Title
Modeling autism spectrum disorders in a dish and the effect of TRPC6 disruption

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Modeling autism spectrum disorders in a dish and the effect of TRPC6 disruption

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

Doctor of Philosophy

in

Biomedical Sciences

by

Allan Jay Acab

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2015
The Dissertation of Allan Jay Acab is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2015
DEDICATION

This work is dedicated to

my mother, brother,

family, friends, colleagues,

and especially,

to my wife Anh,

Thank you.

And also to my dog Choco, thanks buddy.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature Page</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Vita</td>
<td>x</td>
</tr>
<tr>
<td>Abstract of the Dissertation</td>
<td>xi</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>1</td>
</tr>
<tr>
<td>Autism</td>
<td></td>
</tr>
<tr>
<td>Chapter 2</td>
<td>26</td>
</tr>
<tr>
<td>TRPC6</td>
<td></td>
</tr>
<tr>
<td>Chapter 3</td>
<td>36</td>
</tr>
<tr>
<td>Induced pluripotent stem cell technology and disease modeling</td>
<td></td>
</tr>
<tr>
<td>Chapter 4</td>
<td>67</td>
</tr>
<tr>
<td>Modeling non-syndromic autism and the impact of TRPC6 disruption in human neurons</td>
<td></td>
</tr>
<tr>
<td>Chapter 5</td>
<td>147</td>
</tr>
<tr>
<td>Summary and future directions</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2.1</td>
<td>TRPC6 signaling pathways</td>
</tr>
<tr>
<td>3.1</td>
<td>iPSC disease modeling</td>
</tr>
<tr>
<td>3.2</td>
<td>RTT human iPSC-derived neuronal phenotypes</td>
</tr>
<tr>
<td>4.1</td>
<td>Mapping the breakpoints in the ASD individual with the 46, XY, t(3;11)(p21;q22) karyotype</td>
</tr>
<tr>
<td>4.2</td>
<td>TRPC6 channels regulate the expression of neuronal development genes</td>
</tr>
<tr>
<td>4.3</td>
<td>Derivation of NPCs and neurons from iPSCs</td>
</tr>
<tr>
<td>4.4</td>
<td>Alterations in neural cells derived from the TRPC6-mutant individual</td>
</tr>
<tr>
<td>4.5</td>
<td>TRPC6 regulates the neural development of adult-born neurons in the dentate gyrus of the hippocampus</td>
</tr>
<tr>
<td>4.6</td>
<td>Confirmation of TRPC6 disruption and gene expression dysregulation in TRPC6-mutant cells</td>
</tr>
<tr>
<td>4.7</td>
<td>Generation and characterization of iPSCs</td>
</tr>
<tr>
<td>4.8</td>
<td>Electrophysiological recordings and morphological phenotypes of iPSC-derived cortical neurons</td>
</tr>
<tr>
<td>4.9</td>
<td>TRPC6-mut neural cell analysis, MeCP2 promoter occupation, and shRNA validation</td>
</tr>
<tr>
<td>4.10</td>
<td>Principal component analysis and population stratification plots</td>
</tr>
<tr>
<td>5.1</td>
<td>Summary of TRPC6-mut neuronal phenotypes</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Human iPSC models of autism spectrum disorders</td>
<td>53</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Selected functionally relevant genes differentially expressed between the TRPC6-mutant individual and controls</td>
<td>125</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Sequence of the primers used in qPCR experiments</td>
<td>126</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>PCR primers covering all coding regions of TRPC6, designed by RainDance</td>
<td>127</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Rare de novo SNVs and frameshift mutations found in TRPC6-mutant individual</td>
<td>128</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Differentially expressed genes between TRPC6-mutant individual and controls</td>
<td>130</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>Fingerprinting analysis of DPC and iPSC lineages from TRPC6-mutant individual and one control sample</td>
<td>133</td>
</tr>
<tr>
<td>Table 4.7</td>
<td>Summary of the iPSC subjects and clones (C) utilized for each experiment</td>
<td>134</td>
</tr>
<tr>
<td>Table 4.8</td>
<td>Quality metrics of high-throughput sequencing</td>
<td>135</td>
</tr>
<tr>
<td>Table 4.9</td>
<td>Novel nonsynonymous singleton mutations in TRPC6</td>
<td>136</td>
</tr>
</tbody>
</table>
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Acab A, Muotri AR. The use of induced pluripotent stem cell technology to advance autism research and treatment. Neurotherapeutics. (accepted).


ABSTRACT OF THE DISSERTATION

Modeling autism spectrum disorders in a dish and the effect of TRPC6 disruption

by

Allan Jay Acab

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2015

Professor Alysson R. Muotri, Chair

Autism spectrum disorders (ASDs) are a group of complex neurodevelopmental disorders of high prevalence in the United States, affecting 1 in 68 children. ASDs demonstrate a strong genetic component and share a common core set of symptoms, yet are etiologically genetically heterogeneous. An increasing number of genetic variants have been implicated in ASDs, and the functional study of these variants will be critical for the elucidation of autism pathophysiology. The advent of induced pluripotent stem
cell (iPSC) technology has allowed for the generation of human cellular disease models containing individual patient genomes. Here, we report a \textit{de novo} balanced translocation disrupting the TRPC6 gene in a non-syndromic autistic individual. Using multiple models, including dental pulp cells, iPSCs, and mice, we demonstrate that TRPC6 reduction leads to altered neuronal development, morphology and function. The observed phenotypes could then be rescued by restoration of TRPC6 levels and by treatment with insulin-like growth factor-1 or hyperforin, a TRPC6-specific agonist, suggesting that ASD individuals with alterations in this pathway may benefit from these drugs. We also show that \textit{MECP2}, the causative gene in Rett syndrome, affects TRPC6 expression levels, suggesting common pathways among ASDs. Genetic sequencing of TRPC6 in large ASD and control cohorts revealed significantly more nonsynonymous mutations in the ASD population, identifying two instances of loss-of-function mutations with incomplete penetrance. Taken together, these findings suggest that TRPC6 is a novel predisposing gene for ASD. This is the first study to use iPSC-derived human neurons to model non-syndromic ASD and illustrates the potential of iPSCs to model genetically complex sporadic diseases.
Chapter 1

Autism
Introduction

Autism is an etiologically diverse group of complex, heterogeneous neurodevelopmental disorders that share a common core set of symptoms, including impaired social interaction and communication, cognitive deficiencies, and stereotyped repetitive behaviors. Collectively termed autism spectrum disorders (ASDs), these are common disorders of high prevalence, affecting 1 in 68 children in the United States according to the most recent CDC report. Interestingly, boys are five times more likely to be diagnosed with an ASD, than girls. Reported ASD incidence rates have been drastically increasing, with early estimates of about 1-5 in 1000 in surveys performed in the 1990s, to reports estimating 1 in 150 in 2007 and 1 in 88 in 2012. This increasing rate has largely been attributed to improved awareness of and ability to detect ASDs, especially milder cases, rather than a true general increase in incidence. Understandably, this increasing trend in ASD prevalence has garnered great attention from doctors, researchers, and of course parents, as to what is driving the etiology of ASD.

First described by Kanner in 1943, autism was illustrated as a fascinating disorder in which children exhibit significant disturbances in social interaction, cognition and behavior, while appearing essentially, physically normal. Kanner described his patients as displaying an extreme desire for aloneness and tendency of obsessive behaviors. While Kanner pointed out that the often lack of “warmhearted” parents might have contributed, he maintained that because all of his described autistic children had the same desire for solitude and inability to socialize normally from a very early age, this was likely an innate disorder. However, for several decades, the prevailing hypothesis on the cause of autism was poor parenting, as opposed to a biological basis.
The significance of a biological basis was not apparent until the 1980s, when the incidence of autism was associated with the chromosomal abnormality causing Fragile-X syndrome \(^{10}\). In addition, twin studies revealed autism was highly heritable, with high concordance in identical twins vs. fraternal twins, as well as a high recurrence risk for families already with affected children \(^{11-13}\). Now, over decades of research, it is clear that the biology of ASDs have a strong genetic component. Studies have revealed that known mutations, genetic syndromes, and \textit{de novo} copy number variations (CNVs) account for about 10-20\% of all ASD cases \(^{14}\). Interestingly, and perhaps puzzlingly, each defined mutation accounts for only 1-2\% of cases \(^{14}\). ASD etiology provides an interesting conundrum that while ASDs share common symptoms and are highly heritable, there exists no definitive genetic causes or markers that account for the majority of cases. As a result, hypotheses, such as the multiple-hit hypothesis, which states that autism arises from the impairment of multiple genes affecting specific pathways above a certain threshold leads to autism, have become popular ways to view ASD etiology \(^{15-17}\).

**Core symptoms and neurological phenotypes of autism**

ASDs are both etiologically and clinically heterogeneous, yet they share a common core set of behavioral symptoms. ASDs usually develop early, before 3 years of age. While early phenotypes are common, medical attention or diagnosis is not sought until there are clear deficiencies such as delayed language \(^{8}\). The three defining features of ASDs are impaired social interaction, deficiencies in communication, and repetitive behaviors. In a large portion of ASD cases, expressive language in children is significantly delayed and they often fail to develop any meaningful communication skills \(^{18}\). Children with “classical autism” mostly prefer isolation over contact with those around
them. ASD individuals usually fail to make meaningful relationship with other individuals, often viewing them as akin to objects. Speech and communication are often delayed and impaired. Language skills are usually lacking because of this impaired social interaction, with difficulties proper usage and assigning meaning to words. Among the most well known features of ASDs are the repetitive behaviors demonstrated by patients. Stereotyped repetitive behaviors in ASD encompass a wide variety, including hand and finger movements, licking, twirling, stacking, and running. These repetitive behaviors can actually go on for hours, with the individuals usually finding comfort in performing them. However, while these three core features of ASD are common among patients, the actual assortment of symptoms each individual displays is unique and varies widely.

Neurological phenotypes are also apparent in ASD individuals. A wide variety of neurological and neuroanatomical abnormalities have been described, including seizures and gross anatomical changes such as microcephaly. Seizures can be found in about 25% of ASD cases. This is much higher than prevalence in all children in general, about 2%. These cases often represent the more severe forms of ASD, as ASD individuals displaying with seizures are often comorbid for other symptoms such as mental retardation and motor impairments. Both macrocephaly and microcephaly have been described in ASDs, with incidence rates at 16.7% and 15.1%, respectively. While the incidence of macrocephaly is considered high, it is not predictive of other comorbid phenotypes or severity of the disorder. No association was found between macrocephaly and cognitive impairments, seizures, or mental retardation. On the other hand, microcephaly was highly associated with severe ASD cases, often comorbid for mental retardation. Indeed there is a high incidence of microcephaly in some forms of syndromic ASDs, such as Rett syndrome (RTT). Definitely a severe form of ASD, Rett syndrome patients also display with mental retardation, seizures, and ataxia.
Postmortem studies of brains from autistic individuals have also revealed neuroanatomical phenotypes. One of the first histoanatomic studies of an autistic brain by Bauman and Kemper in 1985 revealed several abnormalities, including in the hippocampus, cortex, amygdala, and cerebellum. Subsequent studies revealed only small differences in several regions. Reduced neuronal soma size in the amygdala and hippocampus has been reported, as well as a significant decrease in Purkinje cells in the cerebellum. These studies were all performed on nonsyndromic ASD patients. In terms of syndromic ASD, postmortem studies of brains have also revealed more dramatic neuronal phenotypes. For example, postmortem studies of RTT patients revealed abnormalities in cortical neurons with reduced dendritic arborization.

The genetics of autism

Several lines of evidence point to the clear genetic component of autism and ASDs. First are the aforementioned studies revealing the heritability of autism. Twin studies have indicated that concordance rates between identical twins are much higher (70-90%) than that of fraternal twins (0-10%) \(^\text{13, 27}\). Even more, Jorde et al. demonstrated that the risk of autism increases 25-fold over the general population prevalence in children who have an affected sibling. Finally, the growing number of implicated genes that increase risk of autism and the genetically defined neurodevelopmental syndromes comorbid with autism further support the strong genetic basis of autism. Although studies have demonstrated that ASDs are highly heritable, attempts to identify specific, definitive genetic causes have had limited success. The heterogeneity of the spectrum of autistic symptoms, which varies widely from patient to patient, likely contributes to the lack of a central genetic cause. However, the study of syndromic forms of autism and of known autism-associated genes to understand affected cellular and molecular pathways
has shed light on the biology of autism. These studies focus on known affected genes to determine their functional or phenotypic consequence and overall contribution to the ASD patient. In addition, with the advent of high-throughput genome-wide association studies (GWAS) and next-generation sequencing, the heterogeneous genetic basis of autism has begun to unravel.

Syndromic forms of autism are the ASDs with known genetic causes. These forms of ASD are usually the most severe cases, as the etiologies are not specific or restricted to autism and often induce a variety of neurological defects. Syndromic ASDs comprise about 10% of all cases, and the rest are nonsyndromic, or sporadic/idiopathic, cases, where the etiology remains unknown. Examples of syndromic ASDs are fragile X syndrome (FXS), Angelman syndrome, Timothy syndrome, Down syndrome, tuberous sclerosis, and Rett syndrome (RTT). Substantial progress has been made in studying the pathogenesis of autism, and the genotype-phenotype relationships in the etiology of syndromic ASDs has been employed to identify potential pathways important in causing ASD phenotypes. For example, disorders such as fragile X, Angelman, and Rett syndromes point to synaptic dysfunction as a potential common etiology. Other syndromes such as tuberous sclerosis and Timothy syndrome reveal numerous affected signaling pathways, as well as alterations in calcium signaling. Interestingly, while several of these disorders are caused by genes with multiple functions, potential converging pathways have been identified. For example, mutations and variants in the gene causing RTT, methyl-CpG binding protein 2 (MECP2) have been identified in unrelated autistic patients. In addition, expression of important neuronal genes such as UBE3A and GABRB3 were shown to be reduced in Angelman syndrome, RTT, and also nonsyndromic autism cases. Importantly, these syndromic ASDs induce robust effects in the individual and on affected cell types, which allows for a better genotype-
phenotype functional analysis not provided by genomic alterations with more subtle effects.

Significant progress has also been made in the elucidation of the genetic etiologies of nonsyndromic forms of ASD. Essential to these studies are the large-scale sample collections of genotyped ASD individuals, such as the Simons Simplex Collection, Autism Genome Project, and the Autism Center of Excellence. Large-scale genome-wide studies and the arrival of next-generation sequencing has allowed for the emergence of an ever-growing number of ASD-associated genetic alterations potentially contributing to ASD. Several genome-wide association and linkage studies have been performed, revealing quite a few possible chromosomal regions and genes linked to ASD. Regions such as 5p14.1, 6q27 and 20p13 were identified by GWAS studies as significantly associated with ASDs. While numerous genome-wide studies have been performed for ASDs, the common theme is that the effect size for single variants is small and have low odds ratios (<1.5). Indeed, the recent report form the Autism Genome Project evaluating GWAS SNPs from 2705 families concluded that common variants may affect risk for ASD but each of their individual effects are modest. No single SNP showed a significant association with ASD at a genome-wide level across the families analyzed.

Recent genome-wide studies studying CNVs in ASDs have also revealed several genetic abnormalities potentially contributing to ASD etiology. Sebat and colleagues found a significantly increased amount of CNVs in children from ASD families compared to controls. A whole genome CNV study by Glessner et al. revealed several pathogenic CNVs to important neuronal genes, such as NRXN, CNTN4, NLGN1, and ubiquitin-involved genes, such as UBE3A and FBXO40. SHANK3 and SHANK2 CNVs have also been identified in autistic patients. Common chromosomal CNVs reported in
ASD genome studies include 15d11.2 duplications and 16p11.2 duplications and deletions. Another important CNV is the 7q11.23 region, where deletions cause Williams syndrome and duplications are associated with autistic phenotypes. Interestingly, Williams syndrome is a genetic neurodevelopmental disorder that is characterized by a hypersociability phenotype very contrasting to ASDs. In total, these CNVs are estimated to account for ASD susceptibility in about 1-2% of all ASD cases.

Rare variants

The aforementioned genome-wide investigations, cytogenic studies, and familial ASD genetic analyses have identified a growing number of ASD-associated rare variants (loosely defined as alleles with <1%-5% frequency). Intriguingly, the rare variants identified for ASDs are often related to each other, sharing similar pathways or functions. The most common denominator is that they are usually neurologically important and affect neuronal pathways essential to functions such as neuronal development, chromatin remodeling, synaptogenesis, and calcium signaling. The low occurrence rates of these rare variants, the usually mild effects, and low increased risk of ASD by these variants by themselves, support the multiple-hit hypothesis of autism.

Rare variants in synaptic genes have been identified in several ASD patient studies, such as the genes encoding for neuroligin, neurexin, and SHANK proteins. Neuroligin and neurexin proteins are proteins critical for neuronal and synaptic development. These proteins are synaptic cell-adhesion molecules and binding partners that connect the presynaptic (neurexins) and postsynaptic (neuroligins) terminals of synapses. As such, they are important for proper synaptic transmission, signaling, and function. The five members of the neuroligin proteins are encoded by the genes NLGN1-5, however only a few have been associated with ASDs. Jamain et al.
identified neurexin-3 (NLGN3) and neurexin-4 (NLGN4) as candidate ASD genes. The authors found these genes to be mutated in individuals with autism and with Aspergers syndrome. A related study revealed that the same autism-associated Nlgn3 mutation alters synaptic physiology in mice, causing an increase in inhibitory synapses, as well as impaired social interactions. Several follow up studies identified additional NLGN4 mutations in autistic patients, and also associating with mental retardation and Tourette syndrome. The three genes NRXN1-3 encode for the neurexin proteins, which are essential for proper presynaptic bouton formation and are mediators of neurotransmitter release. Deletions and genetic variants in neurexin-1 (NRXN1), the binding partner of neuroligins, have been observed in autistic patients. Deletions in the NRXN3 region have also been found in some ASD patients, although with a number of which came from seemingly unaffected parents. This supports the multiple-hit hypothesis of ASDs, as perhaps single rare variants by themselves are not enough to induce ASDs, perhaps because of compensatory mechanisms.

The proteins of the SHANK family are encoded by the genes SHANK1-3, are scaffold proteins found in the postsynaptic density important for the proper synaptic organization. SHANK3 has been implicated in several studies as an ASD-associated, and also falls within the chromosomal region 22q13, which is the causative region in the syndromic ASD disorder 22q13 microdeletion syndrome, or Phelan-McDermid syndrome. Durand and colleagues first identified SHANK3, found in dendritic spines, as an ASD-associated gene by observing mutations in ASD patients demonstrating language and communication impairments. Corroborating these findings, additional SHANK3 mutations were identified in ASD individuals in further genetic analysis studies.

In accordance with the identification of synaptic gene rare variants, ASD-associated genes involved in neurotransmission have also been identified. Studies have
revealed genetic variants affecting serotonin, glutamate and GABAergic receptors. While still controversial, some studies have reported linkage between serotonin levels and variants in the serotonin receptor gene \textit{SLC6A4} with ASDs. Jamain and colleagues also observed linkage and ASD-association with glutamate receptor 6 (GluR6). As mentioned previously, the GABA-related gene \textit{GABRB3} has also been implicated in ASDs.

Genes involved in Ca$^{2+}$ signaling have also been identified as ASD-associated variants. Ca$^{2+}$ signaling has been shown to be critical for normal neuronal function, development, and growth. Studies have shown that mutations in genes encoding voltage-gated Ca$^{2+}$ channels can lead to ASDs. These reports identified autistic patients carrying mutations in the gene encoding the L-type voltage-gate Ca$^{2+}$ channel Ca$_{1.2}$ (\textit{CACNA1C}). Mutations in this gene are also known to cause the syndromic ASD Timothy syndrome. Subsequent papers reported autistic patients carrying mutations in the closely related \textit{CACNA1F} and \textit{CACNA1H} genes. Other calcium-related channels shown to be associated with ASDs are the genes encoding sodium channels \textit{SCN1A} and \textit{SCN2A}, as well as the Ca$^{2+}$-activated K$^+$ channel BK$_{Ca}$ gene \textit{KCNMA1}. Mutations in the \textit{SCN1A} and \textit{SCN2A} sodium channels caused dysregulated channel activation times during depolarization. Disruption of the \textit{KCNMA1} gene resulted in a more depolarized membrane potential and reduced neuronal excitability.

Fundamental to these studies of ASD-associated genes was the ability to get samples and/or information from ASD patients and controls. Thus the study of and access to ASD patient cohorts is essential for identifying and investigating these rare variants. Fortunately several outreach programs exist to facilitate community engagement and sample collection, such as the Tooth Fairy Project, a program our lab participates in. This project allows for families to send newly lost baby teeth form...
autistic individuals to researchers, from which dental pulp cells can be extracted and analyzed. Access to these valuable patient cells has provided researchers, such as our lab, to identify even more potential novel rare variants important to ASDs. As a prime example, one of our first samples revealed a chromosomal abnormality that resulted in a translocation disrupting the gene TRPC6, which is important for calcium signaling and neuronal development.

**Mouse models of autism**

The heritability and strong genetic basis of ASDs has stimulated the generation of several mouse models of autism. Identification of candidate genes, ASD-associated rare variants, and CNVs has allowed for the creation of mouse models carrying these specific genetic variants. Because of the behavioral core of ASD symptoms, researchers have developed behavioral and social assays to study these phenotypes in mice. Thus a popular approach to examining ASD candidate genes has been to assess the effect of altering these genes in mice. Interestingly, several of the ASD mouse models actually demonstrate phenotypes paralleling human ASD behaviors. Yet, mouse models of ASD are still limited, as complex human behaviors are difficult to recapitulate in mice.

Taking advantage of the known genetic cause of syndromic forms of ASD, numerous mouse models have been generated, including of RTT (Mecp2), fragile X syndrome (Fmr1), Angelman syndrome (Ube3a), and Phelan-McDermid syndrome (Shank3). RTT is caused by mutations in the X chromosome gene MECP2, causing a reduction or loss of function of the protein. The first mouse models of RTT were generated using knockouts or null alleles of the Mecp2 gene. Fascinatingly, these Mecp2 null mice recapitulated several RTT features, including a delayed onset of
symptoms, motor impairment, hypoactivity, and hind-limb clasping. Additional RTT models, such as the *Mecp2*\textsuperscript{308}, which encodes for a human-found mutation causing truncation, also demonstrates RTT phenotypes such as anxiety, altered social behaviors, and limb clasping\textsuperscript{82}. Even more, researchers have demonstrated the RTT mice have fewer glutamatergic synapses, altered dendritic spines, and impaired synaptic plasticity\textsuperscript{83-85}. FXS mouse models were generated using null alleles of *Fmr1*\textsuperscript{86}. Recapitulating symptoms of human FXS patients, the *Fmr1*-null mice demonstrated abnormal dendritic spines, cognitive defects, and altered social interactions\textsuperscript{87-89}. Angelman syndrome, which is caused by deletions in the chromosome 15q11 region, has been modeled using *Ube3a*-null mice\textsuperscript{90}. Angelman syndrome patients present with mental retardation, seizures, and motor and language impairments\textsuperscript{91}. *Ube3a*-null mice demonstrate motor dysfunction, seizures, and learning and memory deficits. Mice null for *Gabrb3*, the implicated gene in Prader-Willi syndrome, the sister disorder to Angelman, also demonstrate seizures, hyperactivity, repetitive behavior, and learning and memory defects\textsuperscript{92,93}. Interestingly, demonstrating the potential overlap of ASDs, *Mecp2*-null mice demonstrate reduced expression of *Ube3a* and *Gabrb3*\textsuperscript{38}. Finally, the Phelan-McDermid mouse model, which carry *Shank3* null alleles, demonstrate ASD-like behaviors including excessive grooming, reduced social interaction, and anxiety\textsuperscript{81}. The mice also exhibited reduced dendritic arborization and decreased spine density. A recent study revealed that a haploinsufficient *Shank3* mouse demonstrated defects in long term potentiation that could be rescued with IGF-1 treatment\textsuperscript{94}.

Several mouse models of ASD-associated variants have also been generated; some notable ones include *Nlgn4, Neurexin1, Slc6a4, Pten* and *Foxp2*\textsuperscript{76,95-100}. The *Nlgn4*-null mouse demonstrates reduced reciprocal social interactions, reduced vocalization, and low sociability\textsuperscript{95}. A null mutation in the *Neurexin1* gene results in
altered nest-building behavior and repetitive self-grooming\textsuperscript{96}. The \textit{Slc6a4}-null mouse exhibits low sociability and lack of preference for social novelty\textsuperscript{99}. When \textit{Pten} was conditionally ablated in cortical and hippocampal neurons, the mice had reduced reciprocal social interactions, low sociability, and impaired nest-building\textsuperscript{100}. \textit{Foxp2} ablation results in reduced vocalization in pups, demonstrating phenotypes present in only young mice\textsuperscript{98}.

While these mouse models provide valuable information about ASDs, they are still limited in their scope of recapitulating the disorders. Autism is a complex human behavioral and social disorder, thus obviously mice cannot completely replicate these defects. Another limitation is that signaling pathways and biological substrates can differ in humans and animals\textsuperscript{101}. In addition, genes can have different functions or importance across different species, such as the Lesch-Nihan-causing hypoxanthine phosphoribosyltransferase gene\textsuperscript{102}. A source of controversy is the relevance of supposed autism-like phenotypes in mice to actual ASD phenotypes. For example, it remains questionable as to how representative some phenotypes are, such as excessive self-grooming or hyperactivity. While these ASD mouse models provide informative neuroanatomical and molecular results, researchers remain wary of reducing complex human mental disorders to simple behavioral traits in an animal model\textsuperscript{101}.

Though animal models have provided valuable knowledge about specific ASDs, they cannot fully recapitulate human biology and it is also impractical to use them for assessing the role of several rare variants. As mentioned previously, the number of implicated ASD-associated rare variants has been increasing. Hence to examine the relevancy potential rare variants, human cellular models of ASD are more applicable. With the advent of patient-specific stem cell modeling\textsuperscript{103}, generating numerous human cell lines able to generate neuronal cells carrying ASD-associated mutations is possible.
Thus a primary focus in our laboratory was to generate these human cellular models of ASDs to examine cellular phenotypes in syndromic forms such as RTT as well as in nonsyndromic forms to assess potential rare variants such as *TRPC6*. 
References


Chapter 2

TRPC6
Introduction

By participating in the Tooth Fairy Project, our lab was able to obtain primary samples from ASD individuals. We then extracted the dental pulp cells from the baby milk teeth sent by the patient families. Subsequent chromosome karyotype analysis of these patient cell lines revealed a patient carrying a balanced translocation mapping to chromosome 3p21 and 11q22, which causes a disruption in the genes TRPC6 (located in chromosome 11) and VPRBP (located in chromosome 3). We decide to focus primarily on the TRPC6, the more neuronally relevant gene, whereas VPRBP, or VPR (HIV-1) binding protein, is known mainly only for its role in HIV infection.

TRP family

TRPC6, or Transient Receptor Potential Canonical 6, which encodes for the protein TRPC6, is a member of the TRP family of channels. Mammalian TRP channel proteins form six transmembrane cation-permeable channels. The TRP family can be subdivided into six groups, separated based on sequence homology and function: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML. The primary function of TRP channels is to allow for the transmembrane influx of cations, increasing intracellular Ca\(^{2+}\) and Na\(^{+}\) and depolarizing the cell. This influx of Ca\(^{2+}\) into the cell is a strong cellular signaling event leading to several downstream effectors in numerous cell types. TRP channels are generally activated by a variety of stimuli. One example is by receptor-induced activation such as from G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs), whose activation induces downstream effectors that can stimulate TRP channels. Another is by ligand activation by exogenous or endogenous molecules, or by direct activation by temperature or mechanical stimulus.
TRPC family

TRPC6 is a member of the TRPC subfamily of TRP channels. TRPC channels are mostly receptor-operated channels, stimulated by GPCRs and RTKs \(^4\). TRPC1 was the first mammalian TRP reported, and is known to form heteromeric channels with TRPC4 and TRPC5 \(^4,6\). TRPC4 and TRPC5 can also form homomultimers, which along with the TRPC1 heteromultimer, all have distinct electrophysiological properties \(^7,8\). While both TRPC1 and TRPC5 have been shown to function in the CNS, only TRPC5 has been linked to modulating hippocampal growth cones and neurite extension \(^9\). TRPC4 has been shown to be involved in vasoregulation \(^10\). TRPC3, TRPC6, and TRPC7 are closely related, share ~75% identity, and are the rest of TRPC family. Diacylglycerol has been shown to be the endogenous activator of these channels \(^4\). These channels are known to interact and form heteromeric tetramer channels. While these channels are closely related, their electrophysiological properties differ. TRPC3 and TRPC7 channels exhibit elevated basal channel activity, whereas TRPC6 is more tightly receptor-regulated with minimal activity \(^11\). TRPC3 has been shown to be important in the CNS for synaptic transmission and motor coordination, as well as in vasoregulation \(^12\). TRPC7 is expressed in endothelial cells, muscle cells, brain cells, and keratinocytes, and has been shown to be important for Ca\(^{2+}\) signaling regulation \(^13\).

TRPC6 function

TRPC6 has been reported to be an important protein channel in regulation Ca\(^{2+}\) influx and downstream signaling in several cell types and affecting numerous cellular functions. The human TRPC6 gene was first identified in 1998 by D’Esposito and colleagues, revealing it is located on chromosome 11 and contained 13 exons, with the full-length protein 931 amino acids in length \(^14\). The mouse homolog contains the same
number of exons but is located on chromosome 9. The protein structure parallels the
other TRP channels, containing six transmembrane helices and a pore loop between the
fifth and sixth transmembrane region. TRPC6 is a Ca\textsuperscript{2+}-permeable non-selective
cation channel displaying double rectification with a conductance of 28-37 pS and an ion
permeability ratio P\textsubscript{Ca}/P\textsubscript{Na} is about 6. TRPC3, which is closely related to TRPC6, is
considerably less Ca\textsuperscript{2+} selectivity. As mentioned previously, TRPC6 has very little basal
activity and is tightly receptor-operated. TRPC3/6/7 subfamily of the TRPC channels can
be activated by the secondary messenger diacylglycerol (DAG) produced by receptor-
activated phospholipase C. Accordingly, the DAG analog 1-oleyl-1-acetyl-sn-glycerol
(OAG) and the DAG lipase inhibitor RHC80267 increase TRPC6 activity. The most
specific TRPC6 blocker is the compound 8009-5364, which is 2.5x more selective for
TRPC6 than TRPC3. Another pharmacological activator of TRPC6 is flufenamate,
which inhibits TRPC3 and TRPC7 but also stimulates some non-TRPC receptors such
as adrenoreceptors. An additional well-known TRPC6 activator is hyperforin, a
component of the antidepressant St. John’s wort. Hyperforin has been demonstrated to
specifically activate TRPC6 and not TRPC3.

TRPC6 has been demonstrated to have multiple functions and cellular effects. TRPC6
expression is found in almost all cell types, but has been shown to be highly
expressed in the heart, smooth muscle, kidney, and brain. In the heart, TRPC6 has
implicated in cardiac hypertrophy, as Ca\textsuperscript{2+} regulation is very important and tightly
regulated in cardiac cells. In smooth muscle cells, TRPC6 has been revealed to be
involved in vasopressin signaling and critical to pressure-induced depolarization and
constriction of arteries. In addition, TRPC6 also functions in endothelial cells, as it is
involved in vascular endothelial growth factor signaling which induces vascular
permeability and Ca\textsuperscript{2+} entry. TRPC6 is known to complex with TRPC3 in the kidney,
and is found in the glomerulus and collecting duct. Importantly, several reports have implicated TRPC6 mutations in focal segmental glomerular sclerosis, with TRPC6 affecting Ca2+ regulation in podocytes.

**TRPC6 and neurons**

Numerous reports have revealed a critical role for TRPC6 in neuronal function and development. Overall TRPC6 expression in the brain is lower than other TRPC channels, but it is highly expressed in the dentate gyrus of the hippocampus. TRPC6 activation in neurons is known to allow the entrance and subsequent increase of intracellular Ca2+ and Na+, causing an inhibition of the reuptake of neurotransmitters such as serotonin, dopamine, norepinephrine, glutamate and GABA. TRPC3 has a higher general expression in the brain, and has been shown to be essential for metabotropic glutamate receptor signaling in mouse Purkinje cells. Both TRPC6 and TRPC3 are important for brain-derived neurotrophic factor (BDNF) downstream signaling, as down-regulation of both resulted in blocking BDNF protective effects in the cerebellum. In addition Lee et al. demonstrated that TRPC6 and TRPC3 down-regulation blocked BDNF-induced intracellular Ca2+ influx and BDNF-mediated growth cone guidance. TRPC6 expression has been detected in the hippocampus even during embryonic development, and has been shown to promote dendritic outgrowth. Tai and colleagues also reported that TRPC6 activates downstream signaling involving calmodulin kinase IV (CaMKIV) and cAMP response element-binding protein (CREB) resulting in an increase in dendritic spine density in hippocampal neurons. Supporting these findings, Zhou et al. showed that TRPC6 can be detected at excitatory synapses and actually promotes their formation through a CaMKIV-CREB-dependent pathway. Even more, Heiser et al. recently demonstrated that TRPC6-mediated neuronal
outgrowth involves the activation of important critical neuronal pathways such as AS/MEK/ERK, PI3K, and CaMKIV signaling\textsuperscript{30}. In this study, the authors revealed that these TRPC6-induced neuronal effects, including neurite outgrowth and spine density. Interestingly, in a mouse model of RTT, \textit{Mecp2} mutant mice demonstrated impaired activity-dependent BDNF release and TRPC signaling. Supporting this, a recent report showed that MeCP2 levels can affect TRPC6 expression levels\textsuperscript{31}.

Based on the evidence from the literature, TRPC6 is clearly a protein important for neuronal function and development (\textbf{Figure 2.1}). In addition, the implicated functions and pathways TRPC6 is involved in have been shown to be affected in ASDs, such as \textit{Ca}\textsuperscript{2+} signaling and synaptogenesis. Because of this, we decided to explore our ASD sample carrying the disrupted \textit{TRPC6} gene to determine if \textit{TRPC6} could be a novel ASD variant contributing to the phenotypes in the ASD individual.
Figure 2.1. TRPC6 signaling pathways.

TRPC6 has been implicated in the activation of several important neuronal signaling pathways. Influx of Ca$^{2+}$ via TRPC6 pathways has been demonstrated to induce several signal transduction pathways, including the Akt (protein kinase B), CaMKIV (Calcium/calmodulin-dependent protein kinase IV), and ERK (extracellular signal-regulated kinase) pathways. Activation of these pathways eventually leads to the activation of the transcription factor CREB (cAMP-response element binding protein phosphorylation), which has been demonstrated to promote pro-neuronal effects, including neuronal survival, neuronal plasticity, neurite outgrowth, and synaptogenesis. Several TRPC6 agonists have been demonstrated to stimulateTRPC6 and induce Ca2+ influx, including hyperforin and DAG (diacyl glycerol), the endogenous TRPC6 activator. Both the BDNF receptor TrkB (Tropomyosin receptor kinase B) and the IGF-1 receptor can promote downstream signaling that can also activate TRPC6, as well as both the ERK and Akt pathways. Recently, MeCP2 has been shown to bind to the promoter region of TRPC6 and to be important for TRPC6 expression.
References


Chapter 3

Induced pluripotent stem cell technology and disease modeling
Abstract

Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental disorders sharing a core set of symptoms, including impaired social interaction, language deficits, and repetitive behaviors. While ASDs are highly heritable and demonstrate a clear genetic component, the cellular and molecular mechanisms driving ASD etiology remain undefined. The unavailability of live patient-specific neurons has contributed to the difficulty in studying ASD pathophysiology. The recent advent of induced pluripotent stem cells (iPSCs) has provided the ability to generate patient-specific human neurons from somatic cells. The iPSC field has quickly grown, as researchers have demonstrated the utility of this technology to model several diseases, especially neurological disorders. Here, we review current literature using iPSCs to model ASDs, and discuss the notable findings, and the promise and limitations of this technology.
Introduction

Autism spectrum disorders (ASDs) are a set of complex neurodevelopmental disorders sharing a core set of symptoms, including social deficiencies, cognitive impairments, and stereotyped repetitive behaviors. While the exact etiology of ASDs remains unknown, ASDs do demonstrate a strong genetic component. ASDs are categorized as syndromic (caused by a known genetic disorder) or nonsyndromic (idiopathic, unknown genetic cause). Syndromic forms of ASD include Rett syndrome, Timothy syndrome, Fragile X syndrome, Angelman syndrome, and Phelan-McDermid syndrome. These syndromic forms of ASD are caused by defined genetic or chromosomal abnormalities, and are estimated to account for 10-20% of ASD cases. The genetic abnormalities associated with nonsyndromic ASDs, which make up the majority of ASD cases, are being intensively researched, with evidence for both hereditary and de novo mutations. Several different chromosomal loci and genetic variants have been implicated in ASD susceptibility, indicating that while symptoms are shared, these disorders are genetically heterogeneous. Increasing numbers of rare variants are being implicated in ASD, and often presenting modest to low degrees of risk. These studies support the multiple-hit hypothesis of autism, which postulates that some nonsyndromic ASDs are caused by a combination of several genetic abnormalities affecting specific pathways above a threshold level. However, while several ASD-related genes have been implicated, the functional study of these genes and their individual relevance to human ASD etiology remains lacking. Thus the identification and analysis of the functional relevance and cellular contributions of these ASD variants is critical for the elucidation of autism pathophysiology.

The lack of relevant human disease models has hindered the understanding of ASD etiology. Until recently, human neurological disorder researchers have lacked
sufficient amounts of samples to properly study the target cell type, the neuron. Access to the affected cell type is essential for the analysis of cellular and molecular mechanisms driving the disorders. Human postmortem samples have long been used to study phenotypes of neurological disorders, but often present several limitations. These samples often represent only the end-stage of the disease, where secondary symptoms and phenotypes can present problems. Even more, environmental factors such as drug treatments and can also play confounding roles. In addition, the obvious lack of living cells in preserved post-mortem samples essentially prohibits the use of functional assays to study cellular physiology and neural networks.

Animal models have also been long used to model neurological diseases and disorders to study disease etiology. Transgenic and knockout technology using mouse models can provide valuable analysis of genetic disorders in vivo and in vitro. However, they are restricted mostly to monogenetic diseases, which is limiting for genetically complex, heterogeneous disorders such as autism. Disorders characterized by several rare variants, translocations, or large deletions are difficult to model in mice, especially when considering species differences in genetics. In addition, mouse models often do not fully recapitulate complex human diseases, especially social and behavioral disorders such as autism.

Thus, to understand cellular and molecular diseases driving neurological disorders such as ASDs, a human neuronal cellular model able to both recapitulate the causal genetics and produce the target cell type is necessary. The advancement of stem cell technology has allowed for the generation of these human cellular models. Pluripotent human embryonic stem cells (hESCs) arose as promising sources of human cells, able to study early developmental time points as well as generate multiple cell types. However, ethical issues and the scarcity of available disease-specific hESC lines
and have hindered disease modeling progress. The advent of cellular genetic reprogramming has revolutionized human cellular disease modeling. Recently developed, somatic cells such as fibroblasts and dental pulp cells can be reprogrammed into a pluripotent state by the overexpression of specific transcription factors. These induced pluripotent stem cells (iPSCs), can then be differentiated into virtually any target cell type. These iPSCs are isogenic to the original donor cells, and thus recapitulate the genetics of the patient from which they were obtained. Previously not possible, unlimited numbers of human cells such as neurons, even carrying disease-specific mutations, can be generated. Researchers are then able to examine cellular phenotypes, perform functional assays, as well as test drugs for any potential efficacy in ameliorating defects (Figure 3.1).

In this review, we discuss recent iPSC disease models of autism, examine the noteworthy findings, and explore the future implications and challenges toward using these human cellular models for understanding autism etiology.

**iPSC Disease Modeling**

Since the inception of iPSC technology, several diseases have been successfully modeled. Virtually any disorder known to have some genetic basis can be modeled by iPSCs, but the successful identification of cellular phenotypes can be quite variable. Even more, these human cellular models are particularly useful when no good animal model exists. Several human diseases affecting different human tissue types have been modeled, including hematopoietic disorders such as Fanconi anemia. Cardiovascular disorders such as Long QT syndrome and LEOPARD syndrome have also been successfully modeled. Interestingly, while mouse models do not reproduce the human phenotypes, Itzhaki and colleagues found Long QT syndrome iPSC-derived
cardiomyocytes to reproduce the prolonged action potential observed in patients. Importantly, the authors were able to perform a simple screen and demonstrate that β-blockers can improve the affected cardiomyocyte QT interval. These studies demonstrate the capability of iPSC models to effectively recapitulate human phenotypes and allow for the screening of drugs to ameliorate these defects.

While iPSCs can potentially generate any cell type, neurological disorders have been the most frequent targets of iPSC disease modeling. Neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Parkinson’s, and Alzheimer’s disease were among the first to be targeted using iPSCs. While iPSC models of neurodegenerative disorders such as ALS and Parkinson’s disease can generate neurons carrying disease-specific genetics, the lack of understanding of the mechanisms driving neurodegeneration undeniably makes iPSC modeling more challenging. Unlike disorders such as Long QT syndrome that have known hallmark defects, robust phenotypes for neurodegenerative disorders are scarce. Nevertheless, Dimos et al. were able to generate an iPSCs from ALS patients and generate both motor neurons and astrocytes, two cell types specifically affected in ALS. Yet they were unable to observe any novel or robust cellular phenotypes. A subsequent study by Mitne-Neto et al. modeled ALS8 using patients carrying a mutation to the VAPB protein. The authors revealed a potentially exploitable biochemical phenotype, that motor neurons generated from the VAPB-iPSC carried reduced protein levels of VAPB compared to controls. Soldner and colleagues successfully modeled Parkinson’s disease, a neurodegenerative disorder characterized by loss of dopaminergic neurons. The authors were able to generate dopaminergic neurons but failed to see any cellular phenotypes. However, subsequent iPSC models of Parkinson’s were able to identify elevated oxidative stress in the iPSC-derived dopaminergic neurons. Alzheimer’s disease results in progressive
neuronal loss and while no clear disease etiology has been elucidated, hallmarks of the disease are the presence of amyloid-β plaques and neurofibrillary tangles. The altered processing of amyloid-β precursor protein into αβ peptides is thought to play a role in AD and the generation of plaques. Israel et al. demonstrated that neurons generated from Alzheimer's patient iPSCs produced elevated amounts of the pathogenic αβ peptide. These recent reports suggest iPSCs can provide an important model of neurodegenerative disorders to not only generate the affected human cell types but also for the identification of mechanisms contributing to disease etiology.

Neurodevelopmental disorders have become a popular target of iPSC modeling, with recently published models of schizophrenia (SZ), Cockayne Syndrome, and syndromic ASDs such as Fragile X syndrome (FXS), Down syndrome, and Rett syndrome (RTT). Neurodevelopmental disorders are characterized by defects in central nervous system development and growth, and often have a genetic cause. iPSC technology is particularly well suited for modeling genetic disorders because of its ability to capture disease-specific genotypes, which is especially useful for complex genetic disorders. As such, neurodevelopmental disorders are ideal targets because of their strong genetic component, with both monogenetic and complex multigenic forms. In addition, unlike neurodegenerative conditions, neurodevelopmental disorders are often characterized with cellular defects apparent at early stages in life.

For example, SZ is a disabling neurological disorder characterized by paranoia, hallucinations, and cognitive and emotional abnormalities. SZ encompasses a spectrum of phenotypes, including neuroanatomical changes and altered neurotransmission across several neuronal subtypes. While the spectrum of disease is broad and environmental conditions are important, evidence suggests SZ has a genetic basis. The neurodevelopmental hypothesis of SZ suggests that the disease
is caused by the altered interaction of multiple genes affecting important developmental pathways inducing a cascade of neuropathological changes and events during development \(^{47-49}\). Recently Brennand et al. generated iPSCs from four SZ patients carrying complex genetic mutations \(^{35}\). Importantly, the authors were able to demonstrate that the neurons were less complex and contained fewer neurites, recapitulating post-mortem studies \(^{50, 51}\). Even more, they showed further phenotypes including reduced neuronal connectivity, synaptic protein levels, and altered gene expression. Moreover, neuronal connectivity and expression alterations were rescued after treatment with an antipsychotic drug, exemplifying the potential of iPSC models as drug discovery platforms.

**Modeling syndromic autism**

Syndromic forms of autism are the disorders falling under the umbrella of ASDs in which there is a known, usually monogenetic cause. Unlike nonsyndromic, or idiopathic, forms of autism, where the genetic cause is unknown, syndromic forms are associated with specific genes which are known to cause an ASD when mutated. Because the genetic causes are already known, syndromic forms of ASD, including Fragile X syndrome, Timothy syndrome, CDKL5 disorder, and Rett syndrome, were quickly targeted for iPSC modeling (Table 1) \(^{37, 38, 52, 53}\).

**Fragile X syndrome**

Fragile X syndrome (FXS) is characterized by a CGG trinucleotide repeat expansion in the 5' UTR of the fragile X mental retardation 1 (FMR1) gene leading to the hypermethylation and gene silencing \(^{54}\). FXS, which results when the expansion is over 200 repeats, is the most common syndromic form of ASD in the population and patients
display with physical, intellectual, and behavioral phenotypes of varying severity. FMR1 is a gene associated with synaptogenesis, and its translated protein FMRP can be detected at dendritic spines and synapses. An initial human ESC study of FXS demonstrated that FMR1 was unmethylated at the undifferentiated pluripotent stage, allowing for its expression. However, the first reported iPSC FXS model showed that FMR1 remained inactive and retained the epigenetic silencing, highlighting differences between ESCs and iPSCs. A subsequent study showed that multiple reprogrammed patient FXS lines had variable levels of FMR1 silencing and expression. Highlighting its importance to neurodevelopment, lines that demonstrated reduced FRM1 expression resulted in aberrant neuronal differentiation. Another study generated iPSC from FXS pre-mutation individuals (carrying 55-200 CGG repeats), who do not display with classical FXS but suffer from neurodegenerative Fragile X-associated tremor/ataxia syndrome. Interestingly, neurons derived from these iPSCs revealed reduced neurite length, fewer synaptic puncta, reduced synaptic protein levels, and increased calcium transients. A recent report showed that forebrain neurons derived from iPSCs from FXS patients also showed reduced neurite outgrowth. While facing initial hurdles, these reports represent the potential of FXS iPSC models to provide cellular tools that recapitulate disease phenotypes.

**Timothy Syndrome**

Timothy Syndrome (TS) is a rare autosomal dominant neurodevelopmental disorder caused by a mutation in the CACNA1C gene, which encodes for the voltage-dependent calcium channel CaV1.2. TS has been associated with an array of phenotypic manifestations, including heart malformations, arrhythmia, developmental delay, and autism. The TS-causing mutation induces aberrant CaV1.2 function, leading
to loss of the voltage-dependent channel activation and subsequent excess of intracellular \( \text{Ca}^{2+} \). The high prevalence of TS patients with ASD and intellectual disability underscores the importance of \( \text{Ca}_v1.2 \) to neurodevelopment \(^{63, 64}\). Recently, Pasca et al. generated an iPSC model of TS and identified several abnormalities in the derived cells, including neurons \(^{52}\). Pasca and colleagues reported defects in Ca2+ signaling as well as defects in differentiation, with TS cells producing fewer neurons expressing cortical and callosal projection markers, and more neurons expressing tyrosine hydroxylase \(^{52}\). While the connection between TS and ASD symptoms is still unclear, the authors claim the observed reduction in cortical projecting neurons is consistent with the connectivity hypothesis of ASD \(^{65}\). In addition, the increase in tyrosine hydroxylase expressing neurons was ameliorated after treatment with the L-type channel blocker roscovitine, highlighting the potential for a TS iPSC drug-screening assay. A subsequent study of TS iPSCs revealed aberrant activity-dependent dendritic retraction in both the TS derived neurons and rodent neurons \(^{66}\). The authors found that this was a result of RhoA activation and was independent of Ca2+ influx through \( \text{Ca}_v1.2 \). Identifying a novel mechanism, the dendritic retraction phenotype could be rescued by both overexpression of Gem, an inhibitor of RhoA, and treatment with C3 transferase, a Rho inhibitor.

**Angelman syndrome and Prader-Willi syndrome**

Angelman syndrome (AS) and Prader-Willi syndrome (PWS) are neurodevelopmental disorders associated with genomic imprinting and ASD. AD and PWS are considered sister disorders, as they are both caused chromosomal deletion, or an imprinting defect, of the chromosomal region 15q11-13 \(^{67, 68}\). AS is a result of this deletion occurring on the maternal allele, causing the reduced expression of the ubiquitin-protein ligase E3A gene (UBE3A) \(^{67}\). PWS is caused by the deletion occurring
on the paternal allele, resulting in the loss of or reduced expression of seven paternally expressed genes in the affected 15q region\textsuperscript{69}. While AS and PWS do not have identical developmental defects, they do share neurological symptoms such as cognitive, social, and speech disabilities\textsuperscript{70,71}. Chamberlain and colleagues generated the first iPSC model of AS and PWS from patient cells\textsuperscript{72}. Their AS and PWS iPSCs showed no erasure of the DNA imprinting, and found that $UBE3A$ imprinting occurred during neuronal differentiation in the AS cells.\textsuperscript{72} However, the authors found no neuronal phenotypic differences between AS neurons and control neurons.

**Phelan-McDermid syndrome**

Phelan-McDermid syndrome (PMDS), also known as 22q13.3 deletion syndrome is a neurodevelopmental disorder characterized by range of clinical symptoms, including absent or delayed speech, intellectual disability, mental retardation, and autism\textsuperscript{73,74}. PMDS is caused by deletion or loss of genes in the 22q13 region, typically causing loss of the gene $SHANK3$. $SHANK3$ is a scaffolding protein found in excitatory synapses, involved in the organization of the postsynaptic density\textsuperscript{75}. $SHANK3$ mutations have been associated with ASD, and mouse models carrying Shank3 mutations demonstrate synaptic defects and ASD-like behaviors\textsuperscript{76,77}. Shcheglovitov et al. recently generated a PMDS iPSC model using fibroblasts from two PMDS patients carrying large 22q13 deletions that include $SHANK3$\textsuperscript{78}. Neurons generated from the PMDS iPSCs demonstrated altered excitatory electrophysiology and fewer synapses. The authors revealed these neuronal defects could be rescued using expression of $SHANK3$ by lentivirus and by pharmacological treatment using IGF-1\textsuperscript{78}.

**Rett syndrome**
Rett syndrome (RTT) is a monogenic progressive neurological disorder caused by mutations in the X-linked gene methyl CpG-binding protein 2 (MeCP2) [79]. RTT patients are predominantly female as affected males are usually pre-term lethal and those that survive are severely affected [80, 81]. RTT patients have apparently normal development until 6-18 months old, which is followed by progressive neurological abnormalities [82]. This period of regression is often characterized by the deceleration of head growth and loss of acquired motor and language skills [82]. The spectrum of RTT neuropathology includes autistic behavior, stereotyped hand wringing, seizures, microcephaly, hypotonia, ataxia, and loss of speech [83, 84]. Human postmortem analysis revealed neuronal cellular phenotypes such as altered neuronal morphology, reduced soma size, fewer dendritic spines, and reduced dendritic arborization [85, 86]. Revealing the potential role of multiple cell types, several studies have demonstrated the effect of mutant astrocytes in RTT etiology [87-90]. In addition, recent reports have shown that microglia and oligodendrocytes are also important players in RTT pathophysiology [91-93].

While phenotypes have been robust and abundant, how alterations to the MECP2 gene induce this array of abnormalities remains elusive. As a result, an abundant amount of research has been performed to study the function of MeCP2. The causal role in RTT and the ability to rescue defects in RTT mouse models by reintroduction of MeCP2 has demonstrated the importance of MeCP2 to neuronal development and function [94, 95]. MeCP2 has been shown to both activate and repress transcription [96]. Skene and colleagues have also shown that MeCP2 is highly expressed in neurons and acts as a global transcriptional regulator, with a vast number of potential targets [97].

RTT has become a popular target as several RTT iPSC studies have already been reported. Our work was the first to describe an iPSC model of RTT, where we
discovered that neurons derived from RTT iPSC recapitulated several aspects of known RTT neuropathology (Figure 3.2)\(^{38}\). The human neurons were derived from four different RTT patients carrying different MeCP2 mutations. The RTT neurons demonstrated phenotypes paralleling the human postmortem and rodent model findings, such as smaller soma size, reduced dendritic spine density, altered \(\text{Ca}^{2+}\) influx and electrophysiology, and fewer excitatory synapses. To verify the causal role of MeCP2, gain- and loss-of-function assays using MeCP2 re-expression and shRNA targeting MeCP2 validated several of the neuronal abnormalities. Even more, treatment of the neurons with the candidate drug, insulin-like growth factor 1 (IGF-1), was able to rescue the synaptic defects. IGF-1 is a known neurotrophic factor currently in clinical trials for RTT, and has been shown to be able to stimulate neuronal growth and synaptogenesis\(^{98, 99}\). Another report from our laboratory demonstrated that neural progenitor cells derived from RTT iPSCs had increased LINE-1 retrotransposition, showing that MeCP2 regulates these events\(^{100}\). A subsequent study using the RTT iPSC model also observed a reduced soma and nuclear size in affected neurons\(^{101}\). Kim and colleagues observed a neuronal maturation defect in iPSCs derived from RTT\(^{102}\). In a recent report, Williams et al. generated astrocytes from RTT iPSCs and demonstrated that these mutant astrocytes and their conditioned media are enough to induce neuronal abnormalities\(^{103}\). Using IGF-1 and GPE (an IGF-1 peptide), the authors were able to partially rescue the morphological defects.

Exemplifying a prototypical iPSC model, RTT is a monogenetic disorder with known, robust phenotypes validated in numerous models. For RTT, iPSC modeling allowed for the ability to produce different affected subtypes that recapitulated known phenotypes, as well as for the generation of a drug-screening platform.
Modeling nonsyndromic autism

A prevailing theme in the field is the use of syndromic forms of autism to shed light onto nonsyndromic autism. Because the genes driving syndromic autism are known, the idea is to determine how those known mutations induce neuronal phenotypes common among different ASD, and subsequently examine if those same genes or pathways are affected in nonsyndromic cases of autism. Indeed, previous studies have shown shared synaptic phenotypes in syndromic and nonsyndromic mouse models of autism\textsuperscript{104}. Because the large majority of ASD cases are sporadic, models of nonsyndromic autism are essential to the study of ASD etiology. ASDs exhibit a common core set of symptoms and demonstrate a strong genetic component, yet the exact etiology of ASD remains unknown\textsuperscript{3}. ASD susceptibility has been implicated in several different chromosomal loci and genes, indicating genetic heterogeneity\textsuperscript{105-107}. However, a large number of these genes are related and share molecular pathways, including those involved in neurotransmitter pathways\textsuperscript{108-111} or neuron adhesion and junction molecules\textsuperscript{112}. Mutations in Ca\textsuperscript{2+} channels and genes involved in Ca\textsuperscript{2+}-regulated signaling have also been associated with ASD\textsuperscript{63,113,114}. With increasing numbers of rare variants being implicated in ASD, but often presenting modest to low degrees of risk\textsuperscript{9}, it is crucial to identify and study the relevance of these rare variants to nonsyndromic ASD etiology.

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Figure 3.1. iPSC disease modeling

The generation of induced pluripotent stem cells (iPSCs) has allowed researchers to capture the specific DNA of a patient within a pluripotent cell line. Scientists are then able to differentiate these iPSCs into their cell type of choice, usually the affected cell types (e.g., neural cells for neurological disorders). With the affected cell types carrying patient-specific DNA, researchers have a “disease in a dish” model. One can then analyze these affected cells for phenotypes or defects. After identifying phenotypes, researchers can then screen drugs or compounds to identify therapies that might ameliorate any defects. Finally, these drugs can then be used as potential treatments for the original patient and other patients of the same disease or disorder.
Figure 3.2. RTT human iPSC-derived neuronal phenotypes

A human iPSC model of RTT generated by the reprogramming of patient cells. Neurons differentiated from these iPSCs exhibited several cellular phenotypes, including (a) reduced soma size, (b) less dendritic branching, and (c) fewer glutamatergic synapses and (d) dendritic spines. These morphological alterations contributed to the (e) electrophysiological defects in these RTT neurons.
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<th>ASD type</th>
<th>Incidence (chromosome)</th>
<th>Key gene (chromosome)</th>
<th>Genetic mutation</th>
<th>Neuronal differentiation</th>
<th>Relevant neuronal phenotypes</th>
<th>Rescue?</th>
<th>Drug treatment?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTT</td>
<td>1:10,000 (female)</td>
<td>MeCP2 (X)</td>
<td>Nonsense (Q244X)</td>
<td>Yes; TuJ1+, MAP2+, GABA+, Synapsin+, VGLUT1+</td>
<td>Reduced soma size, dendritic spine density and synapses, Altered Ca²⁺ signaling, electrophysiological defect</td>
<td>Yes</td>
<td>IGF-1, gentamicin</td>
<td>Marchetto</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Missense (T158M, R306C)</td>
<td>Yes; MAP2+</td>
<td></td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Null (Axon3-4)</td>
<td>Yes; TuJ1+, SCN1A/B+</td>
<td>Lower expression of mature neuron marker</td>
<td>No</td>
<td></td>
<td>Cheung</td>
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<tr>
<td></td>
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<td></td>
<td>Missense (T158M, R306C)</td>
<td>Yes; TuJ1+</td>
<td></td>
<td>No</td>
<td></td>
<td>Kim</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Nonsense (Q244X)</td>
<td>Yes; TuJ1+</td>
<td>Reduced nuclear size</td>
<td>No</td>
<td></td>
<td>Ananiev,</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Missense (T158M, R306C)</td>
<td>Astrocytes, GFAP+, S100β+</td>
<td>Astrocyte conditioned media adversely affects neuronal morphology</td>
<td>Yes</td>
<td>IGF-1, GPE</td>
<td>Williams</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Nonsense (V247X, R294X)</td>
<td>Astrocytes, GFAP+, S100β+</td>
<td>Astrocyte conditioned media adversely affects neuronal morphology</td>
<td>Yes</td>
<td>IGF-1, GPE</td>
<td>Williams</td>
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<td>FXS</td>
<td>1:4,000-6,000</td>
<td>FMR1 (X)</td>
<td>&gt;200 CGG repeats in 5'UTR</td>
<td>No</td>
<td></td>
<td>No</td>
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<td></td>
<td></td>
<td></td>
<td>&gt;700 CGG repeats in 5'UTR</td>
<td>Yes; TuJ1+</td>
<td>Fewer and shorter neurites</td>
<td>No</td>
<td></td>
<td>Sheridan</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;435 CGG repeats in 5'UTR</td>
<td>Yes; TuJ1+, FOXG1+</td>
<td>Neurite outgrowth defects</td>
<td>No</td>
<td></td>
<td>Doers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>84 CGG repeats in 5'UTR</td>
<td>Yes; TuJ1+, FOXG1+</td>
<td>Reduced synaptic protein expression, shorter neurites, altered Ca²⁺ transients and glutamate response</td>
<td>No</td>
<td></td>
<td>Liu</td>
</tr>
<tr>
<td>Down syndrome</td>
<td>1:700</td>
<td>Chromosome 21</td>
<td>Trisomy 21</td>
<td>No; TuJ1+, VGLUT1+, CuX1+, Bm2+, Satb2+</td>
<td>Increased Aβ42 production, phosphorylated tau accumulation, Neurogenesis defect, reduced MeCP2 expression</td>
<td>No</td>
<td></td>
<td>Park, Li</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Trisomy 21</td>
<td>No; TuJ1+</td>
<td></td>
<td>No</td>
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<td></td>
<td></td>
<td></td>
<td>Trisomy 21</td>
<td>No; TuJ1+</td>
<td></td>
<td>No</td>
<td></td>
<td>Lu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Trisomy 21</td>
<td>No; TuJ1+</td>
<td>Reduced synaptic activity, fewer synaptic puncta</td>
<td>No</td>
<td></td>
<td>Weick</td>
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</table>
Table 3.1. Human iPSC models of autism spectrum disorders, continued.

<table>
<thead>
<tr>
<th>ASD type</th>
<th>Incidence</th>
<th>Key gene (chromosome)</th>
<th>Genetic mutation</th>
<th>Neuronal differentiation</th>
<th>Relevant neuronal phenotypes</th>
<th>Rescue?</th>
<th>Drug treatment?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down syndrome</td>
<td>1:700</td>
<td>Chromosome 21</td>
<td>Trisomy 21</td>
<td>Yes; TuJ1+, MAP2+, Synapsin+</td>
<td>Neural progenitor apoptosis, neuronal maturation defect</td>
<td>Yes</td>
<td>EGCG</td>
<td>Hibaoui</td>
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<td></td>
<td></td>
<td>Trisomy 21</td>
<td>Yes; TuJ1+, Astrocytes, GFAP+, S100B+</td>
<td>Astrocyte conditioned media toxic to neurons</td>
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<td>Minocycline</td>
<td>Chen</td>
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<td>PMDS</td>
<td>Over 600</td>
<td>SHANK3 (22)</td>
<td>Deletion 22q13</td>
<td>Yes; Synapsin+, HOMER1+ PSD-95+, Ctip2+, Satb2+, GAD67+</td>
<td>Impaired excitatory neurotransmission, reduced expression of glutamate receptors, fewer synapses</td>
<td>Yes</td>
<td>IGF-1</td>
<td>Shcheglovitov</td>
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<tr>
<td>TS</td>
<td>20 cases</td>
<td>CACNA1C (12)</td>
<td>Missense (G406R)</td>
<td>Yes; MAP2+, VGLUT1/2+, TH+, GAD65/67+, Ctip2+, FOXP1+, SATB2+,</td>
<td>Defect in Ca^{2+} signaling and electrophysiology, decreased SATB2 expression, increased TH and catecholamine expression Ca^{2+}-dependent dendritic retraction</td>
<td>Yes</td>
<td>Roscovitine</td>
<td>Pasca</td>
</tr>
<tr>
<td></td>
<td>reported worldwide</td>
<td></td>
<td>Missense (G406R)</td>
<td>Yes; TuJ1+, MAP2+, VGLUT1/2+, GAD65/67+</td>
<td></td>
<td></td>
<td>C3 transferase</td>
<td>Krey</td>
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<td>CDKL5-related disorder</td>
<td>80 cases</td>
<td>CDKL5 (X)</td>
<td>Nonsense (Q347X)</td>
<td>Yes; TuJ1+, MAP2+, VGLUT1/2+, GAD65/67+</td>
<td></td>
<td>No</td>
<td>No</td>
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<td>reported worldwide</td>
<td></td>
<td>Missense (T288I)</td>
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<td>AS</td>
<td>1:12,000</td>
<td>UBE3A (15)</td>
<td>Maternal Δ15q11-q13 (including UBE3A)</td>
<td>Yes; TuJ1+, MAP2+, Synapsin+ PanNav+</td>
<td></td>
<td>No</td>
<td>No</td>
<td>Chamberlain</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>PWS</td>
<td>1:15,000</td>
<td>Unknown paternal 15q11-q13 (15)</td>
<td>Paternal Δ15q11-q13 (15;4)(q11.2;q27)</td>
<td>No</td>
<td></td>
<td>No</td>
<td>No</td>
<td>Chamberlain</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes; TuJ1+, MAP2+</td>
<td></td>
<td>No</td>
<td>No</td>
<td>Yang</td>
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<td>Non-syndromic autism</td>
<td>1:29,000</td>
<td>TRPC6 (11)</td>
<td>Translocation t(3;11)(p21;q22)</td>
<td>Yes; TuJ1+, MAP2+, VGLUT1+, Synapsin+, Ctip2+, Tbr1+</td>
<td>Altered neuronal morphology, fewer dendritic spines and synapses</td>
<td>Yes</td>
<td>IGF-1, hyperforin</td>
<td>Gresi-Oliveira</td>
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</tbody>
</table>
References


have adverse effects on wild-type neurons. *Hum Mol Genet* **23**, 2968-2980 (2014).


Chapter 4

Modeling non-syndromic autism and the impact of *TRPC6* disruption in human neurons
Abstract

An increasing number of genetic variants have been implicated in autism spectrum disorders (ASD), and the functional study of such variants will be critical for the elucidation of autism pathophysiology. Here, we report a *de novo* balanced translocation disruption of *TRPC6*, a cation channel, in a non-syndromic autistic individual. Using multiple models, such as dental pulp cells, iPSC-derived neuronal cells and mouse models, we demonstrate that *TRPC6* reduction or haploinsufficiency leads to altered neuronal development, morphology, and function. The observed neuronal phenotypes could then be rescued by *TRPC6* complementation and by treatment with IGF1 or hyperforin, a TRPC6-specific agonist, suggesting that ASD individuals with alterations in this pathway might benefit from these drugs. We also demonstrate that MeCP2 levels affect *TRPC6* expression. Mutations in MeCP2 cause Rett syndrome, revealing common pathways among ASDs. Genetic sequencing of *TRPC6* in 1041 ASD individuals and 2872 controls revealed significantly more nonsynonymous mutations in the ASD population, and identified loss-of-function mutations with incomplete penetrance in two patients. Taken together, these findings suggest that *TRPC6* is a novel predisposing gene for ASD that may act in a multiple-hit model. This is the first study to use iPSC-derived human neurons to model non-syndromic ASD and illustrate the potential of modeling genetically complex sporadic diseases using such cells.
Introduction

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders that are characterized by deficits in reciprocal social interaction and communication as well as the presence of repetitive behaviors and highly restricted interests. While the allelic ASD architecture remains unclarified, there is definitive evidence of a high degree of locus heterogeneity and a contribution from rare and de novo variants. However, determining a contributing role from low-frequency variants is challenging, particularly for variants that are transmitted in a non-Mendelian fashion, carry intermediate risks, and are present in conjunction with a tremendous amount of apparently neutral rare variations in the human genome.

Reprogramming somatic cells to a pluripotent state by transient over-expression of specific factors enables the development of neuronal models of genomes that are predisposed to human diseases. We recently demonstrated the utility of induced pluripotent stem cells (iPSCs) for investigating the functional consequences of mutations in the gene encoding the methyl CpG binding protein-2 (MeCP2) in neurons from patients with Rett syndrome (RTT), a syndromic form of ASD. Neurons derived from RTT-iPSCs display several alterations compared with controls, such as increased frequency of de novo L1 retrotransposition, decreased soma size, altered dendritic spine density, and reduced excitatory synapses. Therefore, functional studies using neuronal cultures derived from iPSCs from ASD individuals are an important tool to explore the contribution of rare variants to ASD etiology. Furthermore, by capturing the genetic heterogeneity of ASDs, the iPSC model might clarify whether ASD individuals carrying distinct mutations in disparate genes share common cellular and molecular neuronal phenotypes.

Here, we characterize the breakpoints of a de novo balanced translocation
t(3;11)(p21;q22) in an ASD individual that disrupts the TRPC6 gene. TRPC6, a gene not previously implicated in ASD, encodes for the canonical transient receptor potential 6 channel, a voltage-independent, Ca^{2+}-permeable cation channel involved in dendritic spine and excitatory synapse formation. The biological impact of the genetic alteration in the index case and its functional relationship to ASD etiology was evaluated through several analyses using the affected individual's dental pulp cells (DPCs), mouse models, and neural cells derived from iPSCs. To test the hypothesis that different ASD-related variants can produce similar biological effects, we compared the neuronal phenotypes of iPSC-derived neurons from the TRPC6-mutant (TRPC6-mut) individual with those of patients with RTT syndrome. Finally, we conducted a large-scale case-control sequence analysis of TRPC6, which revealed a significant association of mutations in this gene with ASD.

Results

Characterization of the t(3;11)(p21;q22) translocation breakpoint and exome sequencing

We identified an 8-year-old male autistic individual carrying a de novo 46, XY, t(3;11)(p21;q22) translocation by G-banding karyotyping of lymphoblastoid cells. No gain or loss of genetic material was observed near the breakpoint areas via a genome-wide array analysis (Figure 4.1a). Only a duplication (104.225.150 bp - 104.339.273 bp) on chromosome 14 was identified, which was previously shown to be a common copy number variant (CNV; http://projects.tcag.ca/variation/). Fluorescent in situ hybridization (FISH) analysis revealed that BAC probes RP11-780O20 and RP11-109N8 span the breakpoint on chromosome 3p21, while probes RP11-3F4 and RP11-1006P7 map distal
and proximal to the breakpoint, respectively (Figure 4.1b, c). This narrowed the breakpoint to an interval of approximately 15 kb spanning the gene encoding the Vpr-binding protein (VPRBP), indicating that this gene was disrupted. Similarly, the breakpoint on chromosome 11q22 was mapped to a region spanned by probes RP11-141E21 and RP11-153K15, distal to RP11-315B9 and proximal to RP11-942D19 (Figure 4.1d, e), suggesting disruption of the TRPC6 gene, which was confirmed by the use of additional strategies.

We first measured the expression levels of TRPC6 exons 4, 6, 12, and 13 in the lymphocytes of the ASD individual, his parents, and six non-affected control individuals by quantitative real-time PCR (qPCR) (Figure 4.6a). In the ASD individual’s parents and in six other individual controls, exons 6, 12, and 13 had similar expression levels as exon 4. In the ASD individual, however, the expression levels of exons 12 and 13 were reduced by 60% compared to exon 4. After sequencing all TRPC6 exons, we found that the individual was heterozygous for two common polymorphisms: one mapping to exon 6 (rs12366144) and the other to exon 13 (rs12805398). However, sequencing of cDNA from the individual’s lymphocytes revealed heterozygosity only for the polymorphism in exon 6 (Figure 4.6b). Parentage was confirmed through genotyping of microsatellite markers (Figure 4.6c). These results demonstrate that TRPC6 has biallelic expression and that the heterozygosity loss in exon 13 in the individual’s cDNA can be explained by TRPC6 disruption. Accordingly, TRPC6 is transcribed up to the breakpoint, which is located between exons 6 and 12. We did not identify any pathogenic change in TRPC6 exons upon sequencing the individual’s DNA (data not shown). We also did not identify any extra band in the protein extracts from individual’s cells using a N-terminal antibody, indicating that a truncated TRPC6 form is unlikely to be a byproduct of the translocation (Figure 4.6d).
Disruption of *TRPC6*, *VPRBP*, and several other unknown genes might contribute to the ASD phenotype. To identify other genetic alterations in this ASD individual, we performed exome sequencing on the individual and compared the result to those for his parents. Exome sequencing analysis revealed 50 *de novo*, rare, nonsynonymous variants and three frameshift insertions/deletions in the individual. Consultation with AutismKB \(^{10}\) indicated that none of the other genes harboring genetic variants are associated with ASD, with the exception of the cyclic adenosine monophosphate-specific (*PDE4A*) gene, for which lower levels of expression have been observed in the brains of autistic individuals \(^{11}\). We also observed an alteration in the *ATXN3* gene, linked to the spinocerebellar ataxia-3 disease in humans. All genetic variants are presented in **Table 4.4**.

**TRPC6 disruption leads to transcriptional alterations and dysregulation of CREB phosphorylation**

To determine gene transcription due to genetic perturbations in the ASD individual carrying the novel chromosomal translocation, we conducted a global expression analysis comparing the individual’s dental pulp cells (DPCs) to six control samples. DPCs can be easily isolated from the deciduous teeth of ASD individuals via a non-invasive procedure \(^{12}\). DPCs have an ectodermic neural crest origin, express several neuronal markers, and have proven to be a useful model to study ASD \(^{13-15}\). We identified 67 differentially expressed genes (DEGs) between the ASD individual and non-affected controls (*P*<0.05; **Table 4.5**). Functional annotation analysis revealed that 16 (24%) of these genes have a role in nervous system development and function (**Table 1**). We confirmed the reduction of *TRPC6* expression (*P*<0.01) but not *VPRBP* (**Figure 4.2a**). The reduced level of *TRPC6* expression is likely due to nonsense-mediated decay
or rearrangement of regulatory elements caused by the translocation. Moreover, PDE4A, ATXN3 and DOCK3 (another neuronal gene present near the break point on chromosome 3, Figure 4.1b) were also not differentially regulated in the individual’s DPCs (Figure 4.2a). TRPC6 is a Ca\(^{2+}\)-permeable, nonselective, cation channel involved in neuronal survival, growth cone guidance, and spine and synapse formation, biological processes that have previously been implicated in ASD etiology. The function of VPRBP (Vpr-binding protein) is less clear and may include DNA replication, S-phase progression, and cellular proliferation. Given the time-consuming nature of additional functional analyses, we elected to focus on additional genetic and functional studies of TRPC6, which has not been previously associated with ASDs.

Using the CREB-target genes database (http://natural.salk.edu/CREB/), we determined that 8 of the 16 functionally relevant DEGs are regulated by CREB, a transcription factor that is activated upon Ca\(^{2+}\) influx through TRPC6. Of the functionally relevant DEGs, we evaluated 6 CREB-target genes (INA, MAP2, NPTX1, CLDN11, PCDH10, and EPHA4) and two other genes (SEMA3A and CDH6) by quantitative PCR (qPCR) to validate the microarray experiments. We measured dysregulated expression of CDH6 (-2.68-fold, P<0.05), INA (-2.64-fold, P<0.05), MAP2 (-2.79-fold, P<0.05), and CLDN11 (4.07-fold, P<0.001) in the individual compared with controls in the same direction observed in the microarray analysis (Table 1). To validate that TRPC6 haploinsufficiency is leading to transcriptional dysregulation of these genes, we treated a control DPC culture with hyperforin plus flufenamic acid (FFA) and measured the expression levels of the same candidate genes over 48 hours (Figure 4.2b, c and Figure 4.6e). Hyperforin specifically activates TRPC6 and FFA increases the amplitude of the currents through this channel. If the candidate genes are regulated through TRPC6 signaling, we expect a change in their expression levels opposite to the
observed change in the TRPC6-mut individual. After a 48-hour treatment, we observed the expected correlation for five of the eight genes. While the expression levels of *SEMA3A*, *EPHA4*, and *CLDN11* were significantly reduced (-28-fold, -3.2-fold and -4.76-fold, respectively), *MAP2* and *INA* displayed 20- and 10-fold increases in expression, respectively. These results validate the microarray data and support the hypothesis that the selected genes are regulated by the TRPC6 pathway.

We measured CREB phosphorylation in the DPCs of the individual and a control to assess the functional effect of TRPC6 disruption. Stimulation of DPCs with hyperforin plus FFA induced a significantly reduced level of increased phosphorylated CREB (p-CREB) in the individual's DPCs (30.3±0.7%) compared to control (48.6±2.3%; *P*<0.005) after 15 minutes. After 30 minutes, p-CREB levels in the individual's DPCs (6.3±2.1%) were also significantly lower compared to the control (20.9±2.1%; *P*<0.05) (Figure 4.2d and e). Taken together, these results demonstrate that several of the functionally relevant DEGs identified in the microarray studies are controlled via TRPC6 signaling, likely through CREB phosphorylation, suggesting that TRPC6 disruption influences neuronal cell function.

**Generation of neural cells from ASD individuals**

To further evaluate the effect of TRPC6 haploinsufficiency on neural cell function, we generated iPSCs from DPCs from the ASD individual and two control individuals (Figure 4.7 and Table 4.6 and 4.7). We chose to reprogram DPCs because these cells develop from the same set of early progenitors that generate neurons. Furthermore, the neurons derived from iPSCs generated from DPCs express higher levels of forebrain genes, many of which are implicated in ASD. We fully characterized three clones from each individual and used at least two different clones for follow up experiments. A
summary of the clones used for each experiment can be found in Table 4.7. Neural progenitor cells (NPCs) and cortical neurons from iPSCs were obtained using a modified protocol from our previous publication \(^6\). Briefly, iPSC colonies on Matrigel were treated with dorsomorphin under FGF-free conditions until confluence. Pieces of iPSC colonies were grown in suspension for 2-3 weeks as embryoid bodies (EBs) in the presence of dorsomorphin (Figure 4.3a). The EBs were then dissociated and plated to form rosettes. The rosettes were manually selected and expanded as NPCs (Figure 4.3a). These NPCs were negative for the pluripotent marker OCT4 and positive for early neural-specific markers such as Musashi-1 and Nestin (Figure 4.3b and Figure 4.8a). To obtain mature neurons, NPCs were plated with ROCK inhibitor and maintained for 3-4 additional weeks under differentiation conditions. At this stage, the cells were positive for the pan neuronal marker Tuj1 (β-III-Tubulin) and expressed the more mature neuronal markers synapsin I (SYN1) and microtubule-associated protein 2 (MAP2; Figure 4.3c). These cells expressed genes typically found in the cortex, including CTIP2, important for the differentiation of subcortical projecting neurons; TBR1, critical for cortical development and ABAT, a marker for GABAergic neurons, encoding for the 4-aminobutyrate aminotransferase protein and responsible for the catabolism of GABA neurotransmitter. (Figure 4.8a). Expression of NESTIN indicates the presence of NPCs and the expression of S100B and GFAP are indicative of glia cells, suggesting a mixed cell population at this stage (Figure 4.8a). In our cultures, the presynaptic SYN1 puncta were frequently adjacent to the postsynaptic marker HOMER1, suggesting the presence of developed synapses (Figure 4.8b). Using immunostaining, we also detected expression of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in 13% of the neurons, and 22% were positive for vesicular glutamate transporter-1 (VGLUT1), a marker for excitatory neurons, in both controls and ASD subjects (Figure 4.3c-e). Our
protocol generated a consistent population of forebrain neurons, confirmed by the co-localization of pan-neuronal and subtype-specific cortical markers, such as 16% of Ctip2 (Layers V and VI) and 6% of Tbr1 (Layers I and VI; Figure 4.3d-e). Expression of peripherin and En1, markers for peripheral and midbrain neurons, respectively, was not detected. We did not observe a significant variability in these subtypes of neurons between the control and ASD backgrounds (Figure 4.8a). Next, we determined the functional maturation of the iPSC-derived neurons using electrophysiological methods. Whole-cell recordings were performed using cells that had differentiated for at least 6 weeks in culture. Both controls and ASD- neurons showed action potentials evoked by somatic current injections (Figure 4.3f-h and Figure 4.8c and d). Therefore, our data indicate that somatic cell reprogramming did not affect the ability of iPSC-derived neurons to mature and become electrophysiologically active.

**TRPC6 disruption does not affect NPC proliferation**

*TRPC1*, another member of the transient receptor potential channel family, is involved in NPC proliferation mediated by FGF. Therefore, we investigated whether reduction of *TRPC6* expression levels affects the cell cycle profile. No difference was observed when comparing the percentage of cells in G1 (56.2±5.0% and 47.8±10.5%, P>0.2), S (30.6±3.0% and 36.0±6.4%, P>0.2), and G2/M (10.1±1.4% and 14.3±4.8%; P>0.2) phases between control and TRPC6-mut iPSC-derived NPCs, indicating that TRPC6 likely does not play a role in NPC proliferation, in contrast to TRPC1 (Figure 4.9a).

**Ca^{2+} influx is reduced in TRPC6-mutant NPCs**
The role of TRPC6 in dendritic spine formation depends on a pathway that involves Ca\(^{2+}\) influx through the channel \(^8\). To test if changes in intracellular Ca\(^{2+}\) levels might be altered in TRPC6-mut neural cells upon TRPC6 activation, we stimulated iPSC-derived NPCs from the TRPC6-mut individual and a control with hyperforin plus FFA. This combination of drugs induced transient and repetitive increases in intracellular Ca\(^{2+}\) concentrations in both TRPC6-mut- and control-derived NPCs. The TRPC6 activation-induced Ca\(^{2+}\) oscillation peak was significantly higher in control NPCs compared with TRPC6-mut NPCs (Figure 4.4a). The average amplitude of the Ca\(^{2+}\) increase over baseline in the 100 cells analyzed was reduced by 30% in the TRPC6-mut NPCs (1.9±0.08-fold) compared with the control sample (2.7±0.2-fold; \(P<0.001\)) when stimulated with hyperforin and FFA (Figure 4.4b).

**TRPC6 signaling regulates gene expression in neuronal cells**

To validate our DPC findings, we examined the expression of some neuronal genes in NPCs in response to TRPC6 activation (Figure 4.8b). After a 48h hyperforin treatment, SEMA3A expression was reduced (0.6±0.05-fold, \(P<0.05\)), whereas INA and MAP2 again showed increased expression (2.6±0.09-fold and 1.8±0.1-fold; \(P<0.001\)). These results parallel our DPC expression analysis and support the hypothesis that TRPC6 signaling is important for the regulation of genes involved in neuronal function.

**TRPC6 disruption alters the neuronal phenotype**

To determine if TRPC6 disruption influences spine formation and synaptogenesis, we investigated neurons derived from TRPC6-mut and control iPSCs. To avoid variability from reprogramming, all experiments were performed with different iPSC clones and independent experiments. All biological replicates and iPSC clones
used in each experiment are summarized in Table 4.7. The neurons derived from this ASD individual exhibited a 60% reduction (P<0.01) in TRPC6 protein levels as measured by western blot (Figure 4.4c and d). We first examined neuron morphology by infecting cells with a previously described lentiviral vector containing the EGFP sequence under the control of the synapsin gene promoter (syn::EGFP) 6. By measuring the size of neurites and their ramifications, we verified that the TRPC6-mutant neurons are shorter in total length (1782±101.2 and 2666±153.7 pixels; P<0.001) and less arborized (3.7±0.2 and 8.7±0.5 vertices; P<0.001) than the controls (Figure 4.4e-g). Moreover, the density of dendritic spines in TRPC6-mutant neurons was reduced (7.4±0.5 spines per 20µm of dendrite length) compared with control neurons (12.9±0.8 spines; P<0.001) derived from several individuals (Figure 4.4h-j, Figure 4.8e). TRPC6 expression was previously shown to regulate spine density 8. Thus, to confirm that the alterations observed in this ASD individual were caused by TRPC6 haploinsufficiency, we downregulated TRPC6 expression in control neurons using a specific, pre-validated shRNA in a lentiviral vector. Neurons derived from control iPSCs expressing shTRPC6 exhibited a significant reduction in spine density (6.0±0.5 spines) compared with control neurons expressing a scrambled shRNA (12.5±0.7 spines; P<0.0001; Figure 4.4j). Even further, restoring TRPC6 expression in the TRPC6-mut neurons using a lentiviral vector expressing wild-type TRPC6 (Figure 4.9c-d) rescued these morphological alterations, increasing total neuronal length (3051±133.4 pixels; P<0.001), arborization (8.9±0.6 vertices; P<0.001), and dendritic spine density (11.9±0.9 spines; P<0.001) to control levels (Figure 4.4e, f, and h). Interestingly, the specific activation of the wild type TRPC6 in mutant neurons by hyperforin was also sufficient to rescue these morphological phenotypes in our culture conditions (Figure 4.4e, f and h).
TRPC6 is mainly expressed in glutamatergic synapses and its loss interferes with synapsin I cluster density in pre-synaptic sites of hippocampal neurons, suggesting that this gene has an important role in the regulation of excitatory synapse strength. Quantifying VGLUT1 puncta in MAP2-labeled neurons confirmed that the TRPC6-mutant neurons had a significantly lower density of VGLUT1 puncta (4.6±0.3 puncta per 20µm of dendrite length) compared with independent clones isolated from several independent controls (10.3±0.4 puncta; P<0.001) (Figure 4.4k, Figure 4.8g). To determine if TRPC6 haploinsufficiency contributed to the lower density of VGLUT1 puncta, we treated TRPC6-mut neurons with hyperforin to specifically stimulate TRPC6. After 2 weeks of treatment, the neurons exhibited a significant increase in the number of VGLUT1 puncta compared with untreated cells (7.4±0.5 puncta; P<0.05) (Figure 4.4k). Control neurons expressing shTRPC6 also exhibited a lower density of VGLUT1 puncta, indicating that loss of TRPC6 function affects the formation of glutamatergic synapses (P<0.01) (Figure 4.4m-n). In addition, overexpression of TRPC6 in the TRPC6-mut neurons was able to rescue synapse numbers (8.0±0.6 puncta per 20µm of dendrite length; P<0.001) to control levels, as measured by synapsin I puncta (Figure 4.4m-p). Finally, electrophysiological recordings revealed that the Na+ currents of TRPC6-mutant neurons (28.38±7.5 pA) were impaired compared to controls (154.4±45.9 pA; P<0.0001) (Figure 4.4q-r, Figure 4.8e).

TRPC6 and MeCP2 share a similar molecular pathway

Certain neuronal phenotypes (reduction of spine density and glutamatergic synapses) associated with TRPC6 function loss are similar to those previously described for loss of MeCP2 function in human neurons. MeCP2 genetic alterations have been recognized in several non-syndromic ASD individuals, and reduced MeCP2
expression in brains of autistic individuals has been reported \cite{34, 35}. In addition, two independent studies have reported that MeCP2 regulates TRPC6 expression in the mouse brain, likely through an indirect mechanism \cite{36, 37}. Thus, we investigated whether MeCP2 acts upstream of TRPC6 in human neurons. We used two iPSC clones from a female RTT patient carrying the T158M MeCP2 mutation, which results in persistent X chromosome inactivation \cite{6}. Each clone expresses a different MeCP2 allele, a wild type or mutant version of the MeCP2 gene. We then differentiated both clones into neurons and evaluated TRPC6 protein expression levels. The TRPC6 expression level was reduced by 40\% in the clone carrying the non-functional version of MeCP2 compared to the wild type control clone (61.67±6.0\% and 99.3±1.2\%; \(P<0.01\)), indicating that MeCP2 levels affect TRPC6 expression in human neurons (Figure 4.4s). This observation supports MeCP2 acting upstream of TRPC6 in the same molecular pathway to affect neuronal morphology and synapse formation. We next investigated whether MeCP2 could occupy regions of the human TRPC6 promoter. Chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) revealed high levels of MeCP2 in association with the TRPC6 promoter region in human neurons, suggesting a potential mechanism of transcriptional regulation (Figure 4.4t, Figure 4.9f).

Our data suggest that the molecular pathway involving MeCP2 and TRPC6 is a rate-limiting factor in regulating glutamatergic synapse number in human neurons. Administration of insulin-like growth factor-1 (IGF-1) promotes the reversal of RTT-like symptoms in a mouse model \cite{38} and of molecular alterations in RTT human neurons \cite{6}, and is currently in clinical trials for RTT patients. To investigate whether the potential convergence of molecular mechanisms underlying RTT and non-syndromic autism suggests shared therapeutic benefits, we treated TRPC6-mutant neurons with full-length IGF-1 (10 μg/mL). Interestingly, we observed a significant increase in TRPC6 protein
levels after treatment. Moreover, Psd-95 and synapsin I protein levels were also upregulated by IGF-1 ($P<0.01$; Figure 4.4c and d). IGF-1 treatment also rescued the glutamatergic synapse number in TRPC6-mutant neurons as measured by VGLUT1 puncta (9.2±0.6 puncta per 20µm of dendrite length; $P<0.01$), suggesting that the drug treatment could correct this neuronal phenotype (Figure 4.4k).

**TRPC6 downregulation compromises neuronal development in vivo**

*In vitro* experiments in rodent primary neurons have shown that Trpc6 levels affect spine density and excitatory synapses. To corroborate our findings from human derived neurons, we looked to examine the effect of Trpc6 loss in a rodent model. We validated two shRNAs (#1 and #3) against mouse Trpc6 by western blot analysis and used both for further experiments (Figure 4.9g-h). Using this shRNA targeting Trpc6, we transduced mouse primary hippocampal neurons. The neurons expressing shRNA targeting Trpc6 demonstrated reduced spine density (7.5±0.5 and 11.4±0.8 spines per 20µm of dendrite length; $P<0.001$) and fewer synapses (5.6±0.5 and 7.7±0.5 puncta per 20µm of dendrite length; $P<0.01$) versus neurons transduced with a shRNA scramble control (Figure 5a and b). Thus, as described above, we determined that TRPC6 downregulation causes similar neuronal alterations in human and rodent neurons. We next looked to validate the cell autonomous effect of TRPC6 loss of function *in vivo* by taking advantage of adult neurogenesis in the hippocampus. Using retroviruses to target newborn neurons, we delivered the shRNAs against mouse Trpc6. Trpc6 downregulation led to migration defects and reduced neuronal dendritic arborization (Figure 5c-f). Moreover, whole-cell patch clamping to record action potentials revealed a significant reduction in the firing rate of neurons expressing shRNAs against Trpc6 compared with controls (Figure 5g-i). To demonstrate the
contribution of Trpc6 to these phenotypes in vivo, we rescued the migration defects by co-transfecting the shRNA with an expression construct for an shRNA-resistant form of TRPC6-WT (TRPC6-WTR; **Figure 5d, Figure 4.9h**).

TRPC6 knockout (KO) mice display reduced exploratory activity in a square open field and elevated star maze compared with control siblings. Limited environmental exploration is commonly associated with ASD individuals. Thus, we decided to investigate whether the TRPC6 KO mouse displays other ASD-like behaviors. We assessed the social interaction and repetitive behaviors of these animals, but observed no significant differences between wild-type controls (WT) and heterozygotes (HET) or WT and KO mice (**Figure 5j**). Together, these data demonstrate loss or reduction of TRPC6 in a rodent model induces neuronal abnormalities paralleling our findings in the TRPC6-mut iPSC-derived neurons, such as reduced neuronal arborization, spine density and synapse numbers.

**Mutation screening of TRPC6**

Based on the initial observation of TRPC6 disruption by a chromosomal breakpoint, we established a narrow hypothesis focusing on TRPC6 to conduct a single gene case/control association study. We screened targeted high-throughput sequencing data from all coding exons and splice sites of TRPC6 in 1041 ASD cases from the Simons Simplex Collection (SSC) and 942 ancestrally matched controls from the NINDS Neurologically Normal Caucasian Control Panel (http://ccr.coriell.org/Sections/Collections/NINDS/). A summary of the quality control metrics of the high-throughput sequencing is presented in **Table 4.8**. We focused on novel splice sites, missense, and nonsense mutations that were observed only once across all of our cohorts and not present in the dbSNP137 and 6503 exomes available.
from the Exome Variant Server (EVS, v.0.0.15). We reasoned that these variants were most likely to be deleterious and subject to purifying selection. Moreover, the study of variants observed only once, in combination with case-control matching for ancestry, represents a more rigorous approach to protecting against population stratification.

Table 4.9 lists all such variants in TRPC6. We observed significantly more novel nonsynonymous singleton mutations in cases compared with controls (10/1041 cases versus 1/942 controls; \( p = 0.013 \), \( OR = 9.127 \), 95% CI = 1.211-191.027, Fisher exact test, two-tailed). To confirm the low mutation rate observed in this control sample, we examined the whole exome-sequencing data from an in-house database and identified an additional 1930 northern European (NE) controls who clustered tightly with the HapMap CEU cohort. We evaluated the coding exons and splice sites of TRPC6 and, to maximize sensitivity, did not set a minimum read threshold to identify all novel nonsynonymous singleton variants, which are listed in Table 4.6. An omnibus analysis revealed an even more significant over-representation of such variants in cases (10/1041 cases versus 4/2872 controls; \( p = 0.001 \), \( OR = 6.954 \), 95% CI = 2.008-26.321, Fisher exact test, two-tailed). Because our results indicate that TRPC6 disruption leads to haploinsufficiency of the corresponding protein, two of the case variants are particularly noteworthy: M1K, which disrupts the start codon; and Q3X, which is a very early premature stop codon. Unfortunately, live cells from these individuals were not available for follow-up functional studies. No TRPC6 mutations affecting the start codon or nonsense mutations were identified in a total of 7445 controls: 942 NINDS neurologically normal Europeans and 6503 exomes from the EVS (4300 European-American, 2203 African-American). Segregation analysis of the case variants revealed that each was inherited from an apparently unaffected parent, suggesting that these variants are incompletely penetrant, as has been previously observed for a wide range
of ASD mutations such as Shank3\textsuperscript{46} and CNTNAP2\textsuperscript{47}. Thus, although these genetic variations cannot be considered as causal mutations, they might represent risk factors for ASD. No \textit{TRPC6} CNVs have been described in ASD (http://projects.tcag.ca/autism_500k).

Discussion

A rapidly increasing number of ASD risk regions are being identified, and there is now considerable effort focused on moving from gene discovery to understanding the biological influences of these various mutations\textsuperscript{2-4,48-50}. The development of relevant human-derived cellular models to study ASDs represents a complementary strategy to link genetic alterations to molecular mechanisms and complex behavioral and cognitive phenotypes\textsuperscript{51}. Here, we identified the disruption of the \textit{TRPC6} gene by a balanced \textit{de novo} translocation in a non-syndromic ASD individual. \textit{TRPC6} is involved in the regulation of axonal guidance, dendritic spine growth, and excitatory synapse formation\textsuperscript{8,9,18}, processes that have been consistently implicated in ASD etiology\textsuperscript{52-55}. To explore if \textit{TRPC6} disruption could result in such neuronal alterations, we made use of several different cellular models.

Global transcriptional studies of DPCs derived from the ASD individual and expression analysis upon activation of \textit{TRPC6} in DPCs and NPCs suggested that \textit{TRPC6} signaling regulates the transcription of genes involved in neuronal adhesion, neurite growth, and axonal guidance. The abnormal dysregulation found in the ASD individual might be triggered, at least for some genes, by reduced levels of phosphorylated CREB, a transcription factor activated by \textit{TRPC6} signaling\textsuperscript{8}. CREB controls a complex regulatory network involved in memory formation, neuronal
development, and plasticity in the mammalian brain, processes that are compromised in ASD \(^{56-58}\).

Reprogramming the DPCs from the ASD individual to a pluripotent state allowed us to explore the functional consequences of \(TRPC6\) disruption in human neuronal cells. \(\text{Ca}^{2+}\) influx was aberrant in NPCs derived from the ASD individual, suggesting that \(\text{Ca}^{2+}\) signaling-dependent mechanisms were compromised in these cells. \(\text{Ca}^{2+}\) signaling pathways have previously been implicated in ASD etiology; mutations in different voltage-gated \(\text{Ca}^{2+}\) channels and \(\text{Ca}^{2+}\)-regulated signaling molecules have been identified in ASD individuals \(^{59-62}\). This result, combined with the measured protein levels, reveals that disruption of \(TRPC6\) leads to a functionally relevant haploinsufficiency, making the existence of a novel disease-relevant protein resulting from a \(TRPC6\) and \(VPRBP\) combination unlikely.

In human neurons, \(TRPC6\) haploinsufficiency causes other functional and morphological alterations that reflect defects in axonal and dendritic growth, such as shortening of neurites, a decrease in arborization, and a reduction in dendritic spine density. Alterations in these phenotypes were already been described for post-mortem or iPSC-derived ASD neurons \(^{63, 64}\). Analysis of neurons derived from the ASD individual's iPSCs also revealed a reduction of VGLUT1 puncta density, in agreement with previous work demonstrating that \(TRPC6\) expression levels can modulate glutamatergic synapse formation in rat neurons \(^9\). Alterations in glutamatergic neurotransmission have been identified in individuals with syndromic forms of ASD: dysregulation of the metabotropic glutamate receptor 1/5 (mGluR1/5) pathway has been well documented in Fragile-X syndrome, and neurons derived from RTT patient iPSCs also present a reduction in the number of VGLUT1 puncta \(^6, 65, 66\). In addition, a reduction in glutamatergic transmission was observed in Shank3 heterozygous mice, an ASD
mouse model \(^{67}\). Finally, Na\(^+\) currents were also decreased in ASD individual’s neurons. This result is in agreement with previous findings that demonstrate that TRPC6 channels participate in Na\(^+\) cell entry \(^{21}\). Decreased Na\(^+\) current densities have previously been reported in other ASD models \(^{68}\).

Due to the high degree of locus heterogeneity, it is challenging to identify additional individuals carrying similar rare variants in the ASD population. Therefore, we used complementary functional assays such as loss-of-function experiments and mouse models to validate the observation that reduction in TRPC6 expression levels leads to abnormal neuronal phenotypes and is important for neuronal homeostasis. Moreover, we have demonstrated that several of the phenotypic alterations seen in the TRPC6-mut neurons could be rescued by both using hyperforin, which activates the channel, and expressing wild-type TRPC6. These TRPC6 loss-of-function and complementation assays underscore its importance for neuronal homeostasis. Based on the results obtained from our different cellular models, this is likely due to TRPC6 influence on Ca\(^{2+}\)-signaling dependent mechanisms and neuronal transcriptional regulation. The common altered neuronal phenotypes shared by the TRPC6-mutant individual and RTT patients support the idea that ASD caused by different genetic mechanisms affect common pathways. Indeed, our data suggest that MeCP2 may act upstream of TRPC6, regulating its expression. Previous mouse studies \(^{36,37}\) suggested similar findings but failed to show a direct link between MeCP2 and the TRPC6 promoter through ChIP assays, likely due to the poor conservation between the promoter regions in these two species. Additional studies using large samples of idiopathic ASD individuals will help address this hypothesis.

Our findings also provide insights supporting the testing of novel drugs in ASD such as hyperforin, a drug that specifically activates TRPC6 \(^{21,69}\), or IGF-1, which might
increase not only TRPC6 protein levels but also other synaptic components. Therefore, individuals with alterations in this pathway might benefit from these drugs. These defects could also be rescued by activating the AKT/mTOR pathway using IGF-1. The TRPC6 KO mice exhibit reduced exploratory interest, a typical ASD-like behavior, but no impaired social interaction or repetitive movements. The lack of some ASD-like behaviors in mouse models is common and can be attributed to the inherent differences between human and mouse genetic backgrounds and neural circuits \cite{70-73}. Alternatively, other genetic alterations may be required to develop the full autistic phenotype in this mouse model. Accumulating evidence favors the multiple-hit model in a significant proportion of ASD individuals as well as in the case of the ASD individual described here \cite{47, 74-77}. In fact, while our functional data demonstrate that TRPC6 has a crucial role in synaptogenesis and is involved in pathways previously associated with ASD, our mutation screening data suggest rare TRPC6 variants may have a more moderate contribution to the disease. Our sequencing findings revealed TRPC6 loss-of-function mutations in two ASD families with incomplete penetrance of the phenotype, supporting the multi-hit hypothesis for ASD. Indeed, the individual studied here also presents other rare genetic variants, such as in the ASD associated gene PDE4A or even VPRBP, that might contribute to his phenotype. However, this does not diminish the impact of TRPC6 to the phenotype, as our experiments using hyperforin or TRPC6 complementation rescued the observed cellular alterations. This suggests that while other genetic alterations present in the individual might augment the observed phenotypes, TRPC6 disruption is the predominant contributor to the abnormal neuronal function in this ASD individual.

Thus, our results suggest TRPC6 as a novel predisposing gene for ASD that likely acts in combination with other genetic variants to contribute to autistic phenotypes.
Our work demonstrates that individual-specific iPSC-derived neurons can be used to correlate novel variants in ASD individuals to the etiology of these highly complex disorders.

**Materials/Subjects and Methods**

**Patient ascertainment**

*ASD individual F2749-1 (TRPC6-mutant):* The 8-year-old proband is the only child of non-consanguineous healthy parents. He was born at term after an uncomplicated pregnancy with no malformations recognized at birth. He was noted to have delayed motor skills development and poor social responsiveness and was brought for medical examination at 2 years of age. His hearing was tested and found to be normal. He did not suffer from any other chronic medical conditions, and there was no history of head trauma or seizure. On examination, the individual met the DSM-IV criteria for autistic disorder, and the diagnosis was supported by the administration of the Childhood Autism Rating Scale (CARS). The electroencephalogram and magnetic resonance imaging were normal. The individual did not have dysmorphic features, except for synophrys, which is also present in other members of the father’s family. A molecular test for Fragile-X Syndrome was normal. Karyotype analysis revealed a balanced translocation (46, XY, t[3;11] [p21;q22]) in the proband that was not found in the parents. Parenthood was confirmed through genotyping of microsatellite markers.

**Controls:** As controls, we used six non-affected individuals that are non-related to the individual. Cells from two control individuals (USC1 and P603) were selected for reprogramming follow up studies. This project was approved by the Ethics Committees
of the institutes at which the study was conducted. After a complete description of the study, written informed consent was provided by the parents.

**Analysis of genomic copy number variations**

Genomic DNA was hybridized to the HumanHap300 Genotyping BeadChip from Illumina (La Jolla, CA, USA) according to manufacturer’s protocol to detect possible CNVs in the ASD individual. The data were analyzed using PennCNV \(^78\) and QuantiSNP \(^79\) software, and the results were compared to the database of genomic variants (http://projects.tcag.ca/variation/) to classify the identified CNVs as rare or common variants.

**Fluorescent in situ hybridization**

Chromosomes for fluorescent in situ hybridization (FISH) analysis were prepared from colchicine-treated lymphocytes of the proband. Bacterial artificial chromosomes (BACs) encompassing the genomic regions of interest were selected from the RPCI-11 library (Roswell Park Cancer Institute) using the UCSC genome browser (http://genome.ucsc.edu/, assembly Mar. 2006, NCBI36/hg18). The BACs were fluorescently labeled by nick translation and hybridized to the metaphase spreads using standard protocols \(^80\).

**Exome sequencing**

Exome sequencing and analysis were performed by BGI Tech (Shenzhen, China). Briefly, genomic DNA samples were randomly fragmented into segments with a base-pair peak of 150 to 200 bp, and library enrichment for exonic sequences was performed using Agilent SureSelect Human All Exon 51M (for individual and mother) or
Agilent SureSelect Human All Exon 71M (for the father). The captured libraries were loaded on Hiseq2000, and the sequences of each individual were generated as 90-bp paired-end reads. The coverage for the three individuals was 80-fold. Burrows-Wheeler Aligner (BWA) was used for the alignment. Single nucleotide polymorphisms (SNPs) were identified by SOAPsnp, small insertion/deletion (InDels) were detected by Samtools/GATK, and single nucleotide variants (SNVs) were detected by 1/35 Varscan.

Isolation and culture of human DPCs

DPC lineages were obtained as described elsewhere. Briefly, dental pulp tissues were digested in a solution of 0.25% trypsin for 30 minutes at 37°C. The cells were cultivated in DMEM/F12 media (Gibco) supplemented with 15% fetal bovine serum (Hyclone, TX), 1% penicillin/streptomycin, and 1% non-essential amino acids and maintained under standard conditions (37°C, 5% CO2). The DPC control lineages used for the whole-genome expression analysis were donated by Dr. Daniela Franco Bueno and Gerson Shigueru Kobayashi of the University of São Paulo. One of the DPC control lineages used for iPSC generation was a kind gift from Dr. Songtao Shi (University of Southern California).

RNA extraction

RNA samples were extracted from lymphocytes, DPCs, and iPSCs using Trizol reagent (Invitrogen, CA) and treated with Turbo DNA-free (Ambion). Sample concentrations and quality were evaluated using a Nanodrop 1000 and gel electrophoresis.

Microarray studies
For microarray experiments, 100 ng of RNA was converted to cDNA, amplified, labeled, and hybridized to the Human Gene 1.0ST chip from Affymetrix following the manufacturer’s protocol. The chips were scanned using the GeneChip® Scanner 3000 7G System, and a quality control was processed using Affymetrix® Expression Console™ Software. The data were normalized using the robust multi-array average (RMA) method, and the differentially expressed genes were selected with the significance analysis of microarrays method (SAM) and RankProd. To select DEGs, we used a p-value < 0.05 adjusted for the false discovery rate (FDR) and 3,000 permutations. Functional annotation, canonical pathways, and networks analyses were performed using Ingenuity Pathways (http://www.ingenuity.com/). The CREB target genes database (http://natural.salk.edu/CREB/search.htm) was used to determine whether the DEGs found are regulated by the transcription factor CREB.

**Gene expression analyses by qPCR**

RNA samples were reversed transcribed into cDNA using the Super Script III First Strand Synthesis System (Invitrogen, CA) according to the manufacturer’s instructions. The reactions were run on an Applied Biosystem 7500 sequence detection system using SYBR Green master mix (Applied Biosystems, CA). The primers were designed using PrimerExpress v. 2.0 software (Applied Biosystems, CA), and specificity was verified by melting curve analysis using 7500 System SDS v. 1.2 Software (Applied Biosystems, CA). Quantitative analysis was performed using the comparative threshold cycle method. GeNorm (www.medgen.ugent.be/genorm/) was used to determine the stability of the reference genes GAPDH, HPRT1, SDHA, and HMBS and to generate a normalization factor for the expression values of the target genes. The principles of analysis of geNorm have been described. Microarray validation was performed using
the one-tailed unpaired t-test with Welch’s correction to compare the qPCR expression values obtained for the ASD individual and controls. A concentration of 10 µM hyperforin was used to treat the DPCs of a control sample for 15 and 30 minutes and 1, 3, 6, 24, and 48 hours. The samples were prepared in triplicate, and the results were normalized by the values obtained for an untreated sample. Primers used on this work are described in Table 4.2.

**Western blotting**

Rabbit anti-TRPC6 (ProScience, 1:250; Sigma, 1:1000); mouse anti-TRPC6 (Abcam, 1:1000); rabbit anti-CREB (Cell Signaling, 1:500); rabbit anti-P-CREB (Cell Signaling, 1:500); and mouse anti-β-actin (Ambion, 1:5000) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse (Promega, 1:2000) antibodies were used as secondary antibodies. ECL Plus (Amersham) was used for signal detection. For Semiquantitative analysis of p-CREB signal, intensity was corrected with respect to CREB/β-actin relative quantification. A paired t-test analysis with a p-value < 0.05 was used to compare the control and ASD individual p-CREB signal intensity normalized data.

**Cellular reprogramming**

The iPSCs were obtained from the DPCs of the ASD individual and a control. Briefly, DPCs were transduced with retroviruses containing OCT4, SOX2, KLF4, and MYC to induce overexpression of these genes. Two days after transduction, the cells were transferred to a co-culture system with murine embryonic fibroblasts (mEFs) maintained with DMEM/F12 (Invitrogen, CA), 20% Knockout Serum Replacement (Invitrogen, CA), 1% non-essential amino acids, and 100 µM beta-mercaptoethanol and
treated with 1 mM valproic acid (Sigma) for 5 days. The iPSC colonies were identified after approximately 2 weeks in this culture system, transferred to Matrigel (BD Biosciences)-coated plates, and maintained in mTeSR media (Stem Cell Technologies).

**Immunocytochemistry**

The cells were fixed with PBS containing 4% paraformaldehyde for 10 minutes and then incubated at room temperature for 1 hour in a blocking solution containing 5% donkey serum and 0.1% Triton X-100. The primary antibodies were incubated overnight at 4°C, followed by incubation with secondary antibodies (Jackson ImmunoResearch) for 1 hour at room temperature. Images were captured with a Zeiss microscope. The primary antibodies used included the following: Tra-1-81 (1:100, Chemicon); Nanog and Lin28 (1:500, R&D Systems); Sox2 (1:250; Chemicon); human Nestin (1:100, Chemicon); Tuj1 (1:500, Covance); MAP2 (1:100; Sigma); VGLUT1 (1:200, Synaptic Systems); GABA (1:100, Sigma); Musashi (1:200, Abcam); Ctip2 (1:200, Abcam); and Tbr1 (1:200, Abcam).

**Teratoma formation**

iPSC colonies from five semi-confluent 100 mm dishes (1-3 x10⁶ cells) were harvested after treatment with 0.5 ng/mL dispase, pelleted, and suspended in 300 µL Matrigel. The cells were injected subcutaneously into nude mice; 5 to 6 weeks after injection, teratomas were dissected, fixed overnight in 10% buffered formalin phosphate, and embedded in paraffin. The sections were stained with hematoxylin and eosin for further analysis. The protocols were approved by the University of California San Diego Institutional Animal Care and Use Committee.
Fingerprinting and karyotype

Standard G-banding karyotype and DNA fingerprinting analysis were performed by Cell Line Genetics (Madison, WI).

Neuronal differentiation

The iPSC colonies were plated on Matrigel (BD Biosciences)-coated plates and maintained for 5 days in mTSeR media (Stem Cell Technologies). On the 5th day, the media was changed to N2 media [DMEM/F12 media supplemented with 1X N2 supplement (Invitrogen) and 1 µM dorsomorphin (Tocris)]. After 2 days, the colonies were removed from the plate and cultured in suspension as embryoid bodies (EBs) for 2-3 weeks using N2 media with dorsomorphin during the entire procedure. The EBs were then gently dissociated with accutase (Gibco), plated on Matrigel-coated dishes, and maintained in NBF media (DMEM/F12 media supplemented with 0.5X N2, 0.5X B7 supplements, 20 ng/mL FGF and 1% penicillin/streptomycin). The rosettes that emerged after 3 or 4 days were manually selected, gently dissociated with accutase, and plated in dishes coated with 10 µg/mL poly-ornithine and 5 µg/mL laminin. This NPC population was expanded using NBF media. To differentiate the NPCs into neurons, the cells were re-plated with 10 µM ROCK inhibitor (Y-27632, Calbiochem) in the absence of FGF, with regular media changes every 3 or 4 days.

Ca²⁺ influx studies

Intracellular Ca²⁺ levels were monitored using Fluo-4 AM. The cells were incubated for 45 minutes at 37°C with 2.5 µM Fluo-4 AM and superfused for 5 minutes with HBSS buffer before the beginning of the recording. A concentration of 10 µM hyperforin (a kind gift from Dr. Willmar Schwabe GmbH & Co, Karlsruhe, Germany) was
used in combination with 100 µM FFA (Sigma-Aldrich) for TRPC6 activation. Images were captured at 6-second intervals for 30 minutes using a Biorad MRC 1024 confocal system attached to an Olympus BX70 microscope. The drugs were applied at the 3rd minute using a perfusion system. A triplicate of each individual was analyzed. The average fluorescence of the individual cells was quantified and normalized to the resting fluorescence level for each cell. The plugins MultiMeasure and MeasureStacks from ImageJ software were used to measure fluorescence intensity. The analyses were performed blinded to avoid bias.

**Cell cycle analysis**

A total of $1 \times 10^6$ NPCs were harvested from a single-cell suspension with PBS washing buffer (PBS and 1% serum) and fixed in 75% EtOH for at least 2 hours at 4°C. After washing twice with washing buffer, the cells were stained with 200 µL propidium iodine (PI) solution (20 µg/mL propidium iodide, 200 µg/mL RNase A, and 0.1% Triton X-100). Multiple NPC samples from the TRPC6-mutant individual and controls were analyzed by fluorescence-activated cell sorting (FACS) on a Becton Dickinson LSRI, and cell cycle gating was examined using FLOWJO-Flow Cytometry Analysis Software.

**Quantification of neuronal morphology and synaptic puncta**

Neuronal tracing was performed on neurons for which the shortest dendrite was at least three times longer than the cell soma diameter using a semi-automatic ImageJ plug-in (NeuroJ). Spines and VGLUT1 puncta were quantified after three-dimensional reconstruction of z-stack confocal images. The same density of neurons was plated in each condition. Final cell density was confirmed by DAPI and Synapsin-EGFP-positive cells. Only Synapsin-EGFP positive neurons with spines were scored. Images were
taken randomly for each individual and from two different experiments, using at least two different clones. Quantification was performed blind to the cell genotype. The total dendritic length includes the summed length of all dendrites per neuron and dendritic segment count represents the total number of dendritic segments per neuron. No distinction was made between different types of spines due to the unviability of this assessment using the presented method. All experiments were performed with independent clones and different controls. All analyses were performed blinded to avoid bias. For the rescue experiments, 10 ng/mL IGF1 (Peprotech) or 0.5 µM hyperforin was added to neuronal cultures for 2 weeks.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed following the manufacturer’s protocol using a ChIP assay kit (Active Motif). The antibodies used were anti-MeCP2 and IgG (both from Upstate). We validated our antibody conditions for the ChIP assay with a previously characterized MeCP2 target, brain-derived neurotrophic factor (*Bdnf*) promoter in exon IV, and a negative region in another region of the promoter region as previously described. The input was 5% for all samples. All ChIP assays were controlled by performing parallel experiments with either no antibody or with anti-IgG pull downs. After IP, the recovered chromatin fragments were subjected to qPCR using primers for the human TRPC6 promoter region. The primers used for human TRPC6 promoter ChIP were as follows: forward primer 1, 5’AACAGCTTGGAAACGTGGGA3’; reverse primer 1, 5’AAAGAGGCCAACAACCTGCT3’; forward primer 2, 5’TCGCAGTGACGGAAGGAAAA3’; and reverse primer 2, 5’AAACGCCCAGATGTCCAGT3’. The qPCR values were normalized to the IgG precipitation and are shown as fold enrichment. All experiments were performed in
Construction and characterization of retroviruses

Self-inactivating murine oncoretroviruses were engineered to express short-hairpin RNAs (shRNAs) under the control of the U6 promoter and green fluorescent protein (GFP) or the Discosoma sp. red fluorescent protein DsRed under the control of the Ef1 alpha promoter. shRNAs against TRPC6 and a non-silencing scrambled control shRNA were cloned into retroviral vectors as previously described. The following shRNA sequences were selected and cloned into retroviral vectors:

- shRNA-control, 5'-TTCTCCGAACGTGTCACGT-3';
- shRNA-TRPC6-1, 5'-TCGAGGACCACGACATACAT-3'; and
- shRNA-TRPC6-3, 5'-CTCAGAAGATTATCATT-3'.

For rescue experiments, a resistant form of murine TRPC6 (TRPC6-WT<sup>R</sup>) was engineered to harbor six silent mutations in the region targeted by shRNA-TRPC6-1. The TRPC6 targeting sequence was mutated from AA TCG A GA CCA GCA TAC ATG to AA C GC GGC CCT GCT TAT ATG by site-directed mutagenesis. The resistant form of TRPC6 was cloned into a retroviral vector driven by the ubiquitin promoter followed by a bicistronic expression of GFP and a WPRE stabilization sequence. The specificity and efficiency of shRNA-control, shRNA-TRPC6-1, shRNA-TRPC6-3, and the TrpC6-WT constructs were verified by co-transfection into HEK-293 cells. Cell lysates were collected and analyzed by western blot analysis with anti-TRPC6 antibodies (Sigma).

Primary hippocampal cultures

Hippocampal neuronal cultures were prepared from C57BL/6 E18 embryonic mice. Briefly, hippocampi were dissected, dissociated with trypsin, and plated at a
density of 300 cells/mm² on glass coverslips coated with poly-L-lysine and laminin. The hippocampal neurons were maintained in Neurobasal medium (Gibco) supplemented with B27 (Invitrogen). Neurons were treated with either shRNA scramble control or shRNA targeting TRPC6 at DIV12-14 and were fixed for further analysis at DIV21.

**In vivo stereotaxic injection of engineered retroviruses into the dentate gyrus of adult mouse hippocampus**

High titers of engineered retroviruses were produced by cotransfection of retroviral vectors and vesicular stomatitis viral envelope into the 293 GP cell line as described previously. Supernatants were collected 24 hours post transfection, filtered through 45-µm filters, and ultracentrifuged. The viral pellet was dissolved in 14 µL of PBS and stereotaxically injected into the hilus of anesthetized mice at four sites (0.5 µL per site at 0.25 µL/minute). The following coordinates were used: posterior = 2 mm from the bregma, lateral = ±1.6 mm, ventral = ±2.5 mm; posterior = 3 mm from the bregma, lateral = ±2.6 mm, ventral = ±3.2 mm. Adult C57BL/6 mice (6-8 weeks old, female) were used for the study. All procedures followed institutional guidelines.

**Immunostaining and confocal analysis**

Coronal brain sections (40 µm thick) were prepared from retrovirus-injected mice. Images of GFP⁺ cells were acquired on a META multiphoton confocal system. Neuronal positioning was analyzed by acquiring a single-section confocal image of a GFP⁺ cell body stained with DAPI and assigning it to one of the four domains as illustrated. A minimum of 10 GFP⁺ cells were randomly chosen from the each animal, and at least three animals were used for each experimental condition, as previously described. Statistical significance was determined by ANOVA. Dendritic development was analyzed
by via a three-dimensional reconstruction of the entire dendritic tree from Z-series stacks of confocal images. The images were converted to two-dimensional projections for analysis of dendritic length and branch number using NIH ImageJ software and the NeuronJ plugin, as described previously. As a measure of arborization, Sholl analysis was performed by counting the number of dendritic crossings at a series of concentric circles at 10-µm intervals from the cell body using the Sholl analysis plugin.

**Slice electrophysiology**

Mice housed under standard conditions were anesthetized at 3 weeks post-retroviral injection, and acute coronal slices were prepared as previously described. The brains were removed and placed in an ice-cold cutting solution containing the following: 110 mM choline chloride; 2.5 mM KCl; 1.3 mM KH₂PO₄; 25 mM NaHCO₃; 0.5 mM CaCl₂; 7 mM MgCl₂; 10 mM dextrose; 1.3 mM sodium ascorbate; 0.6 mM sodium pyruvate; and 5 mM kynurenic acid. Slices were cut into 300-µm-thick sections with a vibratome (Leica VT1000S) and transferred to a chamber containing ACSF: 125 mM NaCl; 2.5 mM KCl; 1.3 mM KH₂PO₄; 25 mM NaHCO₃; 2 mM CaCl₂; 1.3 mM MgCl₂; 1.3 mM sodium ascorbate; 0.6 mM sodium pyruvate; and 10 mM dextrose (pH 7.4, 320 mOsm), saturated with 95% O₂, 5% CO₂ at 35°C for 20 minutes. The slices were then maintained at room temperature for at least 45 minutes prior to placement in the recording chamber. The slices were maintained at room temperature and used for the following 4 hours. Electrophysiological recordings were performed at 34°C. Microelectrodes (4–6 MΩ) were filled with a solution containing the following: 120 mM potassium gluconate; 15 mM KCl; 4 mM MgCl₂; 0.1 mM EGTA; 10.0 mM HEPES; 4 mM MgATP; 0.3 mM Na₃GTP; and 7 mM phosphocreatine (pH 7.4, 300 mOsm). The whole-cell patch-clamp configuration was used in the current-clamp mode. Approximately 10-
20 giga-ohm seals were obtained with borosilicate glass microelectrodes. The electrophysiological recordings were obtained at 32-34°C. Neurons and dendrites were visualized through differential interference contrast microscopy. The data were collected using an Axon Instruments 200B amplifier and acquired via a Digidata 1322A at 10 kHz.

Electrophysiology recordings using cultured human iPSC-derived neurons

Whole-cell patch clamp recordings were performed using cells cultured in the absence of astrocytes after approximately 6 weeks of differentiation. Before the recordings, the growth media were removed and replaced with a bath solution comprising the following: 130 mM NaCl; 3 mM KCl; 1 mM CaCl\textsubscript{2}; 1 mM MgCl\textsubscript{2}; 10 mM HEPES; and 10 mM glucose (pH 7.4) at room temperature (22-24°C). The electrodes for whole-cell recordings were pulled on a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument, Novato, CA) from filamented borosilicate capillary glass (1.2 mm OD, 0.69 mm ID, World Precision Instruments, Sarasota, FL). The electrodes were fire polished, and the resistance values were typically 2–5 MΩ for the voltage-clamp experiments and 7–9 MΩ for the current-clamp experiments. The pipette solution contained the following: 138 mM KCl; 0.2 mM CaCl\textsubscript{2}; 1 mM MgCl\textsubscript{2}; 10 mM HEPES (Na\textsuperscript{+} salt); and 10 mM EGTA, (pH 7.4). The osmolarity of all solutions was adjusted to 290 mOsM. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), with the exception of MgCl\textsubscript{2} (J.T. Baker, Phillipsburg, NJ). Current traces in voltage clamp were leak-subtracted. Liquid junction potentials were nulled for each individual cell with the Axopatch 1C amplifier (Molecular Devises, Sunnyvale, CA). The analyses were performed in a double-blinded manner to avoid bias.

Behavioral tests in mice
The three-chamber test was used to evaluate the social behavior of TRPC6 wild type (WT), heterozygous (HET) and knockout (KO) mice. To evaluate repetitive behavior, the mice were initially observed for 10 minutes in the dark, and the time spent in grooming and freezing behavior was measured. After 5 minutes of habituation under a light condition, a small cage with a never-met animal was introduced to one side of the box, and an empty cage was introduced to the other side. The time spent in each chamber and the time spent during nose-to-nose interaction between the animals was measured. Adult mice (6-8 weeks old, male) with a C57BL/6 background were used for the study. At least 12 animals per group were utilized as biological replicates. The experimenter was blind to the genotypes. The data were analyzed using the non-parametric Kruskal-Wallis ANOVA. The analyses were performed in a double-blinded manner. All procedures followed institutional guidelines.

**Mutation screening of TRPC6**

**Cohorts:** The clinical characteristics of the Simons Simplex Collection (SSC) have previously been described in detail. The following exclusion criteria were used to filter the cases: 1) ineligible/ancillary status as per SSC Family Distribution List v13; 2) missing genotyping data; 3) genotyping call rate < 95%; 4) discrepancy of genotyping data with recorded gender; 5) Mendelian inconsistencies or cryptic relatedness (up to and including second-degree relatives); and 6) non-European ancestry. A total of 1041 of 1195 cases were included in the final case cohort. The National Institute of Neurological Disorders and Stroke (NINDS) Neurologically Normal Caucasian Control Panel of unrelated adult controls do not have a personal or family history (first-degree relative) of neuropsychiatric illness (http://ccr.coriell.org/Sections/Collections/NINDS/DNAPanels.aspx?Pgld=195&coll=ND).
Of the 953 samples from the DNA panels NDPT020, 079, 082, 084, 090, 093, 094, 095, 096, 098, and 099, 942 passed the quality control checks described above. Additional sequence data for TRPC6 were derived from unrelated northern European (NE) adults present in an exome-sequencing database in our laboratory. Genotyping and whole-exome data were obtained for 2076 individuals, of which 1930 passed the quality control checks described above.

**Mutation screening:** For 1031 SSC cases and all 942 NINDS controls, amplification of the coding exons and splice sites was performed using lymphoblastoid cell line-derived genomic DNA via multiplex PCR using RainDance technology (Table 4.3; Lexington, MA, USA). The resulting PCR products were subjected to high-throughput sequencing on a Genome Analyzer IIx (Illumina, San Diego, CA, USA) at the Yale Center for Genomic Analysis. An in-house script was used to generate a list of variants (see Supplementary Materials for more details). Whole-exome data for 10 additional SSC cases were available and filtered for nonsynonymous singleton variants with a SAMtools SNP quality score $>50$. Variant confirmation was performed on blood-derived genomic DNA for the cases because it was available and on lymphoblastoid cell line-derived genomic DNA for NINDS controls using conventional PCR and Sanger sequencing. Segregation analysis was performed on blood-derived genomic DNA for cases for which family members were available. Chromatograms were aligned and analyzed for variants using the Sequencher v4.9 program (Gene Codes, Ann Arbor, MI, USA). For the NE controls, whole-exome sequencing data were filtered by the same parameters used for the 10 SSC cases: nonsynonymous singleton variants with a SAMtools SNP quality score $>50$. No read threshold was used to maximize sensitivity over specificity. These variants were not confirmed by Sanger sequencing, but the filtering parameters typically lead to a 70% confirmation rate in our experience. Therefore, we
have included the maximum possible number of variants from the NE control cohort. To obtain the exome data, genomic DNA from both the 10 SSC probands and 1930 NE controls was enriched for exonic sequences using NimbleGen capture and sequenced by the Illumina Genome Analyzer IIX or HiSeq2000. The novelty and singleton status of all variants were determined by comparing all three cohorts and screening dbSNP137 and Exome Variant Server v.0.0.15 (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA, URL: http://evs.gs.washington.edu/EVS/), accessed 11/01/2012. All \( p \) values for mutation burden are two-tailed and calculated from Fisher’s exact test.

**Multiplex PCR**

Lymphoblastoid cell line-derived genomic DNA was quantitated using PicoGreen dye (Invitrogen, Carlsbad, CA, USA) on a Synergy HT fluorometer (BioTek, Winooski, VT, USA). DNAs were then pooled by case/control status, 500ng/individual, such that all pooled samples were 8 cases or 8 controls for a total of 4\( \mu \)g input DNA. Pooled samples were sheared on a Covaris S2 to approximately 3kb (Covaris, Woburn, MA, USA), then cleaned by Qiagen Min-Elute columns (Qiagen GmbH, Hilden, Germany) with minor modification of the protocol (10\( \mu \)L 3M\( \text{NaC}_2\text{H}_3\text{O}_2 \) was added to the 5:1 PB:sample mix to facilitate proper pH-driven DNA binding and the mix was left on the membrane for 3 minutes before spinning). Samples were dry spun after the PE wash for 2 minutes and eluted with 9.0\( \mu \)L EB buffer to generate a final volume of 7.7\( \mu \)L, the input volume of DNA for the RDT1000. Successful shears were determined with the DNA7500 protocol by an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

The sheared genomic DNA pools were combined with RainDance microemulsion PCR master mix prepared according to the protocol. The microemulsion droplet merges were run on the RDT1000 machine (Raindance Technologies, Lexington, MA, USA). All
merges were at least 85% efficient (85% of PCR master mix droplets merged successfully 1:1 with a library primer pair droplet); if not, new DNA pools were sheared and the merge was redone to at least 85% efficiency (considered the threshold for "very good" by RainDance support staff). Successful merges were amplified under the following conditions:

<table>
<thead>
<tr>
<th>Step</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94.0°C, 2 min</td>
</tr>
<tr>
<td>55 cycles: Denaturation</td>
<td>94.0°C, 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>54.0°C, 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>68.0°C, 60 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>68.0°C, 10 min</td>
</tr>
</tbody>
</table>

Samples were then purified on Qiagen MinElute columns with 17µL EB buffer. Eluted product was run on an Agilent Bioanalyzer according to the DNA1000 protocol to determine successful amplification.

PCR product was brought to a volume of 19µL with the Tris-EDTA buffer. 2.5µL blunting buffer, 2.5µL 1mM dNTPs, and 1µL blunting enzyme were added (NEB, Ipswich, MA, USA). This reaction mix was incubated at 22°C for 15 minutes to blunt, 70°C for 5 minutes to inactivate the enzyme, and subsequently held at 4°C. Directly after blunting and without cleanup, the PCR products were concatenated into longer DNA fragments; this step was necessary since the range of amplicon sizes in the microemulsion library makes sequencing uniform fragment lengths impossible without first concatenating and then shearing. Concatenation was performed by adding 25µL NEB Quick Ligase buffer and 5µL NEB Quick Ligase, mixing thoroughly by pipetting, and transferring to a thermal cycler holding at 22°C for at least 24 hours. An additional 3µL of Quick Ligase was added, the samples were mixed again, and incubated at 37°C for 1 hour and held at 4°C.
Concatenated samples were sheared on a Covaris S2 to a mean size of ~200bp and subsequently processed according to the Illumina multiplexed library preparation protocol. Samples were quantitated on an Agilent Bioanalyzer 2100 (DNA1000 protocol). They were barcoded using Illumina’s standard protocol with the barcodes randomly allocated to pools of cases and controls.

**High-throughput sequencing**

Two barcoded pools (one of cases, one of controls) were combined in a 1:1 equimolar ratio (quantitated and size-evaluated using the Agilent Bioanalyzer DNA 1000 protocol) and submitted for high-throughput sequencing on a single lane of an Illumina Genome Analyzer IIx (GAIIx). 75bp single-end reads were generated according to the standard GAIIx protocol; a Phi-X control was run on lane one of each flowcell to ensure accurate and consistent base calls. The data were run through Illumina’s Cassava pipeline to generate FASTQ files.

**Alignment and SAMtools conversion**

Rescaled FASTQ format data were aligned to unmasked human genome build 18 (NCBI 36) using the Burrows-Wheeler Aligner (BWA) with the default settings using the following command: bwa aln -t 8 ‘BWA_reference’ ‘Fastq_input’ > ‘Output.sai’. Aligned reads were converted to SAMtools format using the following command: bwa samse ‘BWA_reference’ ‘Output.sai’ ‘Fastq_input’ > ‘Output.sam’.

**Trimming read ends**
Analysis of the error rates per base pair for each position within the 75bp read revealed a higher error rate at the start and at the end of the aligned reads than is seen for conventional sequencing. This is likely due to the concatenation and shearing step and reflects reads that cross the boundary between two amplicons. This explanation is also consistent with the low percentage of reads that align to the genome (89% in this experiment compared to 98% seen in whole-exome sequencing). Since the detection of variants is sensitive to variation in error rate, the 75bp aligned reads were trimmed using an in-house script to remove the first three base pairs and the last eight base pairs in each read resulting in a 64bp read. The SAM CIGAR string was modified accordingly.

Filtering to target

The aligned reads were filtered to remove reads outside the target amplicons using an in-house script. If any read overlapped at least 1bp of a target amplicon then the read was considered ‘on-target’. The total target was 501,959bp of non-overlapping amplicons (not including primers) of which 230,697bp were regions of interest within the amplicons (Table 4.8).

Pileup conversion

The filtered aligned data was converted to a sorted binary format (BAM) using SAMtools on the default settings. The following command was used: samtools view -bSt ‘SAM_reference’ ‘Input.sam’ | samtools sort – ‘Output.bam’. The aligned and filtered SAM file was then converted to pileup format using SAMtools with the default settings: samtools pileup -cAf ‘Reference’ -t ‘SAM_reference’ ‘Input.sam’ > ‘Output.pileup’.
Variant detection

To determine the optimal thresholds for variant detection within a pool of 8 samples the accuracy of detection was compared with genotyping data (Illumina 1Mv1 BeadArray, 166 SNPs) and Sanger sequencing of the gene PCLO (15,266bp). The PHRED-like score of bases predicting a variant were seen to follow a bimodal distribution with the data clustered below a score of 10 and above a score of 20. Accordingly, a threshold of 20 was set. The other threshold considered was the frequency of reads representing the variant allele; since the data represented pools of 8 individuals the expected frequency of reads representing a rare heterozygous allele in a single individual was 6.25% (rather than 50% with a single individual). However, due to random variation in genomic DNA, droplet dynamics, and sequencing representation, a substantial proportion of rare heterozygous alleles will have a frequency below the expected threshold. By varying this detection threshold with a PHRED-like score of 20 the optimal detection frequency was determined to be 3.5%. At these thresholds the sensitivity was estimated at 89% for a variant present on a single allele within the pool; the observed positive predictive value was 75% giving an estimate of specificity of 99.9988%. Variants were detected using an in-house script that filtered at these thresholds and was blind to case/control status.

Variant annotation and filtering

Variants were annotated against the UCSC gene definitions to determine the effect on the resulting amino acid sequence. Where multiple isoforms were present, the most-deleterious interpretation was selected. If the specific variant was present in dbSNPv132 (converted to hg18) the variant was marked. To generate a list of variants
of interest for confirmation, variants were filtered to those at allele frequency ≤2% in the dataset and those which are missense, nonsense, or alter the 2bp splice donor/acceptor sites.

**Confirmation of variants**

Performed by a standard PCR in 25μl volume:

10 ng genomic DNA  
1X PCR PreMix D (Epicentre Biotechnologies, Madison, WI, USA)  
0.5 M betaine  
0.48 μM forward primer  
0.48 μM reverse primer  
0.36 ul Taq polymerase (synthesized in-house)  
0.072 ul PFU (synthesized in-house)

DNA was amplified in a Tetrad 2 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following cycling parameters:

Initial denaturation 95.0°C, 5 min  
40 cycles:  
Denaturation 95.0°C, 30 sec  
Annealing 54.0°C, 30 sec  
Extension 72.0°C, 60 sec  
Final extension 72.0°C, 10 min

PCR products were visualized by agarose gel electrophoresis and sent to the Yale Keck Biotechnology Resource Laboratory for Sanger sequencing. Chromatograms were aligned and analyzed for variants using the Sequencher v4.9 program (Gene Codes, Ann Arbor, MI, USA).

**Case/control population matching and quality control**

**Single nucleotide polymorphism (SNP) genotyping**

Blood-derived genomic DNA was used from SSC cases; only lymphoblastoid cell line-derived genomic DNA was readily available and used from NINDS controls. SSC cases (n=1195) were genotyped using the IlluminaHuman1M-Duo v1, Human 1M-Duo
v3, or HumanOmni2.5 BeadChips, according to the standard Illumina protocol. NINDS controls (n=953) were genotyped using the Illumina HumanOmniExpress12v1. All genotyping was performed at the Yale Keck Biotechnology Resource Laboratory.

SNP quality control

Sample genotypes were analyzed using PLINK\textsuperscript{94} and removed from the analyses if: 1) sample call rate was less than 95%, 2) genotypes were inconsistent with recorded gender, or 3) Mendelian inconsistencies or cryptic relatedness were detected by assessing inheritance by descent (IBD). The following PLINK commands were used:

- plink --bfile<Samplefile> --check-sex
- plink --bfile<Samplefile> --mendel
- plink --bfile<Samplefile> --extract <Hapmap_LD.prune.in> --mind 0.05 --geno 0.1 --maf 0.01 --hwe-all --make-bed --out <Samplefile.indep>
- plink --bfile<Samplefile.indep> --genome --min 0.05 --out <Sample.IBD.Result>

‘Hapmap_LD.prune.in’ is a pre-defined list of 129,932 independent SNPs to ensure consistency of results across samples of different sizes. This SNP list was derived from 120 Hapmap individuals with 1Mv1 Illumina data using the command:

- plink --bfileHapmapfileindep-pairwise 50 5 0.2 --out Hapmap_LD.prune.in

No instances of cryptic relatedness were detected.

Population outlier exclusions

Golden Helix SNP and Variation Suite v7.5.4 (Golden Helix, Bozeman, MT, USA) was used to perform a genotype principal component analysis (PCA) of the SSC cases
and NINDS controls, using 8117 consensus SNPs common to all array platforms and not found to be in high linkage disequilibrium. Based on visualization of a scree plot (Figure 4.10a), Eigenvalues of the first three principal components were plotted against one another (Figure 4.10b), and the interquartile range (IQR) distance around the median of the study population cluster was calculated. A threshold that included all NINDS controls was determined to lie at 6 IQRs from the third quartile, and SSC cases beyond this threshold were excluded as ancestral outliers (Figure 4.10c) for calculation of mutation burden. PCA of genotyping data using Eigenstrat of the 1930 neuropsychiatrically unscreened NE controls used in the omnibus analysis revealed that these controls cluster tightly with the HapMap CEU cohort.

**Acknowledgments**

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Chapter 4, in full, is a reprint of the material as it appears in *Molecular Psychiatry* 2014. Karina Griesi-Oliveira, Allan Acab, Abha R. Gupta, Daniele Y. Sunaga,
Thanathom Chailangkarn, Xavier Nicol, Yanelli Nunez, Michael F. Walker, John D. Murdoch, Stephan J. Sanders, Thomas V. Fernandez, Weizhen Ji, Richard P. Lifton, Estevão Vadasz, Alexander Dietrich, Dennis Pradhan, Hongjun Song, Guo-li Ming, Xiang Gu, Gabriel Haddad, Maria C. N. Marchetto, Nicholas Spitzer, Maria Rita Passos-Bueno, Matthew W. State and Alysson R. Muotri. The dissertation author was the primary investigator and author of this paper.
Figure 4.1. Mapping the breakpoints in the ASD individual with the 46, XY, t(3;11)(p21;q22) karyotype.

(a) The allele frequency distribution plot for chromosomes 3 and 11 generated by SNP array genotyping showed no gain or loss of genetic material on these chromosomes. (b) The schematic view of the BAC probes used and the surrounding breakpoint area on chromosome 3. RP11 probes marked in red span the breakpoint, while the black ones do not. The shared region between probes RP11-780O20 and RP11-109N8 narrows the breakpoint area to a region inside the VPRBP gene. The blue arrows indicate open reading frames. (c) FISH imaging showing that RP11-780O20 probe (red signal) binds to normal and derivative chromosome 3 and to derivative chromosome 11, indicating that the probe spans the breakpoint (arrows). (d) A schematic view of the BAC probes used and the surrounding areas on chromosome 11. A shared region between probes RP11-153K15 and RP11-141E21 places the breakpoint in TRPC6. (e) FISH image showing the BAC probe RP11-153K15 (green signal) bound to normal chromosome 11 and both derivatives chromosomes 3 and 11 (arrows).
Figure 4.2. TRPC6 channels regulate the expression of neuronal development genes.

(a) Differential gene expression in the controls and ASD individual cells of candidate genes located in the translocation region or detected by exome sequencing. Only TRPC6 displayed a significant reduction in mRNA levels ($P<0.01$). (b) Decreasing expression of candidate genes upon TRPC6 stimulation with hyperforin/FFA. (c) Genes upregulated in the TRPC6-mut genetic background after hyperforin/FFA treatment. (d) Representative western blot showing increased CREB phosphorylation after 15 and 30 minutes of hyperforin stimulation normalized to non-stimulated cells. (e) The level of CREB phosphorylation in DPCs from the TRPC6-mut individual after TRPC6 activation with hyperforin is significantly lower compared with the control sample ($n = 3$, $P< 0.05$; t-test). The error bars in all panels show the s.e.m. *$P<0.05$; **$P<0.01$; ***$P<0.001$. 
Figure 4.3. Derivation of NPCs and neurons from iPSCs.

(a) Representative images depicting morphological changes during neuronal differentiation from control and TRPC6-mutant iPSCs. Bar = 100 µm. (b) NPCs are positive for the neural precursor markers Musashi-1 and Nestin. Bar = 50 µm. (c) Representative images of cells after neuronal differentiation. iPSC-derived neurons express neuronal markers such as GABA, MAP2, and synapsin I. (d) Examples of distinct cortical neuronal subtypes present differentiating cultures after 3 weeks. Bar = 30 µm. (e) We obtained 30% neurons in our cultures with this protocol, as measured by MAP2 staining and infection with the syn::EGFP lentiviral vector. Most MAP2-positive cells expressed VGLUT1, in contrast with 12% of neurons expressing GABA. Ctip2-positive neurons were more abundant (16%), whereas Tbr1-positive neurons were present in a small percentage in the population (6%) at the end of the differentiation protocol. (f) Morphology of neurons patched for electrophysiological recording. (g) Representative recordings of evoked action potentials in iPSC-derived neurons in response to current steps under current patch clamps. (h) Representative Na⁺ and K⁺ currents in iPSC-derived neurons. The error bars in all panels show the s.e.m.
Figure 4.4. Alterations in neural cells derived from the TRPC6-mutant individual.
(a) Ca\(^{2+}\) influx dynamics through TRPC6 channels activated by hyperforin plus FFA were reduced in the TRPC6-mut cells. Oscillations generated by hyperforin and FFA treatment were normalized to the fluorescence of the resting level (F\(_{0}\)), synchronized, and averaged. (b) The average peak of Ca\(^{2+}\) influx in the 100 cells analyzed was reduced by 30% in the TRPC6-mut NPCs compared with the control sample when the cells were stimulated with hyperforin and FFA (n=3; P<0.001; ANOVA). (c) Representative western blot of neurons derived from a clone of a control and a TRPC6-mutant iPSC line treated or not treated with IGF-1. (d) TRPC6-mutant neurons displayed low levels of TRPC6 and synaptic proteins Psd95, and synapsin I. IGF-1 treatment significantly increased the protein levels of TRPC6, Psd-95, and synapsin I in TRPC6-mutant neurons (n=3, P<0.01; t-test). (e) Bar graphs showing that the total length (microns) and (f) number of vertices (neuronal branch points) of TRPC6-mutant neurons is reduced compared with controls. Treatment with hyperforin or restoring TRPC6 expression levels rescued these defects (P<0.01; ANOVA). (g) Representative images of TRPC6-mutant and control neurons before and after neuronal tracing. The neuronal morphology was visualized using the syn::EGFP lentiviral vector. Bar = 50 µm. (h) Bar graphs showing that the spine density in TRPC6-mutant neurons was reduced compared with the controls and could be rescued after hyperforin treatment or restoring TRPC6 expression levels (P<0.01; ANOVA). (i) A specific shRNA against TRPC6 (shTRPC6) was used to confirm that the phenotype was caused by loss of TRPC6 function (P<0.01; ANOVA). (j) Representative images of neuronal spines in control and TRPC6-mutant neurons. (k) The bar graphs show that the number of glutamate vesicles in TRPC6-mutant neurons was significantly reduced compared to controls. IGF-1 and hyperforin treatment for 2 weeks increased the number of VGLUT1 puncta in TRPC6-mutant neurons (P<0.01; ANOVA). (l) Representative images of neurons stained for VGLUT1 and MAP2. (m) Control neurons expressing an shRNA against TRPC6 (shTRPC6) exhibited reduced numbers of VGLUT1 puncta compared with the neurons expressing a scrambled shRNA (shScramble). (n) Representative image of a control neuron expressing an shRNA against TRPC6. Bar = 5 µm. (o) The bar graphs show the number of synaptic puncta, as measured by synapsin I staining. Synaptic puncta counts in TRPC6-mutant neurons were reduced compared to controls. TRPC6-cDNA treatment of TRPC6-mut neurons was sufficient to increase synapses to control levels (P<0.01; n=20; ANOVA). (p) Representative image of TRPC6-mut neurons with empty vector and with vector expressing wild-type TRPC6 stained for MAP2 and synapsin I. Bar = 5µm. (q) The whole-cell Na\(^{+}\) current of TRPC6-mutant neurons was significantly less than that of the control (P<0.01; ANOVA). (r) The Na\(^{+}\) current density of TRPC6-mutant neurons was also significantly less than that of the control (P<0.01; ANOVA). (s) TRPC6 protein levels were reduced in neurons derived from an RTT iPSC clone expressing a non-functional version of MeCP2 compared with an isogenic control expressing the functional MECP2 gene. (t) Recruitment of MeCP2 on the TRPC6 promoter region by ChIP. Extracts of formaldehyde-fixed neurons were precipitated with a MeCP2 antibody and analyzed by quantitative PCR using two distinct primers for the TRPC6 promoter. The data show enrichment over the IgG control precipitation. The primers for the BDNF promoter were used as controls. The numbers of neurons analyzed (n) are shown within the bars in graphs. The error bars in all panels show the s.e.m. For the iPSC clones used in each experiment, refer to Table 4.5. *P<0.05; **P<0.01; ***P<0.001.
Figure 4.5. TRPC6 regulates the neural development of adult-born neurons in the dentate gyrus of the hippocampus.

(a) Mouse primary hippocampal neurons revealed reduced spine density in neurons treated with shRNA targeting TRPC6 compared to neurons treated with shRNA scramble control ($P<0.01$; $n=14$; t-test). (b) Mouse primary hippocampal neurons demonstrated reduced synaptic puncta numbers in neurons treated with shRNA targeting TRPC6 compared to neurons treated with shRNA scramble control ($P<0.01$; $n=15$; t-test). Synaptic puncta were labeled using synapsin I antibodies and counted along MAP2+ neuronal dendrites. (c) Representative confocal images of neurons expressing shRNA-control and shRNA-TRPC6-1 at 28 dpi (days post retroviral injection). Green, GFP; blue, DAPI. Bar = 50 mm. Also shown are the divided areas of the dentate gyrus: 1, inner granule cell layer; 2, middle granule cell layer; 3, upper granule cell layer; and 4, molecular layer. (d) Summary of cell body localization of GFP+ newborn neurons under different experimental conditions at 28 dpi. The cell migration phenotype was rescued by expression of TRPC6-WT$^+$ at 14 dpi. Retroviruses co-expressing GFP and TrpC6-WT$^+$ were co-injected with retroviruses co-expressing dsRed and shRNA-TRPC6-1 into the adult mouse dentate gyri. The cell body localization of the GFP$, DsRed$, and GFP$^+$DsRed$^+$ neurons are quantified. The values represent the mean ± s.e.m. (n = 3; $P<0.01$; ANOVA). (e) A 3-dimensional confocal reconstruction of dendritic trees of GFP+ dentate granule cells expressing shRNA-control or shRNA-TRPC6-1 at 14 dpi. Scale bar, 20 mm. (f) Sholl analysis of the dendritic complexity of GFP+ neurons at 14 dpi. Number of crossings refers to the number of dendrites intersecting concentric circles spaced 10µm apart starting from the cell body. The error bars in all panels show the s.e.m. (n = 3; $P<0.05$; ANOVA). (g) A sample DIC image of a newborn neuron patched in whole-cell configuration in an acute slice of the hippocampus. (h) The firing rate of repetitive action potentials of GFP+ neurons under current clamp in response to 1-s current injection steps at 21 dpi. Shown on the left is a sample trace of a GFP+ neuron expressing shRNA-control; a GFP+ neuron expressing shRNA-TRPC6#1 is shown on the right. (i) Summary of the mean firing rate of newborn neurons. The values represent the mean ± s.d. (n = 3; $P<0.01$; ANOVA). A minimum of 10 GFP+ cells was randomly picked from each animal, and at least three animals (n) under each experimental condition were used. (j) Behavioral analysis of Trpc6 KO and HET mice. The mean body weight and defecation and urination episodes during the test revealed no physiological differences between the wild type (WT), heterozygote (HET), and knockout (KO) Trpc6 animals. Evaluation of time spent in freezing behavior and in grooming behavior revealed no significant differences between the groups. Social interaction was assessed by evaluating the time spent with a novel object or in nose-to-nose contact with a strange animal. Adult mice (6-8 weeks old, male) in a C57BL/6 background were used for the study. At least 12 animals per group were utilized in biological replicates. The experimenter was blind to the genotypes. The data were analyzed using non-parametric Kruskal-Wallis ANOVA. The error bars in all panels show the s.e.m. All procedures followed institutional guidelines. *$P<0.05$; **$P<0.01$; ***$P<0.001$. 
Figure 4.6. Confirmation of TRPC6 disruption and gene expression dysregulation in TRPC6-mutant cells.

(a) qPCR data showing the levels of expression of TRPC6 exons 6, 12 and 13 relative to exon 4 for the ASD individual, parents and mean of 6 controls. The ASD individual is the only one that has a decrease of more than 50% on the levels of expression of exons 12 and 13. (b) Genotyping of rs12366144 SNP in TRPC6 exon 6 (left) and rs12805398 SNP in exon 13 (right). The control sample maintains heterozygosis for both SNPs at the transcriptional level (arrows). On the other hand, the ASD individual does not present one of the alleles in exon 13 when the cDNA is sequenced, indicating that TRPC6 is transcribed until the breakpoint on the disrupted chromosome. (c) Examples of microsatellite genotyping for parenthood confirmation. (d) Western blot with total cellular protein extracts from a control individual and the TRPC6 mutant individual. Full length TRPC6 protein (~109Kb) is indicated. No evidence for a truncated protein is present when using a N-terminal antibody. (e) Differential expression of SEMA3A, CLDN11, EPHA4, INA and MAP2 genes in DPCs of TRPC6-mut individual and controls after hyperforin treatment over time.
Figure 4.7. Generation and characterization of iPSCs.

(a) Cells emerging from the dental pulp. (b) Established dental pulp cells lineage. (c) iPSC colony emerging from the co-culture system with mEFs. (d) Isolated iPSC colony. (e) iPSC colony stained for pluripotency markers SOX2 and Lin28. (f) iPSC colony stained for pluripotency markers TRA-1-81 and Nanog. Bar = 100 µm. (g) Karyotypes of TRPC6-mut iPSCs and WT iPSCs showing the stable karyotype of these cells after more than 20 passages. Arrows point to the de novo translocation between chromosomes 3 and 11. (h) Hematoxin-eosin stained slices of the teratomas obtained after iPSC injection in nude mice. The presence of tissues containing the three different embryonic layers indicates that the DPCs were fully reprogrammed to a pluripotent state. Bar = 250 µm. (i) Pearson’s correlation coefficients of microarray profiles of triplicate WT DPC, TRPC6-mut DPC, WT-iPSC clone 1, TRPC6-mut iPSC clones 4 and 6 and the hESC line HUES6. Color bar indicates the level of correlation (from 0 to 1), with color bar reporting log2 normalized expression values (red/blue indicates high/low relative expression). (j) Hierarchical cluster obtained from expression microarray data: the iPSCs lineages obtained clustered with hESCs, indicating that these cells have a similar expression profile, while DPCs lineages clustered all together in a separate group. Three different clones from the ASD individual were used for microarray analysis and all the samples were run in triplicate.
Figure 4.8. Electrophysiological recordings and morphological phenotypes of iPSC-derived cortical neurons.

(a) Expression of cell-type specific markers. mRNA expression level of several cell-type specific markers was assessed by RT-qPCR in iPSCs, NPCs, and iPSC-derived TRPC6-mut and control mixed neuronal cultures (n=3 for each). mRNA expression was normalized to GAPDH. Pluripotent marker: OCT4. NPC marker: NESTIN. Neuronal markers: NEUN, MAP2, CTIP2, TBR1, SYN1, VGLUT1, ABAT. Glial markers: S100ß, GFAP. (b) Images of iPSC-derived neurons immunostained with antibodies against MAP2, showing adjacent localization of the pre-synaptic marker synapsin (SYN) and the post-synaptic marker (HOMER1). Scale bar = 5um. (c) Representative recordings of Na\(^+\) current from an iPSC-derived neuron that was blocked by 10 µM Tetrodotoxin (TTX). (d) K\(^+\) current was blocked using 20 mM tetraethylammonium (TEA). (e) Representative traces of Na\(^+\) currents from control and TRPC6-mut neurons. 16 different voltage steps from -70 to +80 mV were used to evoke Na\(^+\) currents. The TRPC6-mut neurons demonstrate a reduced downward trace, indicating smaller Na\(^+\) currents compared to control neurons.
Figure 4.9. TRPC6-mut neural cell analysis, MeCP2 promoter occupation, and shRNA validation.

(a) NPC cell cycle analysis indicated that there was no significant difference in the percentage of cells in the G1, S, and G2 phases of the cell cycle between the ASD individual and control (n = 3 for each clone analyzed; ANOVA). (b) Gene expression in the TRPC6-mut genetic background after 0.5µM hyperforin treatment in NPCs. EPH4A shows no significant changes after treatment, whereas SEMA3A expression is reduced 0.6-fold. CLDN11, INA, and MAP2 expression are significantly increased after treatment (2.7-, 2.8-, and 1.8-fold, respectively) (n=3; ANOVA). (c) Western blot showing 293T cells before and after transduction with lentivirus expressing the shTRPC6 or TRPC6 cDNA used in human neuron experiments. Bar graph shows protein quantification with a 0.3-fold decrease after shTRPC6 treatment and a 2.2-fold change increase after TRPC6 cDNA treatment. (d) Bar graphs show that spine density in TRPC6-mut neurons (F2749-1; black bars) is reduced compared to controls. (e) Bar graphs show that the number of glutamate vesicles (measured by VGLUT1 puncta along MAP2-positive processes) in TRPC6-mut neurons (black bars) is significantly reduced compared to several controls. WT iPSC-derived controls neurons (white bars) were previously described in Marchetto et al, 2010. (f) A schematic view of the TRPC6 promoter region showing the primers used for ChIP. (g) Validation of the efficacy of shRNAs against TRPC6 in vitro. The retroviral constructs expressing different shRNAs were co-transfected with an expression construct for TRPC6 into HEK-293 cells. The lysates were subjected to Western blot analysis for TRPC6 and GAPDH (a sample image is shown at top). Also shown is the knockdown efficacy quantification. The densitometry measurement was first normalized to GAPDH and then to the shRNA-control expression sample. The values represent the mean ± S.D. (n = 3; P<0.01; ANOVA). (h) Western analysis of TRPC6 protein levels under different conditions. Retroviral constructs expressing different shRNAs were co-transfected with an expression construct for an shRNA-resistant form of TRPC6-WT (TRPC6-WT<sup>R</sup>) into HEK-293 cells. The lysates were subjected to Western blot analysis of TRPC6 and GAPDH expression. Data shown as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.
Figure 4.10. Principal component analysis and population stratification plots.

(a) Principal component analysis. Scree plot of the first 200 components identifies the first three as contributing the greatest amount of variation. (b) Population stratification control plots. The three largest principal components of genotypes for SSC cases (green) and NINDS controls (blue) were plotted against one another (PC = principal component, EV = Eigenvalue). (c) Removal of ancestral outliers. The interquartile range (IQR) distance around the median of the study population cluster was calculated. A threshold that included all the NINDS controls was determined to lie at 6 IQRs from the third quartile, and SSC cases beyond this threshold were excluded as ancestral outliers. Included samples are in blue, and excluded samples (outliers) are in green.
Table 4.1. Selected functionally relevant genes differentially expressed between the TRPC6-mutant individual and controls.

<table>
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<tr>
<th>Gene</th>
<th>Fold change*</th>
<th>Gene Ontology</th>
<th>Regulation by CREB**</th>
<th>qPCR validation (p value)</th>
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<td>INA</td>
<td>-2.639988194</td>
<td>Nervous system development; neurofilament cytoskeleton organization</td>
<td>ChIP-on-chip</td>
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<td>MAP2</td>
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<tr>
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* Logarithmic gene expression difference between ASD individual and controls

** Evidence of gene transcription regulation by the transcription factor CREB according to the database http://natural.salk.edu/CREB/search.htm. Zhang and colleagues (2005) used three different strategies to identify the genes regulated by CREB: *in silico* analysis, chromatin co-immunoprecipitation followed by microarray analysis (ChIP-on-ChIP) and expression analysis of genes induced by forskolin (array). The genes for which no evidence of CREB regulation was found are annotated as "no evidence"; those for which no information is available in the analyzed database are annotated as "no data available".
Table 4.2. Sequence of the primers used in qPCR experiments.

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Table 4.3. PCR primers covering all coding regions of *TRPC6*, designed by RainDance.

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Table 4.4. Rare de novo SNVs and frameshift mutations found in TRPC6-mutant individual.

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Table 4.4. Rare de novo SNVs and frameshift mutations found in TRPC6-mutant individual, continued.

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<td>T</td>
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<td>rs34889393</td>
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<td>rs34059106</td>
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<tr>
<td>ATXN3</td>
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<td>5392537853</td>
<td>G</td>
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<tr>
<td>CHST15</td>
<td>NM_014863:c.1366delC:p.Q455V</td>
<td>Frame</td>
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<td>chr10</td>
<td>125780583</td>
<td>125780583</td>
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<td>chr4</td>
<td>140811084</td>
<td>140811084</td>
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Table 4.5. Differentially expressed genes between TRPC6-mutant individual and controls.

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Name</th>
<th>Regulation by CREB**</th>
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<tbody>
<tr>
<td>RGS4</td>
<td>-3.424461441</td>
<td>regulator of G-protein signaling 4</td>
<td>In silico</td>
</tr>
<tr>
<td>PRRX1</td>
<td>-2.374475207</td>
<td>paired related homeobox 1</td>
<td>In silico; arrays</td>
</tr>
<tr>
<td>PTGS2</td>
<td>-3.49316255</td>
<td>prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)</td>
<td>ChIP-on-chip</td>
</tr>
<tr>
<td>INA</td>
<td>-2.639988194</td>
<td>regulator of G-protein signaling 4</td>
<td>In silico</td>
</tr>
<tr>
<td>ITGA7</td>
<td>-3.173058661</td>
<td>alpha interneuron intermediate filament protein, alpha</td>
<td>ChIP-on-chip</td>
</tr>
<tr>
<td>NPTX1</td>
<td>-2.855578291</td>
<td>neuronal pentraxin 1</td>
<td>In silico</td>
</tr>
<tr>
<td>ANGPTL4</td>
<td>-3.15182498</td>
<td>angiopoietin-like 4</td>
<td>In silico</td>
</tr>
<tr>
<td>MAP2</td>
<td>-2.789671289</td>
<td>microtubule-associated protein 2</td>
<td>ChIP-on-chip</td>
</tr>
<tr>
<td>EPHA4</td>
<td>2.362428255</td>
<td>EPH receptor A4</td>
<td>ChIP-on-chip; In silico</td>
</tr>
<tr>
<td>PMEPA1</td>
<td>-3.901062426</td>
<td>prostate transmembrane protein, androgen induced 1</td>
<td>In silico</td>
</tr>
<tr>
<td>CLDN11</td>
<td>4.066602785</td>
<td>claudin 11</td>
<td>In silico</td>
</tr>
<tr>
<td>PCDH10</td>
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<td>protocadherin 10</td>
<td>ChIP-on-chip; In silico</td>
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<tr>
<td>ESM1</td>
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<td>endothelial cell-specific molecule 1</td>
<td>In silico</td>
</tr>
<tr>
<td>DSP</td>
<td>-3.879025353</td>
<td>desmoplakin</td>
<td>ChIP-on-chip</td>
</tr>
<tr>
<td>FLNC</td>
<td>-2.328384225</td>
<td>filamin C, gamma</td>
<td>in silico</td>
</tr>
<tr>
<td>CLDN1</td>
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<td>claudin 1</td>
<td>In silico</td>
</tr>
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<td>SEMA3A</td>
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<td>semaphorin 3A</td>
<td>No evidence</td>
</tr>
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<td>CDH6</td>
<td>-2.67546301</td>
<td>cadherin 6, type 2, K-cadherin (fetal kidney)</td>
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</tr>
<tr>
<td>CFH</td>
<td>3.295805</td>
<td>complement factor H</td>
<td>No evidence</td>
</tr>
<tr>
<td>CTSK</td>
<td>2.538857806</td>
<td>cathepsin K</td>
<td>No evidence</td>
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<tr>
<td>TNFSF18</td>
<td>2.364541617</td>
<td>tumor necrosis factor (ligand) superfamily, member 18</td>
<td>No evidence</td>
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<tr>
<td>IFIT1</td>
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<td>IGF2</td>
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<td>insulin-like growth factor 2 (somatomedin A)</td>
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<tr>
<td>CASP1</td>
<td>2.545250054</td>
<td>caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)</td>
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<tr>
<td>CRYAB</td>
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<td>crystallin, alpha B</td>
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</tr>
<tr>
<td>PDE3A</td>
<td>-3.350362985</td>
<td>phosphodiesterase 3A, cGMP-inhibited</td>
<td>No evidence</td>
</tr>
<tr>
<td>LUM</td>
<td>2.412407948</td>
<td>lumican</td>
<td>No evidence</td>
</tr>
<tr>
<td>EPSTI1</td>
<td>3.159449161</td>
<td>epithelial stromal interaction 1 (breast)</td>
<td>No evidence</td>
</tr>
<tr>
<td>LTB2P2</td>
<td>-2.717385789</td>
<td>latent transforming growth factor beta binding protein 2</td>
<td>No evidence</td>
</tr>
<tr>
<td>VCAM1</td>
<td>4.546975557</td>
<td>vascular cell adhesion molecule 1</td>
<td>No evidence</td>
</tr>
<tr>
<td>ACAN</td>
<td>-4.199956627</td>
<td>aggrecan</td>
<td>No evidence</td>
</tr>
<tr>
<td>CCL2</td>
<td>2.41655874</td>
<td>chemokine (C-C motif) ligand 2</td>
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</tr>
<tr>
<td>RAB27B</td>
<td>2.89104252</td>
<td>RAB27B, member RAS oncogene family</td>
<td>No evidence</td>
</tr>
</tbody>
</table>
Table 4.5. Differentially expressed genes between TRPC6-mutant individual and controls, continued.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change*</th>
<th>Name</th>
<th>Regulation by CREB**</th>
</tr>
</thead>
<tbody>
<tr>
<td>KYNU</td>
<td>3.411663783</td>
<td>kynureninase (L-kynurenine hydrolase)</td>
<td>No evidence</td>
</tr>
<tr>
<td>EFEMP1</td>
<td>2.641250593</td>
<td>EGF-containing fibulin-like extracellular matrix protein 1</td>
<td>No evidence</td>
</tr>
<tr>
<td>MX2</td>
<td>2.490495298</td>
<td>myxovirus (influenza virus) resistance 2 (mouse)</td>
<td>No evidence</td>
</tr>
<tr>
<td>MX1</td>
<td>2.63120465</td>
<td>myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)</td>
<td>No evidence</td>
</tr>
<tr>
<td>MME</td>
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<td>membrane metallo-endopeptidase</td>
<td>No evidence</td>
</tr>
<tr>
<td>PTX3</td>
<td>3.998647561</td>
<td>pentraxin-related gene, rapidly induced by IL-1 beta</td>
<td>No evidence</td>
</tr>
<tr>
<td>PCOLCE2</td>
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<td>procollagen C-endopeptidase enhancer 2</td>
<td>No evidence</td>
</tr>
<tr>
<td>TLR3</td>
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<td>toll-like receptor 3</td>
<td>No evidence</td>
</tr>
<tr>
<td>SULT1B1</td>
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</tr>
<tr>
<td>PLAC8</td>
<td>2.728492911</td>
<td>placenta-specific 8</td>
<td>No evidence</td>
</tr>
<tr>
<td>ASB5</td>
<td>2.862047519</td>
<td>ankryin repeat and SOCS box-containing 5</td>
<td>No evidence</td>
</tr>
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<td>A kinase (PRKA) anchor protein 12</td>
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<tr>
<td>AEBP1</td>
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<td>AE binding protein 1</td>
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<td>LRRC17</td>
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<tr>
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<td>tissue factor pathway inhibitor 2</td>
<td>No evidence</td>
</tr>
<tr>
<td>PODXL</td>
<td>-3.832320219</td>
<td>podocalyxin-like</td>
<td>No evidence</td>
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<tr>
<td>PTGS1</td>
<td>-3.737081399</td>
<td>prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)</td>
<td>No evidence</td>
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<td>IFI44L</td>
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<td>myocardin</td>
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<td>SLFN11</td>
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<td>schlafen family member 11</td>
<td>No data available</td>
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<td>SNORD35A</td>
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<td>small nucleolar RNA, C/D box 35A</td>
<td>No data available</td>
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<td>ABI family, member 3 (NESH) binding protein</td>
<td>No data available</td>
</tr>
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<td>PCDH18</td>
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<td>C4orf49</td>
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<td>C9orf150</td>
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<td>C9orf150</td>
<td>3.202042462</td>
<td>chromosome 9 open reading frame 150</td>
<td>No data available</td>
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</table>
Table 4.5. Differentially expressed genes between TRPC6-mutant individual and controls, continued.

*Logarithmic gene expression difference between TRPC6-mutant individual and controls.
**Evidences of gene transcription regulation by the transcription factor CREB according to the database http://natural.salk.edu/CREB/search.htm: Zhang and colleagues (2005) used three different strategies to identify the genes regulated by CREB: in silico analysis, chromatin co-immunoprecipitation followed by microarray analysis (ChiP-on-chip) and expression analysis of genes induced by forskolin (array). The genes for which no evidence of CREB regulation was found are annotated as "no evidence"; those for which no information is available in the adopted database are annotated as "no data available".
Table 4.6. Fingerprinting analysis of DPC and iPSC lineages from *TRPC6*-mutant individual and one control sample.

<table>
<thead>
<tr>
<th>Marker</th>
<th>TRPC6mut DPC</th>
<th>TRPC6mut iPSC</th>
<th>WT DPC (USC1)</th>
<th>WT iPSC (USC1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amelogenin</td>
<td>X Y</td>
<td>X Y</td>
<td>X Y</td>
<td>X Y</td>
</tr>
<tr>
<td>vWA</td>
<td>17 18</td>
<td>17 18</td>
<td>16 18</td>
<td>16 18</td>
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<td>D8S1179</td>
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<td>11 15</td>
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<td>TPOX</td>
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<td>8 11</td>
<td>9 12</td>
<td>9 12</td>
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<td>FGA</td>
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<tr>
<td>D3S1358</td>
<td>15 15</td>
<td>15 15</td>
<td>16 16</td>
<td>16 16</td>
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<tr>
<td>THO1</td>
<td>6 9.3</td>
<td>6 9.3</td>
<td>9 9</td>
<td>9 9</td>
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<tr>
<td>D21S11</td>
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<td>31 31.2</td>
<td>30.2 32</td>
<td>30.2 32</td>
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<tr>
<td>D18S51</td>
<td>10 14</td>
<td>10 14</td>
<td>19 19</td>
<td>19 19</td>
</tr>
<tr>
<td>Penta E</td>
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<td>5 11</td>
<td>11 12</td>
<td>11 12</td>
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<td>12 12</td>
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<td>D13S317</td>
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<td>D7S820</td>
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<td>12 13</td>
<td>11 11</td>
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<td>12 13</td>
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<tr>
<td>CSF1PO</td>
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<td>10 11</td>
<td>11 11</td>
<td>11 11</td>
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<tr>
<td>Penta D</td>
<td>9 9</td>
<td>9 9</td>
<td>9 11</td>
<td>9 11</td>
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</table>
Table 4.7. Summary of the iPSC subjects and clones (C) utilized for each experiment. Numbers represent experimental replications for each individual clone. The clones utilized in neuronal differentiation experiments were determined by availability at the end time-point.

<table>
<thead>
<tr>
<th>Study/cell line</th>
<th>F2749-1 (TRPC6-mut)</th>
<th>USC1 (control)</th>
<th>P603 (control)</th>
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<tbody>
<tr>
<td></td>
<td>C1</td>
<td>C4</td>
<td>C6</td>
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<tr>
<td>Pluripotency assays</td>
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<td>1</td>
<td>1</td>
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<td>iPSC microarray</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>NPC cell cycle</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>NPC Ca2+ influx</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Neuronal arborization</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>VGlut1 puncta</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
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<td>Electrophysiology</td>
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<td></td>
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<tr>
<td>Spine density</td>
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<td>2</td>
<td>3</td>
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Table 4.8. Quality metrics of high-throughput sequencing.

<table>
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<tr>
<th></th>
<th>Cases (mean)</th>
<th>Controls (mean)</th>
<th>Cases (std dev)</th>
<th>Controls (std dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individuals</td>
<td>1195</td>
<td>953</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Total passed reads/pool</td>
<td>19,172,412</td>
<td>17,715,906</td>
<td>4,201,928</td>
<td>4,105,181</td>
</tr>
<tr>
<td>Mean coverage/pool</td>
<td>1,793</td>
<td>1,756</td>
<td>399</td>
<td>382</td>
</tr>
<tr>
<td>% Bases at 80x coverage</td>
<td>93.54%</td>
<td>93.06%</td>
<td>3.88%</td>
<td>3.36%</td>
</tr>
<tr>
<td>% Bases on-genome</td>
<td>88.43%</td>
<td>89.37%</td>
<td>2.37%</td>
<td>2.25%</td>
</tr>
<tr>
<td>% Bases on-target</td>
<td>74.79%</td>
<td>78.55%</td>
<td>7.01%</td>
<td>5.33%</td>
</tr>
<tr>
<td>Mean error rate</td>
<td>0.51%</td>
<td>0.49%</td>
<td>0.07%</td>
<td>0.06%</td>
</tr>
</tbody>
</table>
Table 4.9. Novel nonsynonymous singleton mutations in TRPC6.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Variant</th>
<th>Coordinate (hg19)</th>
<th>Ref&gt;Var</th>
<th>ID</th>
<th>Gender</th>
<th>Father</th>
<th>Mother</th>
<th>Proband</th>
<th>Sib(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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*aAll variants are heterozygous.*
References


morphology in hippocampal pyramidal neurons by activating Ca(2+) -permeable TRPC6 channels. *Hippocampus* (2012).


Chapter 5

Summary and future directions
Summary

Autism spectrum disorders are a group of neurological disorders sharing common cognitive and behavioral phenotypes. While these ASDs share a core set of phenotypes (impaired social interaction, communication, repetitive behaviors), the additional behaviors and phenotypes present in the patient can vary immensely. Complicating matters even more, ASDs are highly heritable yet known ASD-associated genetic mutations only account for about 10% of all cases. As a result, a popular explanation is the multiple-hit hypothesis, which states that the accumulation of multiple genetic abnormalities or rare variants affecting specific pathways above a certain threshold causes ASD. The number of implicated genetic abnormalities associated with ASDs is continually increasing, with mutations affecting a variety of pathways and cellular functions such as synaptogenesis, spinogenesis, neurite outgrowth, synaptic transmission, and Ca\textsuperscript{2+} signaling. Thus a critical area of research is to identify rare variants associated with ASDs and determining their functional relevance to a certain pathway or cellular function. The field has shifted from trying to find a catchall genetic mutation causing the majority of ASDs to studying individual mutations and the specific phenotypes they may be causing. Fortunately, the advent of iPSCs has allowed for patient-specific disease modeling, causing a burst of studies using iPSCs to model diseases and disorders such as ALS, Parkinson’s, and even syndromic forms of ASD. Yet before our work, no human iPSC model of nonsyndromic autism had been demonstrated. The goal of this thesis was to generate a human iPSC model of nonsyndromic autism, and examine a potential rare ASD variant in TRPC6 to determine its functional relevance to ASD phenotypes.

Thus as a proof-of-principle, our laboratory recently generated an iPSCs model of nonsyndromic autism to investigate cellular and molecular phenotypes \textsuperscript{1}. The proband
presented with classical autism, delayed motor skills development, and poor social responsiveness. In this model, we generated iPSCs from an ASD individual carrying a de novo balanced translocation disrupting the TRPC6 gene, which encodes for the protein channel Transient Receptor Potential Canonical 6. This translocation resulted in TRPC6 haploinsufficiency the ASD individual (TRPC6-mut). Previously unassociated with ASD, TRPC6 is a voltage-independent, Ca\(^{2+}\)- permeable cation channel. TRPC6 has been implicated in neuronal growth cone guidance, spinogenesis, and synaptogenesis, processes known to be affected in ASD \(^{2-4}\). Furthermore, TRPC6 has been shown to activate important pathways important for neuronal development and function, including the BDNF, CAMKIV, Akt and CREB signaling pathways \(^{2,5,6}\) (Figure 5.1). Using iPSCs, we investigated the functional consequences of this TRPC6 haploinsufficiency.

Neurons derived from TRPC6-mut iPSCs revealed neuronal morphological and functional alterations compared to control neurons. Global gene expression analysis of TRPC6-mut cells revealed that several CREB-targeted neuronal genes important for neurodevelopment were differentially regulated. Analysis of TRPC6-mut neurons demonstrated altered morphology, including reduced total length and dendritic arborization. Key neuronal functions were also affected, such as fewer dendritic spines and synapses, and impaired calcium dynamics. Importantly, these TRPC6-mut-dependent phenotypes were validated using shRNA targeting TRPC6 as well as reexpression of TRPC6 cDNA. Moreover, using shRNA targeting TRPC6 in mice, both in vivo and in vitro, demonstrated phenotypes paralleling the iPSC results, such as reduced neuronal arborization and fewer spines and synapses. We were also able to rescue several of the neuronal abnormalities using the candidate drugs hyperforin and IGF-1. Our premise was that hyperforin, a specific activator of TRPC6 channels, might rescue
phenotypes caused by haploinsufficiency by increasing TRPC6 signaling. As mentioned previously, IGF-1 has been used to rescue neuronal defects in RTT iPSCs models and is also used in ongoing clinical trials for ASD and other CNS disorders\(^7\). Hyperforin and IGF-1 were able to ameliorate neuronal complexity and increase dendritic spine density and synaptogenesis. Interestingly, we also observed MeCP2 affected TRPC6 expression and occupied the TRPC6 promoter region. This potential interaction reveals possible common pathways affected in syndromic and nonsyndromic ASD. Finally, to further investigate TRPC6 as a novel ASD associated gene, mutation analysis of sequencing data from 1041 ASD individuals and 2872 controls revealed significantly more nonsynonymous mutations in the ASD population.

This study brings valuable information for ASD, as we demonstrated that an iPSC model of nonsyndromic ASD reveals striking neuronal phenotypes. These phenotypes and affected pathways represent potentially novel ASD biomarkers and the ability to rescue these abnormalities provides the basis for potential drug screening platforms. While this study is the first to describe an iPSC model of nonsyndromic autism, numerous more lines from nonsyndromic ASD individuals must be generated to validate common phenotypes and affected pathways, and to eventually create effective diagnostic tools.

**Common mechanisms of disease?**

The iPSC models of RTT, SZ, FXS, PMDS, and our nonsyndromic ASD model have demonstrated common phenotypes in neurons generated from these cell lines. All of these disorders demonstrated neuronal abnormalities such as altered morphology and synaptic deficits. And because all of these disorders fall under the umbrella of ASD, they share a common core set of symptoms. These observations suggest that there may be
common molecular mechanisms and pathways driving the observed phenotypes and symptoms in these disorders. Thus, iPSCs models represent a unique opportunity to examine and compare human neurons derived from different ASD at the cellular and molecular level.

Our recent report examined the potential relationship between MeCP2 and TRPC6. MeCP2 has previously been reported to affect TRPC6 levels. Hippocampal neurons from RTT mouse models were reported to have impaired activity-dependent BNDF release and TRPC signaling. Furthermore, TRPC6 has been shown to be necessary for certain BDNF-induced neurite growth cone guidance. Indeed, MeCP2 has been shown to regulate BDNF expression. Another shared feature between these iPSCs models has been the ability of IGF-1 to rescue neuronal phenotypes, as IGF-1 treatment ameliorated the synaptic defects in both RTT iPSC- and nonsyndromic ASD iPSC-derived neurons. These findings suggest MeCP2 and TRPC6 may be a part of common molecular pathway important for neuronal development and function (Figure 5.1). The presence of common phenotypes as well as shared mechanisms of drug rescue implies common mechanisms and pathways driving pathogenesis among these different ASDs. More than anything, iPSCs modeling provides the platform by which these analyses and comparisons can be made. Still, more work needs to be done, as more models of ASD need to be generated in order to verify and better characterize genuine ASD cellular phenotypes.

Limitations of iPSC disease modeling

As with any model for disease, iPSC technology has definite limitations. To begin with, cells are grown in culture, which is a departure from true physiological conditions. As such, the components of the media may be overestimating or underestimating key
signaling molecules, which can affect cell function. Thus to verify that phenotypes observed in vitro are genuine, they must be validated in vivo or using other models. In addition, the field currently lacks the ability to generate a wide variety of specific neuronal subtypes. This is particularly important because certain neuronal subtypes are more severely affected by disorders such as ASD. For example, RTT pathophysiological studies revealed that the pyramidal neurons in cortical layer V are especially affected and display fewer dendritic spines. Protocols are yet to be generated to produce such a specific subtype of cells. Another problem that arises because of this is the cellular heterogeneity in the iPSC-derived cultures. When generating cell types such as neurons, several other cell types remain present in the culture that could introduce artifacts. While some protocols exist for the FACS purification of cells such as neurons, more work is needed to be able to isolate all disease-relevant cell types. Another issue that arises with iPSC modeling is the problem of proper controls, especially with ASD. Currently, most reported ASD iPSC models use unaffected individuals or family members as controls. This can be problematic because each individual contains unique mutations and genetic differences that could potentially affect observable phenotypes. The ideal controlled experiment would be two lines containing the exact same genome, with the only difference being the mutation in question. Fortunately the field is moving toward addressing this concern with the use of genome editing such as TALEN and CRISPR technology. Using genome editing, researchers are able to generate isogenic lines, differing only by the specific targeted mutation. This highlights another important consideration: the types of disorders iPSCs can effectively model. Certainly, iPSCs modeling is most effective for genetic disorders with robust phenotypes. To maximize the potential of iPSCs, it is best if the genetics of the particular patient in question are
known so that any phenotypes can be attributed to a specific genetic cause, and if phenotypes are robust enough or readily detectable.

**Future directions**

Our work has revealed the functional relevance of a potential novel ASD-associated rare variant in *TRPC6*. Yet while we have identified several cellular phenotypes caused by loss or reduction of TRPC6, several follow-up studies could be performed to strengthen our findings. The primary limitation of our work is lack of patient replicates. Due to the nature of rare variants, it is difficult to find another ASD patient carrying the same genetic abnormality. I would propose several lines of research to follow-up studies to our work, such as increasing our experimental “n” with more biological replicates, determining the downstream effectors of TRPC6 signaling, studying the connection with MeCP2, and exploring the potential of hyperforin as a drug treatment.

To address the issue of additional replicates, instead of finding patients with similar genetic abnormalities, I would propose to generate isogenic iPSCs carrying a loss-of-function mutation in *TRPC6*. Taking advantage of genome editing technology, CRISPR could be used in control lines to generate lines carrying only the *TRPC6* mutation. This would provide strong support for our conclusion that *TRPC6* contributes to ASD if phenotypes were recapitulated. In fact, because our TRPC6-mut individual is carrying mutations other than *TRPC6*, this experiment would be even stronger evidence of *TRPC6* as an ASD-associated rare variant.

After identifying a neurologically relevant effect of *TRPC6* disruption, the next obvious question is the mechanism by which this occurs. Activation of TRPC6 channels leads to influx of Na$^+$ and Ca$^{2+}$ and subsequent stimulation of downstream activators
such as Akt, CaMKIV, and ERK. Several of these TRPC6-induced pathways lead to activation of the transcription factors such as CREB. Thus an interesting follow-up study would look at the genes activated by TRPC6 signaling. An example would be to collect RNA from neurons before and after stimulation of TRPC6 with hyperforin, and then performing RNA-seq analysis to identify TRPC6 signaling targets.

Another interesting finding in our work is the potential connection between TRPC6 and MECP2. As mentioned previously, recent work has pointed indirectly to a potential relationship between the two genes. Because we saw a reduction in TRPC6 in our MECP2-null RTT patient lines, it will be interesting to examine if increasing TRPC6 signaling or expression can rescue phenotypes in the RTT patient neurons. In addition, another study would be to assess the direct regulation of TRPC6 by MeCP2. An example experiment would be to perform a luciferase assay using the TRPC6 promoter region in MECP2-WT and MECP2-KO cell lines, in combination with shRNA targeting MECP2 and MECP2-expressing constructs.

Finally, a possible line of research would be to examine hyperforin as a potential drug treatment in ASDs. Our work revealed a potential therapeutic benefit in our ASD individual. Hyperforin by itself has not yet been tested in ASDs or other neurological disorders. However, St. John’s wort, in which hyperforin is a component, is an herbal remedy widely used for treating depression. Hyperforin is one of several components in St. John’s wort, but studies have shown its significance to treatment. A single trial for ASD has been attempted using St. John’s wort. Clinicians reported no patient improvement in behavioral symptoms, however it was an open trial and only had three patients. A better study could use hyperforin instead of St. John’s wort and a much larger patient population. Even more, if patients could be screened first for mutations in TRPC6 or reduced TRPC6 expression using patient-specific neurons, a better test for
Hyperforin efficacy can be performed. Indeed, in our RTT patient neurons, although the patients did not carry TRPC6 mutations, TRPC6 levels were still reduced. Using this example, future pre-clinical trials could involve the use of patient-specific cells to first examine if certain biomarkers exist to determine which treatments may be applicable to the individual.

**Future implications of research**

The iPSC-disease modeling strategy represents a significant step in ASD research and treatment. The most useful applications of these models are for the identification of cellular phenotypes, the elucidation of affected molecular pathways, and for the generation of new therapeutic strategies. This is particularly evident for syndromic forms of autism, as the genetics are known and can be attributed to the observed phenotypes. Yet our work denotes the first step in modeling nonsyndromic autism, which represents the majority of ASD but lacks clear, defining symptoms or cellular phenotypes. By taking advantage of next generation genomics, one can map all of the genetic abnormalities and use iPSCs to analyze their impact on neuronal cells. Allowing for the development of personalized medicine, these iPSC models can take advantage of specific cellular phenotypes for drug screening purposes to identify potential therapeutic drugs tailored to an individual. While iPSC modeling shows great promise for ASD research, more work is needed. More iPSC models of nonsyndromic autism are necessary to generate a library of iPSC models from numerous autistic individuals to identify phenotypes and molecular pathways common to ASD. Fortunately several outreach programs exist to facilitate community engagement and sample collection, such as the Tooth Fairy Project 1, 19. This project allows for families to send newly lost baby teeth form autistic individuals to researchers, from which dental pulp cells can be
extracted and iPSCs generated. Finally, the comprehensive molecular and functional characterization of the iPSC-derived neurons from autistic individuals will be essential for the reliable discovery of true ASD phenotypes and molecular mechanisms driving ASD etiology.

Acknowledgments

Chapter 5, in part, is a manuscript submitted for publication in *Neurotherapeutics* 2015. Allan Acab, Alysson R. Muotri. The dissertation author was the primary investigator and author of this paper.
Figure 5.1. Summary of TRPC6-mut neuronal phenotypes.

IPSC-derived TRPC6-mut neurons revealed a number of phenotypes, including reduced spine density and fewer synapses. The TRPC6-mut neurons also exhibited morphological defects such as reduced dendritic arborization. In addition, TRPC6-mut neural cells demonstrated functional phenotypes such as altered electrophysiology and calcium imaging.
References


