level was still higher in the proband cells, suggesting that differential proteasomal degradation is not the mechanism of increased total GSK3β (Figure 2.7b). We also measured the GSK3B mRNA levels using qRT-PCR, and found no significant difference between the proband and the parental neurons, excluding a transcriptomic mechanism (Figure 2.2). Also, the levels of GSK3α, a closely related protein from a homologous gene, were not significantly different among the cell lines (Figure 2.7b).

2.3.7. Inhibition of GSK3β partially normalizes the dendritic phenotype in proband neurons

A tightly regulated GSK3β signaling is required for normal dendritic growth and
complexity (Hur & Zhou, 2010; Rui et al., 2013). We asked whether the overactive GSK3β contributed to the reduced dendritic complexity in the proband neurons. We treated the growing neurons with IM-12, a selective GSK3β inhibitor and a canonical WNT activator, or with DMSO. Since IM-12 has not been previously used in this type of neurons, we used different concentrations and monitored the neurons as they matured. To achieve the highest level of GSK3β inhibition, we titrated IM-12 using different final concentrations (2 μM, 4 μM, 6 μM, 8 μM) and selected the highest concentration in which neurons remained viable (4 μM), treating neurons on post-plating days 2, 5, and 8. Neuronal media was changed (half-volume replacements with the same drug concentration) on days 4 and 11, and the cells were fixed on day 15.

GSK3β inhibition partially normalized the dendritic phenotype in the proband neurons, increasing the dendritic complexity only in the proximal parts of the dendritic arbor, as the average number of crossings with circles closer to soma (1-10) became similar across the cell lines after IM-12 treatment, but the distal circles remained significantly less branched in the proband neurons. The total dendritic length remained significantly lower in the proband neurons (Figure 2.8). This finding suggests that the overactive GSK3β may partly contribute to the reduced dendritic complexity in the proband neurons.

2.3.8. WNT signaling is suppressed in proband neurons, but is normalized by REELIN pretreatment

WNT signaling is frequently implicated in ASD, and it is one of the few pathways that represent a functional convergence of ASD risk genes (Krumm et al., 2014; Zhang et al., 2014; Packer, 2018). The activity of the canonical WNT signaling is negatively correlated
with the activity of GSK3β (Wu & Pan, 2010), and GSK3β inhibitors such as IM-12 activate the canonical WNT signaling (Wang et al., 2019). Therefore, we hypothesized that the overactive GSK3β is accompanied by an underactive WNT signaling in the proband excitatory neurons. Also, since GSK3β is downstream to REELIN signaling, we hypothesized that REELIN treatment would increase the activity of WNT signaling in the proband neurons.

To find out whether WNT signaling is dysregulated in the proband neurons, we treated excitatory neurons with the canonical WNT signaling ligand, WNT3a (at a final concentration of 2.5 nM) or with PBS. After 24 hours, we extracted the RNA and performed qRT-PCR. Selecting the WNT target genes that have previously been used to show WNT responsiveness in human iPSC-derived cells (TBXT, AXIN2, DKK1, SP5, POU5F1) (Huggins et al., 2016), we calculated the average Ct fold changes with respect to the housekeeping gene, RPLP0 (which was not WNT-responsive itself) and converted them to a scale of 0 to 1.

Compared to the parental neurons, the proband neurons had significantly lower fold changes in response to WNT3a treatment, which suggests a downregulation of canonical WNT signaling in the proband neurons (Figure 2.9a). To rule out a possibility of pre-treatment differential expression among the cell lines, we measured the baseline expression of WNT target genes (without WNT3a treatment, only with PBS). The levels were expectedly low, but they were not significantly different across the cell lines (Figure 2.9c). Since REELIN signaling is known to inhibit GSK3β, which itself inhibits WNT signaling, we asked whether REELIN treatment might restore WNT signaling in the proband neurons.
To find out, we treated neurons with RCM or CCM (same concentrations as used previously) for 48 hours, the second 24 hours of which with a co-treatment of WNT3a, and then RNA was extracted for qRT-PCR. REELIN treatment in the presence of WNT3a increased the expression of WNT target genes in the proband neurons, making their Ct fold changes statistically indistinguishable from those of the parental cells (Figure 2.9b). To rule out a WNT-independent effect of REELIN, we repeated the experiment without the WNT3a treatment (only with RCM). REELIN treatment in the absence of WNT3a did not significantly change the expression of WNT target genes (Figure 2.9d).

2.3.9. The dendritic phenotype is not due to a differentially regulated mTOR signaling

mTOR (mammalian target of rapamycin) is among the molecular pathways implicated in ASD (Ganesan et al., 2019; Winden et al., 2018), and it shares signaling molecules with REELIN signaling (Jossin & Goffinet, 2007). An ASD-linked RELN variant was shown to be associated with mTOR overactivation, the inhibition of which by rapamycin improved REELIN-DAB1 signaling in iPSC-derived neural progenitors cells (Sánchez-Sánchez et al., 2018).

Therefore, we sought to determine if a differentially regulated mTOR signaling contributes to the dendritic phenotype in the proband neurons. To inhibit mTOR signaling, we treated the growing excitatory neurons with rapamycin, and tested a range of concentrations that have been used in similar studies (0.1 μM, 0.5 μM, 1 μM, 2 μM). We selected the highest final concentration of rapamycin in which neurons remained viable (1 μM). Neurons were treated with rapamycin on post-plating days 2, 5, and 8. Neuronal media was changed (half-
volume replacements with the same drug concentration) on days 4 and 11, and the cells were fixed on day 15. Rapamycin did not improve the dendritic complexity or the total dendritic length in the proband neurons (Figure 2.10a,b).

To further investigate any possible role for mTOR dysregulation in the proband, we probed the excitatory neurons for the total and the phosphorylated isoforms of S6, a ribosomal protein used as a readout for mTOR activity which also regulates GSK3β in an mTOR-dependent manner (Zhang et al., 2006). Western blotting on cell lysates from mature neurons did not show significant differences among the cell lines in either S6 or pSer235/236-S6 (Figure 2.10c).

2.3.10. Transcriptomic profiling of iPSCs and inhibitory neurons reveal few differentially expressed genes (DEGs) in the proband

We performed RNAseq on the iPSCs and the iPSC-derived inhibitory neurons. The goal was to find out which genes in the proband cells were differentially expressed compared to the parental cells. However, among the iPSCs only a few differentially expressed genes (DEGs) passed the FDR threshold of 0.1 (EGR1, FST, OTX2, TXNIP, SNHG5, HTR7, SMPD4), and no DEG passed that FDR threshold in the inhibitory neurons. Also, the neuronal DEGs that only passed the simple significance (p < 0.05) with fold changes of ±2 or greater did not make clusters that distinguish the cell lines. Instead, the three clones that we used for each of the three cell lines showed considerable variations which reduced the statistical power (Figure 2.11). This can be partly explained by the fact that we prepared the cDNA libraries in two different times, and although the protocol and all the conditions were the same, some variability must have been introduced to the system.
This is reflected by the principal component analysis (PCA) which showed that there were more clustering by the clone than by the cell line (Figure 2.11). Another possible explanation for the low power is that the control (parental) cell lines have different genomic backgrounds.

We explored the DEGs and the functional enrichment analysis and attempted to validate some relevant findings. We used qRT-PCR to measure the mRNA expression of some significant DEGs as well as some major components of the REELIN signaling. However, we did not reach a conclusion on whether any dysregulation at the transcriptomic level could explain the clinical phenotype in the proband.

WNT signaling was among the enriched pathways in the undifferentiated iPSCs, and we had already shown that WNT signaling was dysregulated in the iPSC-derived excitatory neurons. Therefore, we measured the mRNA expression of WNT target genes in response to WNT3a in iPSCs. We found that the proband iPSCs had higher WNT response compared to the parental cells, which was opposite to the findings in the proband excitatory neurons (Figure 2.11).
Table 2.1. Cell lines under the study by ASD phenotype, RELN genotype, and the clones and technical replicates used for each type of experiment.
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**Figure 2.2.** a. Immunofluorescence staining for neuronal markers and REELIN signaling proteins 2 weeks post-plating for excitatory neurons and 3 weeks post-plating for inhibitory neurons. Both types of neurons expressed neuronal markers MAP2, βIII-Tubulin, and SMI312. The excitatory neurons also expressed VGLUT1, and the inhibitory neurons expressed GAD67 and NPY. Magnification is 40X for VGLUT1, βIII-Tubulin, and REELIN panels, and 20X for the rest. Images are taken from the parental cell lines, but they are representative of all cell lines. b. Relative mRNA expression of RELN in the inhibitory neurons and the excitatory neurons, as well as other genes involved in REELIN signaling and GSK3B in the excitatory neurons. RNA was extracted from inhibitory neurons 3 weeks after plating and from excitatory neurons 2 weeks after plating. Average Ct fold changes with respect to the housekeeping gene (RPLP0) were calculated ($2^{-\Delta\Delta CT}$) and compared across the cell lines. The highest fold change was given a value of 1.0 and others were re-scaled to it. n = 9 wells (3 wells from each of the 3 independently-generated clones) for each of the 3 cell lines (27 wells for each gene in each experiment). One-way ANOVA with Tukey post hoc test, error bars: standard error of the mean. Ptr: paternal line, Mtr: maternal line, Pbd: proband line.
a  

Excitatory neurons

Inhibitory neurons

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Figure 2.3. RXR RELN variants decrease the extracellular and increase the intracellular REELIN. a. HEK293 cells were transfected with WT or RXR RELN variants (R2290C or R2457C). After 48 hours, conditioned media were collected to probe for extracellular REELIN, and the cells were lysed to probe for intracellular REELIN. The extracellular/intracellular REELIN ratio was significantly lower in both RXR variants compared to the WT, suggesting a reduced REELIN secretion. b. iPSC-derived inhibitory neurons were grown for two weeks after plating, and both the conditioned media and the cell lysate were probed for REELIN. Both the proband (Pbd) and the maternal (Mtr) lines carry the R2457C variant, and both had reduced extracellular/intracellular REELIN ratios compared to the paternal (Ptr) line. c. Co-transfection of RXR and WT variants of RELN in HEK293 cells showed no dominant negative effect of RXR variants on REELIN secretion. a: n = 8 wells per construct; b,c: n = 9 wells (3 wells from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total n = 27 wells for the entire experiment). One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean.
Figure 2.4. Dendritic complexity is reduced in the proband excitatory neurons. Sparsely cultured neurons immunostained with dendritic marker MAP2 and analyzed (after blinding) with ImageJ plugin SNT (Sholl analysis). The number of dendritic branches intersecting with the concentric circles were recorded as a measure of dendritic complexity, and the total dendritic length for each neuron was calculated (25 circles, 10 μm intervals).

a. A representative neuron from each cell line with the Sholl circles. (scale bar 10 μm).

b. The number of intersections with each circle was averaged across the traced neurons. n = 30 neurons (10 neurons from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total n = 90 neurons for the entire experiment). Dendritic complexity is significantly lower in the proband (Pbd) neurons (significant difference in circles #1-18).

c. Average total dendritic length is also significantly lower in the proband neurons compared to both parents. One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean.
Figure 2.5. *RELN* gene disruption does not alter dendritic morphology in excitatory neurons. Excitatory neurons were differentiated from *RELN*-KO or unedited iPSCs under the same conditions, and compared in terms of dendritic morphology. **a.** Dendritic complexity (average number of intersections) was not significantly different between each *RELN*-KO line and its unedited isogenic cell line. **b.** Average total dendritic length was also similar between each pair of KO and unedited cells. n = 30 neurons for each of the 6 cell lines (grand total n = 180 neurons for the entire experiment, one clone used for each cell line). Independent samples t-test compared between *RELN*-KO and unedited neurons for each cell line, and one-way ANOVA (with Tukey post hoc test) was used to compare the cell lines within each (KO or unedited) category, * p < 0.05, error bars: standard error of the mean._Ptr: paternal line, _Mtr: maternal line, _Pbd: proband line._
Figure 2.6. REELIN normalizes the dendritic phenotype in the proband excitatory neurons. Neurons were treated with REELIN conditioned media (RCM) or control conditioned media (CCM) from stably transfected HEK293 cells, as described previously (Lammert et al., 2017). RCM was added at a final concentration of half the original media (approximately 360 ng/μl) on post-plating days 2, 4, 6, and 8. Neuronal media was changed as usual, with half-volume replacements on days 4 and 11, and the neurons were fixed on day 15. **a.** Dendritic complexity (average number of intersections) in proband neurons increased to the level of parental neurons after RCM treatment. **b.** Average total dendritic length also became similar across the cell lines after RCM treatment. **c.** and **d.** Dendritic complexity and average total dendritic length remained lower in proband neurons after CCM treatment. Dendritic complexity and average total dendritic length were also compared between RCM and CCM treatment conditions within each cell line, yielding significant differences for the proband, but no significant differences for either parental cell line. *n* = 30 neurons (10 neurons from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total *n* = 180 neurons for the entire experiment). One-way ANOVA with Tukey post hoc test, *p* < 0.05, error bars: standard error of the mean. **Ptr:** paternal line, **Mtr:** maternal line, **Pbd:** proband line.
Figure 2.7. GSK3β protein is increased and overactive (hypo-phosphorylated) in the proband excitatory neurons. a. Immunofluorescence staining of neurons with GSK3β antibody. b. Western blotting on neurons cell lysates. The total and phospho-S9 isoforms of GSK3β are increased and decreased, respectively, in the proband neurons. Treating neurons with the proteasome inhibitor MG132 (100 nM final concentration, for 24 hours) did not differentially affect the GSK3β levels. GSK3α levels were not significantly different among the cell lines. c. Quantification of GSK3β signal by Western blotting, normalized against GAPDH. d. Quantification of GSK3β signal by immunofluorescence. b,c: n = 9 wells (3 wells from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total n = 27 wells for the entire experiment). d: n = 30 neurons (10 neurons from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total n = 90 neurons for the entire experiment). One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean. Ptr: paternal line, Mtr: maternal line, Pbd: proband line.
Figure 2.8. Inhibition of GSK3β partially normalizes the dendritic phenotype in proband neurons. Excitatory neurons were treated with the selective GSK3β-inhibitor IM-12 (final concentration of 4 μM) or with DMSO on post-plating days 2, 5, and 8. Neuronal media was changed (half-volume replacements with the same drug concentration) on days 4 and 11, and the cells were fixed on day 15. a. Dendritic complexity in proband neurons partially increased (in proximal branches) in response to treatment with IM-12. Circles 1-10 had similar number of dendrite crossings among the cell lines. b. Average total dendritic length remained lower in proband neurons after IM-12 treatment. c. and d. Dendritic complexity and average total dendritic length remained lower in proband neurons after DMSO treatment. Dendritic complexity and average total dendritic length were also compared between IM-12 and DMSO treatment conditions within each cell line, yielding significant differences for the proband (circles 1-10), but no significant differences for either parental cell line. n = 30 neurons (10 neurons from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total n = 180 neurons for the entire experiment). One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean. Ptr: paternal line, Mtr: maternal line, Pbd: proband line.
**Figure 2.9.** WNT signaling is downregulated in the proband excitatory neurons, and it is rescued by REELIN treatment. a. Neurons were treated with WNT3a at a final concentration of 2.5 nM or with PBS, and after 24 hours RNA was extracted for qRT-PCR. Average Ct fold changes with respect to the housekeeping gene (*RPLP0*) were calculated ($2^{-\Delta\Delta\text{CT}}$) and compared across the cell lines. The highest fold change was given a value of 1.0 and others were re-scaled to it. Relative expression of WNT target genes in response to WNT3a treatment was significantly lower in the proband neurons, suggesting a downregulation of canonical WNT signaling. b. Neurons were treated with RCM (see Figure 2.5) for 48 hours, the second 24 hours of which with a co-treatment of WNT3a. Relative expression of WNT target genes in response to co-treatment with REELIN and WNT3a increased in the proband neurons, making their Ct fold changes statistically indistinguishable from those of the parental cells. c. Neurons were treated with PBS as a control treatment to measure the baseline expression of the WNT target genes. The expression levels were not significantly different across the cell lines, but they were all lower than those of the WNT3a-treated cells. d. Treating neurons only with RCM (without WNT3a) showed that REELIN in the absence of WNT3a does not significantly change the expression of WNT target genes. n = 9 wells (3 wells from each of the 3 independently-generated clones) for each of the 3 cell lines (27 wells for each gene in each experiment). One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean. Ptr: paternal line, Mtr: maternal line, Pbd: proband line.
**Figure 2.10. Reduced dendritic complexity in proband neurons is not mTOR-dependent.**

Neurons were treated with the mTOR-inhibitor rapamycin (final concentration of 1 μM) on post-plating days 2, 5, and 8. Neuronal media was changed (half-volume replacements with the same drug concentration) on days 4 and 11, and the cells were fixed on day 15. a. Dendritic complexity remained lower in the proband neurons after treatment with rapamycin. b. Average total dendritic length also remained lower in the proband neurons. c. and d. The mTOR pathway ribosomal protein S6 and its phosphorylated form pSer235/236-S6 have similar levels across the cell lines. *n* = 30 neurons (10 neurons from each of the 3 independently-generated clones) for each of the 3 cell lines (grand total *n* = 90 neurons for the entire experiment). One-way ANOVA with Tukey post hoc test, *p* < 0.05, error bars: standard error of the mean. **Ptr**: paternal line, **Mtr**: maternal line, **Pbd**: proband line.
**Figure 2.11.** Analysis of RNAseq data from the inhibitory neurons. Three independently-generated clones from each of the three iPS cell lines (total n = 9 samples) were differentiated to inhibitory neurons as described earlier. RNA was extracted three weeks after plating neurons and cDNA libraries were prepared using QuantSeq 3’ mRNA-Seq Library Prep Kit (Lexogen). Illumina NextSeq 2000 was used for sequencing, and the data were analyzed with Partek Flow. Data were normalized using TPM method, and filtered for transcripts with minimum of 10 copies. Differential analysis was performed using DESeq2 method, comparing the parental cell lines with the proband cell line. 

a. Volcano plot of the differentially expressed genes (DEGs) with p < 0.05 and fold change > ±2. The blue and red dots represent the genes upregulated and downregulated in the proband cells, respectively. (The analysis was originally with respect to the parents, hence the figure legend.) 

b. The heat map of the same list of DEGs in each clone. (blue and red colors as in a., F: paternal, M: maternal, S: proband).

c. The principal component analysis (PCA) by the cell line (left) and by the clone (right).

d. The protein-protein interaction network of the DEGs annotated by the STRING database (Szklarczyk et al., 2023). The significant (FDR < 0.05) molecular function gene ontology (GO) terms were hedgehog family protein binding, patched binding, and natural killer cell lectin-like receptor binding. The top three cellular component GO terms were collagen type I trimer, fibrillar collagen trimer, and complex of collagen trimers.

e. Relative expression of WNT target genes in undifferentiated iPSCs in response to WNT3a (final concentration of 2.5 nM, 24 hours). Average Ct fold changes with respect to the housekeeping gene (*RPLP0*) were calculated ($2^{-\Delta\Delta CT}$) and compared across the cell lines. The highest fold change was given a value of 1.0 and others were re-scaled to it. n = 9 wells (3 wells from each of the 3 independently-generated clones) for each of the 3 cell lines (27 wells for each gene in each experiment).

One-way ANOVA with Tukey post hoc test, * p < 0.05, error bars: standard error of the mean. Ptr: paternal line, Mtr: maternal line, Pbd: proband line.
2.4. Discussion

We characterized iPSC-derived neurons from a patient with ASD (proband) who had a rare inherited RELN variant, as well as neurons from his non-autistic parents. The iPSCs were differentiated into inhibitory neurons to model the inhibitory forebrain neurons that secrete REELIN (Pesold et al., 1998), and into excitatory neurons to model the cortical pyramidal neurons that respond to REELIN (Howell et al., 1999) and require an intact REELIN signaling to develop complex dendrites and to migrate properly and laminate the neocortex (Chai et al., 2015; Sheppard & Pearlman, 1997).

We showed that the iPSC-derived inhibitory neurons from the proband had the lowest ratio of extracellular/intracellular REELIN. The maternal neurons also had reduced extracellular/intracellular REELIN compared to the paternal line, but the reduction was significantly greater in the proband neurons (Figure 2.2b). Given that both the proband and the maternal cells carry the RELN variant (R2457C), the greater extent of reduction in REELIN secretion in the proband cells suggests that those cells have additional genetic liability that may worsen this cellular phenotype and may have contributed to the proband’s clinical phenotype.

The role of the R2457C variant in reducing REELIN secretion was further demonstrated by its heterologous expression in HEK293 cells along with another ASD-linked RELN variant (R2290C), both of which reduced the extracellular/intracellular REELIN ratio compared to the WT RELN construct (Figure 2.2a). Both of these variants belong to a category of RELN mutations that are associated with ASD, and we previously showed that they are enriched in the RXR motif, an evolutionary conserved region in the REELIN
protein repeat domain (Lammert & Howell, 2016).

These findings suggest that the proband has lower levels of extracellular REELIN available for the receiving cells, most importantly the cortical pyramidal neurons, disrupting the REELIN signaling in these neurons.

Modeling the cortical pyramidal neurons with the iPSC-derived excitatory neurons, we demonstrated that the average dendritic length and complexity was significantly lower in the excitatory neurons from the proband compared with the parental neurons (Figure 2.3).

Reduced dendritic complexity has been associated with ASD (Fetit et al., 2021; Fink & Levine, 2018; Martínez-Cerdeño, 2017) and with disruptions in REELIN signaling (Chai et al., 2015; Matsuki et al., 2008; Niu et al., 2004). We showed that the reduced dendritic complexity in the proband excitatory neurons is not due to endogenous REELIN, since despite expressing RELN mRNA, the excitatory neurons do not express a detectable level of REELIN protein. Moreover, we showed that the RELN-KO excitatory neurons had similar dendritic complexity to the unedited neurons (Figure 2.4).

However, the dendritic complexity was normalized with REELIN treatment, making the proband neurons statistically indistinguishable from the parental neurons in terms of dendritic complexity (Figure 2.5). REELIN treatment did not change the dendritic complexity in the parental cells themselves, however, which suggests that REELIN specifically counteracts a factor that underlies the dendritic abnormality in the proband cells.

Another implication of these findings is that the proband excitatory neurons are more
vulnerable to a potential REELIN deficiency. And since REELIN secretion is lower in the proband inhibitory neurons, it is possible that there were additive effects from both inhibitory and excitatory neurons in the proband’s neocortex that contributed to his clinical phenotype.

The vulnerability of the proband’s excitatory neurons must be due to an additional predisposition at a molecular or cellular level, which would have an additive effect on the reduction of dendritic complexity as well as on the clinical phenotype of ASD. We provided evidence that part of this additional liability is due to a downregulation of canonical WNT signaling and an overactivation of GSK3β protein in the proband neurons.

We showed that the expression levels of WNT target genes in response to WNT3a treatment were lower in the proband excitatory neurons compared to those of the parental cells, suggesting that dysregulated WNT signaling may have contributed to the ASD pathophysiology in this patient. Sequencing studies have revealed that WNT signaling is among the few pathways that represent a functional convergence of genes that are implicated in ASD (Krumm et al., 2014; Packer, 2018). And RELN may be one of those genes that interact with the WNT signaling, since we showed that the response of WNT target genes to WNT3a increased in the proband cells after a pre-treatment with REELIN (Figure 2.8). This finding suggests that the altered WNT signaling in the proband cells is due to a factor that is downstream to the REELIN signaling.

We made a case for GSK3β to be such a factor linking the dysregulated REELIN signaling and WNT signaling in the proband cells. GSK3β is regulated by the REELIN/DAB1/PI3K/AKT pathway (Ohkubo et al., 2003), and it is an important regulator
of several molecular pathways, including the canonical WNT signaling (Wu & Pan, 2010), as well as the neurodevelopmental processes relevant to ASD, such as neuronal migration and synaptic function (Hur & Zhou, 2010). We demonstrated that the total GSK3β protein was increased whereas its inactive phosphorylated form pS9-GSK3β was decreased, suggesting augmented GSK3β activity (Figure 2.6).

GSK3β is an unusual enzyme since it is constitutively active, and its signaling effect is through inactivation by phosphorylation, most importantly at the serine-9 residue (Beurel et al., 2015). Overactivation of GSK3β has been associated with autistic-like behaviors in animal models (Mines et al., 2010; Zhang et al., 2012), and with the cellular phenotypes that are associated with ASD, such as dendritic growth (Llorens-Martín et al., 2013; Rui et al., 2013). We showed that the reduced dendritic complexity in the proband neurons may be partially due to an overactivation of GSK3β because treating neurons with a selective GSK3β-inhibitor (IM-12) increased the dendritic complexity in the proband neurons up to the level of parental neurons. However, this effect was seen only in the proximal parts of the dendritic arbor, and the total dendritic length remained lower in the proband neurons after the IM-12 treatment (Figure 2.7).

This partial effect of GSK3β inhibition on increasing the dendritic complexity indicates that GSK3β overactivation is not the only underlying cause of this phenotype. On the other hand, the complete normalization of dendrites in response to REELIN treatment suggests that exogenous REELIN has an overriding effect on all underlying causes including GSK3β signaling. This is not surprising as it has been previously shown that GSK3β is downstream to REELIN signaling (Ohkubo et al., 2003).
Another pathway that both regulates REELIN signaling (Moon et al., 2015) and is regulated by REELIN signaling (Jossin & Goffinet, 2007) is the mTOR pathway. A study by Sánchez-Sánchez et al. (2018) showed that inhibition of mTOR signaling by rapamycin improved the impaired REELIN signaling associated with an ASD-linked RELN variant. We asked whether mTOR overactivation contributed to the dendritic phenotype in the proband neurons. Rapamycin treatment did not improve the dendritic complexity in the proband neurons, suggesting that overactive mTOR signaling is not part of the pathophysiology in the proband neurons (Figure 2.9a,b). This is not surprising because we used a different cell type compared to the aforementioned study (mature neurons vs. neural progenitor cells) and studied a different cellular phenotype in a different patient with a different RELN variant (Sánchez-Sánchez et al., 2018). We provided further evidence against the possibility of mTOR dysregulation in the proband cells by showing that S6, a ribosomal protein used as a readout for mTOR activity (Zhang et al., 2006), is not differentially activated in the proband compared to the parental neurons (Figure 2.9c).

In summary, we characterized iPSC-derived neurons from a patient with ASD (proband) who had a maternally-inherited RELN RXR variant, and neurons from his non-autistic parents. We found several features of iPSC-derived excitatory and inhibitory neurons that may have contributed to the ASD pathophysiology in the proband. We showed that the iPSC-derived inhibitory neurons from the proband had a reduced ratio of extracellular/intracellular REELIN, suggesting a reduced secretion, which was more severe in the proband than in the unaffected maternal inhibitory neurons. We showed that the iPSC-derived excitatory neurons from the proband had reduced dendritic complexity and average total length. This phenotype was rescued by REELIN treatment but was
independent of the RELN gene, since the phenotypes remained unchanged in the RELN-KO excitatory neurons. The proband excitatory neurons also had increased activity and expression levels of GSK3β, and the dendritic complexity was partially rescued by IM-12, which is a selective GSK3β inhibitor and a canonical WNT signaling activator. The WNT target genes had lower expression levels in response to WNT stimulation in the proband excitatory neurons, and this phenotype was normalized with REELIN treatment.

Therefore, we propose a model (Figure 3.2) in which the pathophysiology of ASD in the proband is likely due to a combination of additive risk factors from both the REELIN-secreting inhibitory neurons and the REELIN-responding excitatory neurons, the former with reducing REELIN secretion and the latter with overactive GSK3β and underactive WNT signaling, all of which culminating in reduced dendritic growth and complexity.
2.5. References


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Chapter 3

General Discussion

Contribution:

Ryan Mokhtari did all the experiments and wrote this chapter. Brian Howell generated the RELN KO cell lines, supervised the experiments, and edited the text.
We conceived this study by asking how an RXR RELN variant may have interacted with other genetic risk factors in a patient with ASD. We hypothesized that those risk factors had additive effects with the RELN variant, and that abnormal dendritic complexity may have been the cellular phenotype that contributed to ASD pathophysiology in this patient, since this phenotype is implicated in ASD and also dendritic growth is a REELIN-dependent phenotype.

To address those questions, we used iPSCs from a patient with ASD (the proband) and his non-autistic parents. The proband was heterozygous for a rare RELN variant which he had inherited from his mother. This variant is a missense mutation (R2457C) at a region of REELIN protein that is rich in RXR motifs, and we had previously shown that RXR mutations are associated with decreased REELIN secretion (Lammert et al., 2017). REELIN is secreted from certain types of cells, including a population of inhibitory cortical neurons, and binds to receptors on the cortical pyramidal neurons, which require REELIN for their migration into the developing cortex as well as for the growth and branching of their dendrites (Jossin, 2020). Although we know from the mouse models that REELIN regulates dendritic growth, those models (typically the Reeler mouse) are associated with more severe anatomical and cellular phenotypes than the phenotypes associated with ASD (Amaral et al., 2008; Ecker et al., 2017).

We used iPSCs to generate two types of neurons, the inhibitory neurons to model the REELIN-secreting inhibitory neurons, and the excitatory neurons to model the REELIN-responding cortical pyramidal neurons. We showed that the R2457C variant is associated with reduced REELIN secretion, which we inferred from lower extracellular/intracellular
REELIN ratios in cells carrying the R2457C variant, both in the transfected cells and in the iPSC-derived inhibitory neurons from both the proband and his mother. However, the REELIN secretion was lower in the proband neurons than in the maternal neurons. This suggests that there might be predisposing factors in the proband inhibitory neurons that have an additive effect on the secretion phenotype of R2457C.

The presence of any additive risk factors in the proband cells would not be surprising, given the presence of a clinical phenotype in the proband and lack thereof in his mother. However, we showed that there are also important additive risk factors in the proband excitatory neurons.

The complexity of dendrites was reduced in the proband excitatory neurons, but this phenotype was normalized to the level of parental neurons in response to REELIN treatment. However, the dendritic morphology remained unchanged in the RELN-KO excitatory neurons. This suggests that the dendritic morphology is not due to REELIN produced in the excitatory neurons, but its underlying cause is reversible by exogenous REELIN.

The underlying cause of reduced dendritic complexity in the excitatory neurons of the proband was also related to an overactivation of GSK3β and also possibly due to an under activation of the canonical WNT signaling, since WNT response was compromised and rescued by REELIN pretreatment. GSK3β is downstream to the REELIN signaling (Ohkubo et al., 2003), and its activity is negatively correlated with the activity of canonical WNT signaling (Wu & Pan, 2010). GSK3β is a constitutively active kinase, and its inactivation is necessary for several signaling pathways and cellular processes such as den-
dritic growth (Llorens-Martín et al., 2013; Rui et al., 2013). We showed that the overactive GSK3β in the proband excitatory neurons likely contributed to the dendritic phenotype, since the dendritic complexity partially improved in response to treatment with IM-12, which is both a GSK3β-inhibitor and a canonical WNT activator.

The partial improvement of dendrites (rescue of the proximal branches) can be due to an incomplete inhibition of GSK3β as a result of insufficient drug dose or treatment length. Alternatively, the partial effect may suggest that GSK3β overactivation is only one of the dysregulated pathways that contributed to the dendritic phenotype. It is conceivable that the newly grown proximal dendritic branches would have continued to grow had they been exposed to IM-12 for a longer period of time. However, the same duration of treatment with REELIN completely rescued the dendritic branching, suggesting that the treatment length for IM-12 was not necessarily short.

We also provided evidence for a downregulated canonical WNT signaling in the proband excitatory neurons, inferring from the lower expression of WNT target genes in response to WNT3a, a canonical WNT signaling ligand. The expression levels of WNT target genes were normalized in response to a co-treatment of WNT3a and REELIN. This suggests that dysregulations in WNT signaling and GSK3β activity may be part of the same underlying cause that contributes to the dendritic abnormality and is reversible by exogenous REELIN.

Dysregulation of WNT signaling in the proband cells is a reflection of the findings from genetic analyses on ASD populations, showing that WNT signaling is one of the few pathways that represent a functional convergence of ASD risk genes (Krumm et al., 2014; Packer, 2018). This should not be surprising given the fundamental role of WNT signaling
in regulating the neurodevelopmental processes that are relevant to the ASD pathophysiology. The other aspect of the WNT signaling which makes it relevant to ASD is its extensive involvement in transcription regulation. The largest ASD whole-exome sequencing study so far (Satterstrom et al., 2020) concluded that transcription regulation is one of the two major functional categories that represent the genes associated with ASD, the other category being neuronal communication and synaptic function. Although we did not examine synaptic function in this study, it is conceivable that synaptic deficits might have contributed to the proband’s condition as well, since REELIN signaling (Wasser & Herz, 2017), WNT signaling (McLeod & Salinas, 2018), and GSK3β (Bradley et al., 2012) are all necessary for normal synaptic function.

We propose that both underactive WNT signaling and overactive GSK3β contribute to the sensitivity of proband pyramidal neurons to lower levels of REELIN secreted from his inhibitory neurons. However, we do not know if these two abnormalities are causally related, and if so, which causes the other. Also, the mechanism for increased total GSK3β and suppressed S9 phosphorylation in the proband excitatory neurons remains unknown. Since S9-phosphorylated GSK3β is targeted for proteasomal degradation (McCubrey et al., 2014), our finding that proteasome-inhibitor treatment did not differentially change GSK3β levels rules out proteasome-mediated degradation as possible mechanisms for high GSK3β in the proband cells. On the other hand, similar levels of GSK3B mRNA across the cell lines ruled out transcriptional regulation as possible mechanisms for high GSK3β in the proband cells. However, regulation of GSK3β is complex and involves multiple mechanisms such as localization and sequestration as well as phosphorylation by many kinases (Kaidanovich-Beilin & Woodgett, 2011).
Both the canonical WNT signaling and GSK3β regulate dendritic growth, yet different mechanisms have been proposed for each. Tan et al. (2013) showed that GSK3β inhibition promotes the dendritic growth via upregulation of CRMP2 (collapsin response mediator protein-2), and Viale et al. (2019) showed that canonical WNT signaling promotes dendritic growth in the cortical pyramidal neurons by upregulating NT3 (neurotrophin-3).

Although GSK3β and WNT signaling negatively regulate each other, the overactivation of GSK3β and under activation of WNT signaling in the proband neurons may not necessarily have a common origin. As mentioned earlier, most cases of ASD are caused by many common variants each having a small effect. This is likely to be the case in the proband as well, and it is likely that those common variants have converged on dysregulations of GSK3β and WNT signaling, which have made the excitatory neurons vulnerable to the reduced REELIN secretion, which itself was a result of a rare RELN variant. This RELN variant also increased intracellular REELIN, which might have other detrimental effects, as our lab previously showed that another ASD-linked RXR RELN variant increased intracellular REELIN and activated ER stress response (Lammert et al., 2017).

One reason for the many common variants argument is that we screened the whole-exome sequencing results from the proband and the parental lines, looking for rare variants associated with ASD. We did not find any rare variants of the high-confidence ASD genes (SFARI Gene, 2023) in the proband exome. We found a category-2 gene variant (BIRC6) that we explored for possible interaction with RELN, but our data were inconclusive. We also performed RNA-seq on iPSCs and inhibitory neurons from the three cell lines. However, the list of differentially expressed genes did not include any ASD-linked genes.
What makes common variants conducive to ASD risk in one genome but not in another genome is probably the exact combination of the variants. This is why non-autistic parents may have children with autism (even without de novo risk variants). Common variants in each parent may not be sufficient to cross the clinical threshold, but an unfortunate recombination of their genomes can lead to ASD in their child, and not in other children of theirs. A reflection of this principle is the fact that parents of autistic children are more likely to have subthreshold autistic traits even if they do not have an ASD diagnosis, according to a systematic review by Rubenstein & Chawla (2018). The same study showed that the autistic traits are more common among the unaffected fathers than unaffected mothers (Rubenstein & Chawla, 2018).

As discussed earlier, ASD is on average 4 times more common in men (Maenner et al., 2021; Zeidan et al., 2022) and there is even a higher male bias in high-functioning autism (Brugha et al., 2011; Gillberg et al., 2006). The Female Protective Effect (Jacquemont et al., 2014) refers to findings from population studies concluding that, with similar genetic backgrounds, males have lower thresholds than females for manifesting the clinical autism phenotype. There is also a mother-to-son bias for the inheritance of gene-disrupting variants associated with ASD (Iossifov et al., 2015; Krumm et al., 2015). This implies that a non-autistic mother could transmit a risk variant to her son, making him more vulnerable to ASD, whereas her daughter with the same variant would be less vulnerable. This is probably the case with the family we studied. The R2457C variant is predicted to be likely gene-disrupting, and we showed that it is associated with reduced REELIN secretion. However, any ASD-related risk that R2457C might have conferred to the proband was probably mitigated in his mother.
The underlying mechanism for the female protective effect is still debated (Lawrence et al., 2022; McCarthy & Arnold, 2011). However, two major categories have been historically considered, namely, the sex hormones and the sex chromosomes. Whereas the sex hormones do not typically play a role in an in-vitro study like ours, the role of sex chromosomes, particularly the X chromosome, cannot be overlooked (Mallard et al., 2021). Many X chromosome genes have crucial functions in neurons, including in regulation of synaptic transmission. However, none of those genes were differentially expressed among the three cell lines in our RNAseq analysis.

The protective factors in the proband’s mother could be in part related to GSK3β and WNT signaling in her excitatory neurons, but also partly due to a more efficient secretion of REELIN in her inhibitory neurons compared to the proband neurons. Whereas GSK3β and WNT signaling are not known to be differentially regulated by sex, there is some evidence for interaction between sex and REELIN physiology. Heterozygous Reeler mice lose their Purkinje cells only if they are male (Hadj-Sahraoui et al., 1996), and this phenotype is corrected temporarily by neonatal administration of estrogen (Macri et al., 2010). Male mice have higher REELIN expression and higher Aβ plaques in a mouse model of Alzheimer Disease (Palladino et al., 2017). Reln mRNA expression in the hippocampus increases in response to early life stress and the stress hormone corticosterone, only in the male mice (Gross et al., 2012).

Besides a possible role for sex in modulating REELIN, these studies and others (Fatemi, 2011; Labouesse et al., 2017) suggest that REELIN may be a marker of resilience against stress, and in some contexts or genetic backgrounds males can be more vulnerable. It is
conceivable that the unique genetic background in the proband in our study made him particularly vulnerable to a situation where REELIN needed to be upregulated in response to a stress signal (e.g., early in his development), but the response would have been insufficient since almost half of the REELIN made in his cells are defective for secretion.

The genetic background that has made the proband cells vulnerable is probably related to GSK3β and WNT signaling dysregulations, as our data suggest. There is evidence that a dysregulated WNT signaling itself changes the expression of RELN (Bem et al., 2019; Wang P. et al., 2017), which would further complicate the picture if the RELN variant is associated with reduced REELIN secretion. On the other hand, REELIN signaling is upstream of GSK3β, which itself regulates WNT signaling.

To make sense of these molecular interactions and relate them to the pathophysiology of ASD, it is useful to study a relevant cellular phenotype that is at an intermediate level between the molecular and the clinical phenotype. Dendritic morphology is particularly useful in this context because both REELIN (Chai et al., 2015) and GSK3β (Llorens-Martín et al., 2013) are required for normal dendritic growth, and reduced dendritic complexity is seen in postmortem brains from individuals with ASD (Fetit et al., 2021), in some animal models of autism (Martínez-Cerdeño, 2017), and in iPSC-derived neurons from ASD patients (Fink & Levine, 2018).

We showed that the dendritic complexity and the total dendritic length were reduced in the iPSC-derived excitatory neurons from the proband, and this phenotype was reversible by REELIN treatment, and partially reversible by IM-12, a GSK3β inhibitor and a canonical WNT signaling activator. We argue that the clinical phenotype in the proband might have
been the result of underdeveloped dendrites in his cortical pyramidal neurons, which itself was the result of GSK3β and WNT dysregulations in those neurons as well as reduced REELIN secretion from his inhibitory or Cajal-Retzius neurons. Given that the correction of dendritic phenotype was complete for REELIN treatment, but only partial for IM-12 treatment, it follows that the GSK3β and WNT dysregulations are only part of the vulnerability in the proband excitatory neurons, but REELIN has an overriding effect on the GSK3β/WNT signaling and any other pathway that might have contributed to the dendritic phenotype.

This overriding effect of REELIN on the GSK3β/WNT signaling in relation to dendritic growth may have important implications for translational research on ASD. Whereas GSK3β-inhibitors have been extensively researched for different psychiatric conditions including ASD (Arciniegas Ruiz & Eldar-Finkelman, 2021), possible therapeutic potentials of REELIN have not been explored nearly as much. This is partly due to the challenge of delivering large proteins such as REELIN to the brain, compared to the small molecules such as GSK3β-inhibitors (Ulapane et al., 2019). However, in vitro experiments do not have this limitation and can be used to further explore the potential correcting effects of REELIN on certain molecular and cellular phenotypes associated with ASD.

Our study offers a molecular pathway (GSK3β/WNT signaling) and a cellular phenotype (dendritic complexity) for further exploration in future translational studies. For example, iPSCs from ASD patients with mutations in WNT signaling (Packer, 2018) can be treated with REELIN, and the resulting effects on ASD-related cellular phenotypes can be examined.
An important ASD-related cellular phenotype is the imbalance between proliferation and differentiation in neural progenitor cells (NPCs) in the cortex, leading to increased neurogenesis (Fang et al., 2014), which may partly contribute to brain overgrowth seen in some patients with ASD (Courchesne et al., 2011b). WNT/β-Catenin signaling is critical for the proper balance between proliferation and differentiation in the neocortical progenitor cells (Davidson et al., 2012; Munji et al., 2011), and this role of the canonical WNT signaling appears to be a strong candidate for the mechanism by which WNT signaling abnormalities contribute to the ASD pathophysiology (Packer, 2016). For instance, iPSC-derived NPCs from ASD patients with early brain overgrowth showed dysregulation of a β-Catenin/BRN2 transcriptional cascade (Marchetto et al., 2017).

The role of WNT signaling in regulating neocortical proliferation/differentiation and its dysregulation in ASD may be relevant to our study as well. We found that WNT signaling is upregulated in the proband iPSCs, but after they differentiate to excitatory neurons, WNT signaling becomes downregulated. It is conceivable that this pattern of WNT activity in the proband cells has probably affected the differentiation and maturity of his neurons, contributing to the reduction in dendritic growth and complexity. Interestingly, OTX2, which is an important regulator of stem cell differentiation in early brain development, was among the DEGs in the proband iPSCs.

Another pathway that interacts with REELIN signaling is the mTOR pathway (Jossin & Goffinet, 2007), which is overactive in some patients with ASD (Winden et al., 2018). Therefore, we hypothesized that mTOR overactivation would be the other part of the neuronal vulnerability in the proband. However, we found evidence negating this
hypothesis by showing that inhibiting mTOR with rapamycin treatment did not improve the dendritic complexity in the proband neurons, and the mTOR pathway ribosomal protein S6 (Zhang et al., 2006) was not differentially activated in those neurons.

Exploring a possible role for mTOR signaling was relevant to our study for several reasons. mTOR signaling is among the molecular pathways implicated in the pathophysiology of ASD (Winden et al., 2018), it is required for dendritic growth and branching (Jossin & Goffinet, 2007), and it interacts with both GSK3β (Urbanska et al., 2018) and REELIN signaling (Moon et al., 2015). Recent studies on iPSC-derived neural progenitors cells (NPCs) from ASD patients have shown that certain RELN variants interact with other ASD risk variants (e.g., CACNA1H) in ways that converge on mTOR overactivation and disrupt certain cellular phenotypes such as migration and proliferation of NPCs (Sánchez-Sánchez et al., 2018; Teles e Silva et al., 2022).

However, our study had important differences in methodology and scope with those studies. First, we studied a different RELN variant in a different patient. Second, we differentiated the iPSCs to a more mature neuronal stage as described earlier (Wang C. et al., 2017), and did not use NPCs. Moreover, we focused on a different phenotype, namely, dendritic complexity. We showed that this phenotype was responsive to exogenous REELIN but independent of endogenous REELIN, which the iPSC-derived excitatory neurons do not express at the protein level. Thus, the reported interactions between the RELN variants and mTOR pathway are probably not relevant to the cells we analyzed for dendritic morphology.

The differentiation stage of iPSC-derived neurons makes a significant difference in their
transcriptomic profile (Bardy et al., 2016). In some cases the activity of a pathway (such as WNT signaling) is reversed when iPSCs transition through different stages of maturity (Bengoa-Vergniory et al., 2014), which can make it challenging to interpret the data. Nonetheless, this may explain our finding that the undifferentiated iPSCs of the proband had higher levels of WNT signaling activity compared to the parental iPSCs (Figure 2.10), which is the opposite of what we found in the iPSC-derived mature neurons. Another caveat about the iPSC studies is that the cell line variability is not limited to the obvious differences between cell lines from different individuals. Several studies have shown that even within the clones of the same cell line there is considerable variability (Burke et al., 2020; Volpato et al., 2018).

3.1. Limitations

This study had a few important limitations. First, iPSC-derived neurons, like other in vitro studies, lack the histological complexity and cell type variety in the brain. Second, despite the structural and functional validation of the iPSC-derived neurons, these cells are probably not completely identical to the neurons in vivo, and any imperfect purity in the culture further limits our capacity to extrapolate from the in vitro findings. Another limitation was studying excitatory and inhibitory neurons separately and not using co-cultured neurons of both types, which would have provided more accurate information on the interaction between these neurons. Finally, because the cell lines used in this study were de-identified, we did not have any information on the clinical presentation of the proband or any relevant information on the parents, such as the possibility that one or both parents may have had subclinical autistic traits.
3.2. Future Directions

We studied the excitatory and inhibitory neurons separately, but it would be informative to examine the interaction between them more closely by co-culturing both types of neurons. A simpler preliminary experiment before the co-culture would be adding conditioned media (CM) from the inhibitory neurons to the excitatory neurons, asking whether CM from either parent’s inhibitory neurons rescues the dendritic phenotype in the proband excitatory neurons, or conversely, whether adding the proband inhibitory CM to the parental excitatory neurons decreases their dendritic complexity. Following these experiments, any changes in the activity of DAB1 and other proteins in REELIN signaling can be measured.

To further explore the role of GSK3β in dendritic growth regulation, it would be informative to first distinguish between the GSK3β overactivation (hypo-phosphorylation) and increased total protein levels and determine which one has a more important role in this context. To that end, it would be useful to use the parental cell lines and test whether GSK3β overactivation (using drugs) and/or overexpression (using stable transfection) would recapitulate the dendritic phenotype seen in the proband neurons. Similarly, treating the parental neurons with WNT inhibitor drugs can determine the extent to which WNT downregulation can recapitulate the reduced dendritic complexity. These experiments may also reveal differences between the paternal and maternal cell lines that might give us clues about the role of sex in regulating the pathways implicated in ASD.

Another approach to study the role of WNT signaling in the iPSC-derived excitatory neurons is using the neuronal differentiation protocol published by Nehme et al. (2018)
that showed combining NGN2 expression with dual SMAD and WNT inhibition yields more mature neurons. Dendritic complexity could be re-examined with this differentiation method, and the WNT inhibition can be titrated to determine whether it changes the dendritic phenotype. It is possible that the lower dendritic complexity in the proband neurons is due to immaturity as a result of WNT signaling upregulation. One approach to find out whether the proband dendrites are less complex due to immaturity is to let them grow longer to see if they reach the level of complexity comparable to that of the parental dendrites, and if so, whether manipulation of WNT activity at different stages (during or after neuronal differentiation) can normalize the dendritic phenotype.

Besides immaturity, a differential cell fate may also explain the dendritic phenotype. Even neurons with slight differences in their functional profile can have significant differences in their dendritic morphology (Jacobs et al., 2001). RNAseq on excitatory neurons (we performed RNAseq only on inhibitory neurons and iPSCs) will reveal any fate differences among the cell lines that might underlie the dendritic phenotype. RNAseq can also potentially address other questions that arose from our study but remain unanswered for now.

One such unanswered question is what molecular mechanisms mediate the effects of overactive GSK3β and underactive WNT signaling on the reduced dendritic complexity. Tan et al. (2013) showed that GSK3β inhibition promotes the dendritic growth in cerebellar granule neurons via the upregulation of CRMP2 (collapsin response mediator protein-2). It would be worthwhile investigating whether CRMP2 is also the downstream mediator of GSK3β in the iPSC-derived neurons. Another study by Viale et al. (2019) has shown that
the canonical WNT signaling promotes dendritic growth in the cortical pyramidal neurons by upregulating NT3 (neurotrophin-3). It would be interesting to examine the expression status of this protein in the proband neurons, especially given the fact that we use recombinant NT3 in the differentiation protocol, and if NT3 is differentially expressed, titrating recombinant NT3 might be useful to determine whether it affects the dendritic phenotype.

3.3. Conclusion

We characterized iPSC-derived cortical neurons from a patient with ASD and his non-autistic parents, and proposed a model for the pathophysiology of ASD in this patient. We provided evidence that abnormalities in REELIN signaling, WNT signaling, and GSK3β converged on reduced dendritic growth and complexity, likely contributing to his clinical phenotype. This finding highlights the pivotal role of dendrites and their morphology in relation to ASD, for which there was already some evidence based on postmortem and animal studies. However, human-origin neurons have not been studied extensively in this context.

Recent studies have shown that human neurons have unique biophysical properties compared to neurons of other animals (Beaulieu-Laroche et al., 2021; Kalmbach et al., 2018), and dendrites are probably the most important component that give human neurons specific computational powers (Fişek & Häusser, 2020). For example, Beaulieu-Laroche et al. (2018) recorded layer 5 pyramidal neurons from humans and rats, and concluded that human dendrites have more capacity to functionally compartmentalize the neuron because they are larger and they have lower densities of ion channels. This compartmentalization
may enable dendrites to process the electrical input independently of the soma (Beaulieu-Laroche et al., 2018). Another feature that makes human dendrites computationally independent is a particular form of calcium-mediated action potential that was recently discovered in layer 2/3 cortical neurons, which is believed to facilitate a specific logical operation (exclusive OR) previously thought to be impossible in neurons (Gidon et al., 2020).

These discoveries highlight the importance of studying dendrites and the psychiatric disorders like ASD, in which dendritic abnormalities may be part of the pathophysiology. Despite evidence from animal studies and human studies on postmortem brain and iPSC-derived neurons (Fetit et al., 2021; Fink & Levine, 2018; Martínez-Cerdeño, 2017), there is no consensus whether dendritic abnormalities are a universal feature of autism, or if they are, how they lead to the specific behavioral phenotypes. Future studies can shed light on the role of dendritic abnormalities and other cellular phenotypes, using iPSC-derived neurons from ASD patients. It remains to be seen whether a small number of phenotypes at the level of neurons and circuits can explain the pathophysiology of ASD regardless of its underlying genetic background.
**Figure 3.1.** A simplified illustration of the intersection between three pathways that are implicated in the ASD pathophysiology: the REELIN signaling, the canonical WNT signaling, and the mTOR signaling. Activation of both REELIN signaling and WNT signaling lead to GSK3β inhibition, which is required for the inactivation of the β-Catenin destruction complex (Axin, APC, CK1α, GSK3β) and its membrane translocation. This allows β-Catenin to enter the nucleus, facilitating the expression of WNT target genes. GSK3β is also inhibited by the activation of REELIN/DAB1/PI3K/AKT pathway. The latter pathway has co-regulatory interactions with the mTOR signaling as well.
**Figure 3.2.** We propose that the pathophysiology of ASD in the proband involves an impaired REELIN signaling due to both reduced REELIN secretion from the inhibitory neurons as well as a vulnerability in the REELIN-responding excitatory neurons which has an additive effect. The latter is likely due to an overactive GSK3β signaling and an underactive WNT signaling. These molecular dysregulations culminate in the reduced dendritic complexity, a phenotype that may explain the ASD pathophysiology in the proband.
Model for pathophysiology in proband’s neocortex

Inhibitory neuron

↓ REELIN secretion

Excitatory neuron

↓ WNT signaling
↑ GSK3β activity

↓ Dendritic complexity

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3.4. References


