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Functional characterization of new genes and molecular pathways implicated in developmental brain disorders

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THÈSE DE DOCTORAT

Caractérisation fonctionnelle des nouveaux
gènes et des voies moléculaires impliquées
dans les troubles du développement du
cerveau

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du grade de docteur en** Science de la Vie et
de la Santé d'Université Côte d'Azur
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Caractérisation fonctionnelle des nouveaux gènes et des voies moléculaires impliquées dans les troubles du développement du cerveau

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Résumé en français

Les troubles du développement du cerveau (DBD) englobent un groupe très hétérogène de troubles qui se manifestent par des aberrations cognitives, motrices, neurocomportementales, neuroanatomiques et neurophysiologiques provenant du dysfonctionnement cérébral du développement. Les DBD comprennent, entre autres, la déficience intellectuelle, certaines formes d'épilepsie, le trouble du spectre autistique (TSA), le trouble d'hyperactivité avec déficit de l'attention (TDAH), les troubles de l'apprentissage et du langage et la schizophrénie. Au cours de ma thèse, j'ai participé à deux projets de recherche distincts. Le premier projet visait la caractérisation fonctionnelle de nouveaux gènes impliqués dans la schizophrénie à début précoce (SDP), le trouble du spectre autistique et la déficience intellectuelle. La SDP est un trouble psychiatrique caractérisé par un large éventail de symptômes tels que les délires, les hallucinations et les comportements sociaux anormaux. Cette maladie est souvent accompagnée de troubles du spectre autistique et de déficience intellectuelle, qui sont censés partager un fond génétique commun avec la SDP. Au cours de cette étude, le séquençage de l'exome entier (WES : Whole Exome Sequencing) de 9 trios (enfants touchés et deux parents appartenant à une cohorte de 40 familles partageant un phénotype similaire) a été effectué afin de trouver de nouveaux gènes impliqués dans la pathogenèse de la SDP. Les patients ont été diagnostiqués avec la SDP, qui dans certains cas est associée à d'autres phénotypes tels que le TSA ou la déficience intellectuelle. J'ai trouvé quelques variants très rares qui ont un impact pathologique putatif sur le phénotype des patients selon l'analyse *in silico*. Un variant a été identifié dans le gène **Sérine/thréonine-protéine kinase 33 (STK33)** qui est un membre éloigné du groupe CAMK de sérine/thréonine kinases. Les membres de cette famille participent à la régulation de l'homéostasie calcique qui s'est avérée altérée chez les patients atteints de schizophrénie. Pour évaluer l'impact

pathologique de la mutation dans STK33, j'ai créé un modèle cellulaire dans la lignée cellulaire SH-SY5Y par la technique CRISPR-Cas9 imitant la variante trouvée chez le patient. Pour décrire le phénotype de la lignée cellulaire mutée, j'ai effectué l'analyse de l'expression des gènes et des protéines ainsi que des expériences d'imagerie calcique.

Le deuxième projet de ma thèse a été associé à la caractérisation d'une nouvelle mutation spontanée du gène *Kcc2* chez la souris souffrant de crises tonico-cloniques spontanées à partir de l'âge de quatre mois. Le gène *Kcc2* code le cotransporteur neuronal du chlorure de potassium KCC2, qui participe à la sortie des ions chlorure des cellules, à la protection des réseaux neuronaux contre l'excitotoxicité, à la morphogenèse des dendrites et au maintien de l'équilibre excitation/inhibition. Nous pensons que les variants de ce gène ont un fort potentiel de causer des dysfonctionnements neurodéveloppementaux. La plupart d'entre eux sont associés à l'épilepsie de la petite enfance avec crises focales migrantes (EIMFS), qui appartient à un groupe de syndromes épileptiques rares. Nous avons développé la présente étude dans le but de caractériser le premier modèle d'épilepsie spontanée chez la souris due à une mutation du gène *Kcc2* en corrélant l'activité neuronale, l'apparence des crises et le comportement social et cognitif avec l'expression génique. À ce jour, ces études fonctionnelles n'ont pas été réalisées puisque les souris *Kcc2*-KO meurent quelques jours après la naissance et que les animaux hétérozygotes n'ont jamais été étudiés en détail. La mutation identifiée chez nos souris affecte le même acide aminé qui a été trouvé muté chez un patient EIMFS (R857L), offrant une occasion unique d'étudier l'étiologie de l'EIMFS *in vivo*. Jusqu'à présent, nous avons observé que chez les souris mutantes *Kcc2*, la production de la protéine est fortement diminuée dans le cortex cérébral, l'hippocampe et le striatum. De plus, le cross-linking assay et l'analyse immunohistochimique ont révélé une diminution de la protéine KCC2 au niveau de la membrane. En effectuant une électroencéphalographie intracrânienne (iEEG),

il a été possible d'observer le point typique pour épilepsie chez souris mutantes Kcc2. Pour le moment, des études comportementales ont révélé que les souris Kcc2 mutantes présentaient des déficits d'apprentissage et de mémoire. Ces résultats nous ont donné une solide base pour une évaluation plus approfondie de notre modèle et une possibilité d'obtenir un meilleur aperçu de la pathogenèse des EIMFS avec le but final de définir des interventions thérapeutiques.

En plus de ces deux objectifs principaux, j'ai contribué à l'identification des voies moléculaires qui sous-tendent certaines caractéristiques neurodéveloppementales des neurones corticaux de culture obtenues à partir d'un modèle murin de FXTAS.

Mots-clés : Troubles développementaux du cerveau, schizophrénie, TSA, déficience intellectuelle, gènes candidats, STK33, modèle cellulaire, imagerie calcique, Kcc2, épilepsie, modèle de souris, études comportementales, protéomique, signalisation moléculaire

English abstract

Developmental Brain Disorders (DBDs) encompass a highly heterogeneous group of disorders that manifest through cognitive, motor, neurobehavioral, neuroanatomical and neurophysiological aberrations originating from developmental brain dysfunction. DBDs include, among others, Intellectual Disability (ID), some forms of epilepsy, Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD), learning and language disorders and Schizophrenia (SCZ). During my thesis, I was involved in two distinct research projects. The first project aimed at the functional characterization of new genes implicated in Early Onset Schizophrenia (EOS), ASD and ID. EOS is a psychiatric disorder characterized by a wide range of symptoms such as delusions, hallucinations and abnormal social behavior. This disease is frequently accompanied by ASD and ID, which are believed to share a common genetic background with EOS. During this study, Whole Exome Sequencing (WES) of 9 TRIOs (affected child and both parents belonging to a cohort of 40 families sharing a similar phenotype) was performed in order to find new genes that are involved in the pathogenesis of EOS. Proband was diagnosed with EOS, which in some cases was combined with other phenotypes such as ASD or ID. I found some very rare variants that have a putative pathological impact on the phenotype of the patients according to *in silico* analysis. One variant was identified in the gene ***Serine/threonine-protein kinase 33 (STK33)*** that is a distant member of the CAMK group of serine/threonine kinases. The members of this family participate in the regulation of calcium homeostasis that was shown to be altered in patients with schizophrenia. To further evaluate the pathological impact of mutation in STK33 I created a cellular model in the SH-SY5Y cell line by the CRISPR-Cas9 technique mimicking the potential variant found in the patient. To describe the phenotype of the mutated cell line, I performed the analysis of gene and protein expression as well as calcium imaging experiments.

The second project during my thesis was associated with characterization of a novel spontaneous mutation in the *Kcc2* gene in mouse suffering from spontaneous tonic-clonic seizures starting at the age of four months. The *Kcc2* gene codes the neuronal potassium chloride co-transporter KCC2, which participates in extrusion of chloride ions from cells, protection of neuronal networks against excitotoxicity, dendrite morphogenesis and in the maintenance of the excitation/inhibition balance. Variants in this gene are believed to have a strong potential to cause neurodevelopmental dysfunctions. Most of them are associated with Epilepsy of Infancy with Migrating Focal Seizures (EIMFS), which belongs to a group of rare epileptic syndromes. We developed the present study with the aim of characterizing the first spontaneous epilepsy model in mouse due to a mutation in the *Kcc2* gene by correlating neuronal activity, seizure appearance and social and cognitive behavior with gene expression. To date these functional studies were not performed since *Kcc2*-KO mice die few days after birth and heterozygous animals were never studied in detail. The mutation identified in our mice affects the same amino acid that has been found mutated in an EIMFS patient (R857L), offering a unique opportunity to study the aetiology of EIMFS *in vivo*. So far, we observed that in mutant *Kcc2* mice, production of the protein is strongly decreased in brain cortex, hippocampus and striatum. Moreover, cross-linking assay and immunohistochemical analysis revealed a decrease of the KCC2 protein at the membrane level. By performing intracranial ElectroEncephaloGraphy (iEEG), it was possible to observe the shape of the typical spike for epileptic mouse. For the time being, behavioral studies revealed that mutant *Kcc2* mice exhibit learning and memory deficits. These findings gave us a strong background for further evaluation of our model and a possibility to get further insight into the pathogenesis of EIMFS with the final purpose to define therapeutic interventions.

In addition to these two main aims, I contributed to the identification of molecular pathways underpinning some neurodevelopmental hallmarks of cultured cortical neurons obtained from a murine model of FXTAS.

Keywords: Developmental Brain Disorders, schizophrenia, ASD, ID, candidate genes, STK33, cellular model, calcium imaging, Kcc2, epilepsy, mouse model, behavioral studies, proteomics, molecular signaling.

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Abbreviations

5' UTR - 5' untranslated region
aCGH - array-Comparative Genomic Hybridization
Ach - Acetylcholine
ADHD - Attention Deficit Hyperactivity Disorder
AMPA - A-amino-3-hydroxy-5-Methyl-4-isoxazolePropionate type
APC - APC regulator of WNT signaling pathway
AS - Angelman syndrome
ASD - Autism Spectrum Disorder
ATP13A4 - ATPase type 13A4
Bcl-2 - B-cell lymphoma/leukaemia-2 gene
BDNF - Brain-derived neurotrophic factor
CaMK - Calcium-calmodulin Kinases
CaMKII α - Calcium/Calmodulin-dependent protein Kinase II α
CCCs - Chloride Cotransporters
CCKR - Cholecystinin receptor
CDH - Cadherin
CHD8 - chromodomain helicase DNA-binding protein 8
CLCN2 - chloride channel 2
CNVs – Copy Number Variations
COS - Childhood Onset Schizophrenia
CTNNB1 - β -catenin
DARPP-32 - Dopamine- and cAMP-regulated phosphoprotein of M 32 kDa
DBDs - Developmental Brain Disorders
DISC1 - Disrupted in schizophrenia 1
DKK1 - Dickkopf WNT Signaling Pathway Inhibitor 1
DSM -- The Diagnostic and Statistical Manual of Mental Disorders
DTNBP1 - Dysbindin-1
EGF - epidermal growth factor
Egr4 - Early growth response 4
EIMFS - Epilepsy of Infancy with Migrating Focal Seizures
EMT - Epithelial-Mesenchymal Transition
EOS - Early Onset Schizophrenia
ERKs - Extracellular Signal-regulated Kinases
ExAC - Exome Aggregation Consortium
EXPOI - Fragile X-associated Primary Ovarian Insufficiency
FISH - Fluorescence In Situ Hybridization
FXS - Fragile X Syndrome
FXTAS - Fragile X-associated tremor/ataxia syndrome
FZD7 - Frizzled Class Receptor 7
GABA - Gamma-AminoButyric Acid
GABRB3 - β 3 subunit of the GABA_A receptor
GAD1 - Glutamate Decarboxylase 1
GAP43 - Growth-associated protein 43

GAT - GABA transporter
GSK3 - Glycogen Synthase Kinase 3
GWAS - Genome-Wide Association Studies
HES1 - Hes Family BHLH Transcription Factor 1
ID - Intellectual Disability
iEEG - intracranial ElectroEncephaloGraphy
IGF1 - insulin-like growth factor
JNKs - c-Jun N-terminal kinases
KCC (SLC12A) - Na⁺-independent K⁺-coupled Cl⁻ co-transporters
KCC2 - Potassium-Chloride Cotransporter 2
KCNT1 - Potassium Sodium-Activated Channel Subfamily T Member 1
M1 – M5 - Muscarinic receptors subtypes
MEK - MAP-ERK kinase
MOMP - Mitochondrial Outer Membrane Permeabilization
NCC -Na⁺-Cl⁻ co-transporter
NFATC3 - Nuclear Factor Of Activated T Cells 3
NIHM - National Institutes of Mental Health
NKCC (SLC12A) - Na⁺-dependent Cl⁻ transporters
NMDA - N-methyl-D-aspartate
NMDAR - N-methyl-d-aspartic acid-type glutamate receptors
NRG1 - Neuregulin 1
OPA1 - Optic Atrophy 1
PCP - phencyclidine
PKC - Protein Kinase C
PLA2 - Phospholipase A2
PND - Post-Natal Day
PTEN - Phosphatase and Tensin Homolog
PV - parvalbumin
PWS - Prader-Willi syndrome
RAF - Rapidly Accelerated Fibrosarcoma
RAS - Rat Sarcoma virus
RGS4 - Regulator of G protein signaling-4
SAP97 - Synapse-Associated Protein of 97-kDa molecular weight
SCN1A - Sodium Voltage-Gated Channel Alpha Subunit 1
SCN2A - Sodium Voltage-Gated Channel Alpha Subunit 2
SCN3A - Sodium Voltage-Gated Channel Alpha Subunit 3
WNT - Wnt Family Member 1
SCZ - Schizophrenia
SPAK/OSR1 - Ste20-related Proline Alanine-rich Kinase/Oxidative Stress Response-1 binding motif
STK33 - Serine/Threonine protein Kinase 33
TCF4 - Transcription factor 4
USF - Upstream Stimulating Factors
VGCCs - Voltage-Gated Calcium Channels
VSCCs - L-type Voltage-Sensitive Calcium Channels

Introduction

Developmental Brain Disorders

Developmental Brain Disorders (DBDs) encompass a highly heterogeneous group of disorders that manifest through cognitive, motor, neurobehavioral, neuroanatomical, and neurophysiological aberrations originating from developmental brain dysfunction. DBDs include, among others, Intellectual Disability (ID), cerebral palsy, some forms of epilepsy, Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD), learning and language disorders, motor disorder and schizophrenia (SCZ) (Moreno-De-Luca *et al.*, 2013). Many features characterizing these disorders can describe the same disease and can co-occur in the same patient (Association, 2013). For example, around 16-26% of ID patients suffer from epilepsy (McGrother *et al.*, 2006). It is well-known that many individuals with autism have also ID. Similarly, ADHD patients frequently suffer from a specific learning disorder. Sometimes some separate disease entities such as autism and epilepsy can be included in the features of some genetic disorders such as tuberous sclerosis, Fragile X Syndrome (FXS) and Rett syndrome.

Apart from overlap between phenotypic features, many of these disorders share a common genetic and molecular background. Several studies have demonstrated that some Copy Number Variations (CNVs) or mutations co-occur in ID, ASD, epilepsy or SCZ (Moreno-De-Luca *et al.*, 2013). Moreover, some pathways (*e.g.*, Rho-GTPase, MAPK-signalling, mGluRI, neuronal cell adhesion, cAMP-associated signalling, GABAergic signaling, and neuroinflammation) have been found to be altered in different forms of DBD, both of genetic

and environmental origin. These findings suggest common pathophysiological mechanisms that can be considered as a continuum of developmental brain dysfunction (Antoine-Bertrand *et al.*, 2011; Brooks-Kayal, 2010; Edfawy *et al.*, 2019; Funk *et al.*, 2012; Kelley *et al.*, 2008; Matta *et al.*, 2019; O'Dushlaine *et al.*, 2011; Pucilowska *et al.*, 2015; Wen *et al.*, 2016). The prevalence of ID is around 1% in the worldwide population. The same prevalence is estimated for ASD (Association, 2013). The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) suggested a prevalence between 0.5 – 1% for SCZ (Bell, 1994). On the other hand, DSM-V does not provide information on SCZ accidents. However, it gives 0.2% prevalence estimation for delusional disorder that is closely related with SCZ and schizotypal personality disorder (Association, 2013). Some researchers, basing on multiple population studies, estimate SCZ prevalence to be around 0.4% (Saha *et al.*, 2005).

Most DBDs pose tremendous economic burden on the health care systems worldwide. The costs of medical service and available therapies and treatments often overpass many families' incomes. Out-of-pocket healthcare costs create a major socio-economical issue worldwide (Allers *et al.*, 2015; Chong *et al.*, 2016; Salvador-Carulla and Symonds, 2016; Wang *et al.*, 2013). Thus, there exist an urgent need of new affordable therapies.

Common genetic and molecular background of neurological disorders

a. Common genetic background

ASD, ID, SCZ, Bipolar Disorder (BD) and epilepsy are thought to share a common genetic background (Betancur, 2011; McCarthy *et al.*, 2014; Mefford *et al.*, 2010; O'Connell *et al.*,

2018; Srivastava and Sahin, 2017). Initially, the search for common variants responsible for these disorders came to nothing. The biggest Genome-Wide Association Studies (GWASs) failed in elucidating their genetic causes. Afterwards, researchers found out that *de novo* CNVs are enriched in ASD and SCZ individuals, which revealed that rare, *de novo* variants can be associated with neurological disorders (reviewed in Mitchell, 2011). This finding led to the identification of many rare private mutations (rare mutations that are usually found only in one family) that possibly affect pathogenesis of neurological disorders. Probably, these rare variants undergo negative selection leading to low prevalence in the population. Furthermore, some common Single Nucleotide Polymorphisms (SNPs) were also shown to be shared among some neurological disorders, such as SCZ, BD, ASD, ADHD and depression. However, they are considered to have a small effect on phenotype when stand-alone. Researchers are speculating that common SNPs have to co-occur to contribute to the risk of neurodevelopmental disorders (Consortium, 2013; Doherty and Owen, 2014; Lee *et al.*, 2013). The lack of common causative variants and the identification of many rare mutations revealed the high heterogeneity of neurodevelopmental disorders. This heterogeneity can be also affected by the degree of mutation penetrance, different genetic modifiers, epistatic interactions, and environmental factors (reviewed in Mitchell, 2011). Moreover, the features of each mutation can influence the phenotypic manifestation. Localization of the mutation can impact the severity of the phenotype (*e.g.*, a mutation can affect the different domains of a protein). Tissue-specificity can also play a role in the development of different diseases. It means that mutations in the same gene can cause different phenotypes depending on their tissue-specific expression. Also, the quantity of mutated proteins can influence the phenotypic features. A null allele will lead to entire loss of function, whereas an hypomorphic allele will

cause only partial loss. Zygosity (heterozygosity, homozygosity) may impact the phenotype as well. In order to understand the effects of a particular mutation, functional studies elucidating the interplay between genetic and phenotypic features are essential (Zhu *et al.*, 2014).

There is a growing body of evidence that further exploration of common pathways associated with psychiatric illnesses can provide a deeper insight into the aetiology of neurodevelopmental disorders (Doherty and Owen, 2014). Pathways associated with dopamine, serotonin, voltage-gated calcium ion channels, and folate metabolism have been linked to ASD, SCZ and BD. Moreover, some enrichment was found in gene expression regulation, silencing regulation and hippo signaling pathway that participate in cell proliferation and migration as well as in dendritic maintenance (O'Connell *et al.*, 2018). McCarthy *et al.* (2014) performed WES on 57 TRIOs with SCZ. They found overlap between *de novo* mutations in their SCZ cohort and genes previously related to ASD and ID. The common genes were implicated in epigenetic regulation of transcription (McCarthy *et al.*, 2014).

b. Deeper insight into selected signaling pathways

➤ GABAergic signaling

Transfer of signals in the brain, through electrochemical signaling, is of paramount importance for regulation of its function. These signals are emitted by neurotransmitters and participate in the maintenance of an appropriate balance between excitatory and inhibitory neurons. The inhibitory neurotransmitter Gamma-AminoButyric Acid (GABA) is a crucial agent involved in the maintenance of this balance via GABAergic signaling (reviewed in Kim and

Yoon, 2017). GABAergic signaling influences neural circuit formation, growth and plasticity of neurons (reviewed in Akerman and Cline, 2007). GABA triggers postsynaptic neuron inhibition through binding to the GABA_A and GABA_B receptors (reviewed in Kim and Yoon, 2017). In mature neurons, after GABA binding to the GABA_A receptor, the influx of chloride ions through the membrane increases, leading to its hyperpolarization. On the other hand, activation of the GABA_A receptor in immature neurons causes membrane depolarization. It is the result of the different expression of two main chloride co-transporters during development: *NKCC1* and *KCC2* (reviewed in Akerman and Cline, 2007). For instance, in humans, *KCC2* achieves the peak of its expression between birth and six months after birth while *NKCC1* begins to be expressed at earlier stages of development. Moreover, their expression differs depending on brain regions (Sedmak *et al.*, 2016). Apart from differences in spatiotemporal expression, these two chloride co-transporters have different functions. *KCC2* is responsible for extrusion of chloride ions out of the cell, whereas *NKCC1* is in charge of chloride import. Although in immature neurons *KCC2* levels are lower, its gradual increase starts to affect the processes of depolarization and hyperpolarization of the membrane, pushing it towards hyperpolarization. Changes in this process are associated with a shift in GABA_A receptor reversal potential that is often named “GABA switch” (reviewed in Akerman and Cline, 2007). So far, the exact timing of the GABA switch has not been elucidated (Kirmse *et al.*, 2018). Some data from rat slices suggest that the switch occurs between P2 and P15 (Tyzio *et al.*, 2007).

Alterations in GABAergic signaling are thought to underlie many brain disorders by affecting brain functioning. For instance, mutations in the GABA_A receptor can evoke aberrations in function of post-synaptic neurons (reviewed in Kim and Yoon, 2017). It was

shown that in epileptic patients mutations in the GABA_A receptor could affect GABA current, trafficking, surface expression and cause its retention in the endoplasmic reticulum. Also, mutations in other genes associated with maintenance of chloride homeostasis, such as *chloride channel 2 (CLCN2)* and the potassium chloride co-transporter *KCC3*, were suggested to indirectly influence GABAergic signaling (reviewed in Galanopoulou, 2010). The proper amount of GABA_A and GABA_B receptors in the membrane is important for maintenance of correct functioning of synapses. Also, GABA transporter (GAT) activity affects GABAergic signaling. Loss of GAT transport properties can lead to excess in GABA levels that subsequently will increase inhibitory signaling (reviewed in Kim and Yoon, 2017). Mutations in the *SLC6A1* gene that codes GAT-1 were found in individuals with epileptic encephalopathies. These mutations were predicted to cause GAT-1 loss of function (Carvill *et al.*, 2015). GAT-1-deficient mice have increased levels of extracellular GABA, which cause over-activation of the GABA_A receptor (Jensen *et al.*, 2003).

ASD and epilepsy, especially epileptic encephalopathies, share some common genes that are mainly involved in action potential generation and release of neurotransmitter (ion channel and transporter function), synapse formation, transcriptional regulation, signal transduction, DNA replication, and cell adhesion. Both ASD and epileptic encephalopathies features were suggested to be related to the excitatory/inhibitory imbalance. This imbalance is associated with aberration in sodium and chloride channels and GABAergic signaling (reviewed in Srivastava and Sahin, 2017). Dysfunctions of chloride co-transporters were shown to participate in forming human epileptiform activity. Huberfeld *et al.* (2007) observed aberrations in GABAergic signaling in tissue slices derived from individuals with mesial

temporal lobe epilepsy associated with hippocampal sclerosis. Electrophysiological recordings on these slices revealed the presence of interictal-like activity and depolarizing GABA_A receptor-mediated postsynaptic events in subicular pyramidal cells, suggesting an imbalance in chloride levels. Moreover, by performing *in situ* hybridization, the authors observed the absence of KCC2 in around 30% of calcium/CalModulin-dependent protein Kinase II α (CaMKII α)-positive subicular pyramidal cells (CaMKII α was used to distinguish principal cells from interneurons) (Huberfeld *et al.*, 2007). The GABA_A receptor was shown to be decreased in the cortex and cerebellum brain areas of patients with autism (Fatemi *et al.*, 2008). The GABA_B receptor was also found to be decreased in the cerebellum of autistic patients (Fatemi *et al.*, 2009). Moreover mRNA levels of different subunits of the GABA_A and GABA_B receptors were shown to be changed in different brain areas of autistic patients (Fatemi *et al.*, 2010). Maternal duplication of chromosomal locus 15q11-q13, which encompasses the $\beta 3$ subunit of the GABA_A receptor (*GABRB3*), is very common in ASD patients. Delahanty and collaborators (2011) reported a maternally transmitted variant in *GABRB3* (P11S). Electrophysiological analysis of this variant in HEK293T cells revealed reduced current amplitudes. Moreover, it was shown that the mutated subunit is decreased at the membrane surface, probably due to its intracellular aberrant processing. Altogether, these data demonstrate that the $\beta 3$ subunit of the GABA_A receptor can be an important factor in autism pathogenesis (Delahanty *et al.*, 2011).

Abnormal GABAergic signaling was suggested to underlie the autistic and Rett syndrome phenotypes. It was shown that *Mecp2*-KO mice present many features typical for these disorders. Analysis of GABA-releasing neurons from *Mecp2*-KO mice revealed that

Mecp2 is crucial for their proper function (Chao *et al.*, 2010). GABAergic signaling aberrations were also found in Prader-Willi syndrome (PWS) and Angelman syndrome (AS) that share some features with ASD and epilepsy (Srivastava and Sahin, 2017). Other neurodevelopmental disorders, such as FXS and Dravet syndrome, are also thought to be linked to aberrations in the GABAergic system (Braat and Kooy, 2015). Indeed, in Fragile X KO mouse, the expression of some subunits of the GABA_A receptors were decreased. Moreover, the treatment with a GABA_A receptor agonist restores some behavioral abnormalities observed in this model (Aa and Kooy, 2020).

In SCZ, aberrations in GABAergic signaling were suggested to be implicated in its aetiology (reviewed in Lidow, 2003). Some drugs used in SCZ and BD treatment act upon GABAergic signaling (reviewed in Cherlyn *et al.*, 2010). Moreover, there exists a hypothesis that SCZ origin is linked to alterations in GABAergic signaling. This hypothesis assumes that N-methyl-d-aspartic acid-type glutamate receptors (NMDAR) hypofunction is sufficient to trigger a SCZ-like phenotype (reviewed in Nakazawa *et al.*, 2012). It was previously demonstrated that NMDAR activation participates in GABA-dependent excitatory synapse formation (Wang and Kriegstein, 2008). Moreover, it was observed that NMDA antagonists, such as phencyclidine (PCP), can cause psychosis that encompasses many positive and negative symptoms of the schizophrenic phenotype (Javitt and Zukin, 1991). Selective deletion of NMDARs from mouse cortical and hippocampal GABAergic interneurons, in majority parvalbumin(PV)-positive, evoke a SCZ-like phenotype. PV-positive GABAergic interneurons are in majority fast-spiking neurons that participate in the generation of synchronous gamma oscillations and control of cortical inhibition. Aberration in PV-positive GABAergic interneurons functioning was

suggested to lead to SCZ (reviewed in Nakazawa *et al.*, 2012). Dysfunction of NMDARs were also associated with Potassium-Chloride Cotransporter 2 (KCC2) aberrations. Lee and collaborators (2011) demonstrated that influx of calcium ions and activity of the NMDA receptor cause dephosphorylation of KCC2 Ser940 in dissociated rat neurons. This dephosphorylation results in loss of function of KCC2 leading to deficits in hyperpolarizing GABAergic inhibition (Lee *et al.*, 2011). Latest findings suggest that aiming at restoring the proper levels of GABAergic neurons is a promising target in SCZ treatment. Piyabhan and collaborators (2019) tested an Ayurvedic herb – Brahmi (*Bacopa monnieri*) – in a rat model of SCZ. Their results indicated that Brahmi treatment can lead to improvements in cognitive deficits and can have neuroprotective effects on GABAergic neuronal density (Piyabhan *et al.*, 2019).

➤ Wnt/ β -catenin/chromatin remodeling pathways

Aberrations in Wnt signaling are related to pathogenesis of different neurodevelopmental diseases such as SCZ, ASD and BD. The Wnt pathway participates in many processes such as regulation of gene transcription, apoptosis, proliferation and migration of the cells, and cytoskeletal dynamics. It plays an important role in Central Nervous System (CNS) development, in particular in the differentiation of neural progenitors, synaptogenesis, neuronal migration and plasticity. Mutations in Wnt signaling genes have been associated with many psychiatric illnesses. Genes, such as *APC regulator of WNT signaling pathway (APC)*, *Chromodomain Helicase DNA-binding protein 8 (CHD8)*, *Disrupted in schizophrenia 1 (DISC1)*, *Phosphatase and Tensin Homolog (PTEN)*, *Wnt Family Member 1 (WNT1)*, *WNT2*, *WNT3*,

WNT7A, β -catenin (*CTNNB1*), *Transcription Factor 4 (TCF4)*, and *TCF7* were linked to ASD. The Wnt/ β -catenin loci were identified in a genomic analysis of SCZ patients and some of them were suggested to be a risk factor for this disorder. Among them: *CHD8*, *CTNNB1*, *DISC1*, *TCF4*, *DKK1* (*Dickkopf WNT Signaling Pathway Inhibitor 1*), and *DKK4* (Mulligan and Cheyette, 2016). Hoseth *et al.* (2018) showed that the mRNA expression level of Wnt signaling members - *Frizzled Class Receptor 7 (FZD7)* and *Nuclear Factor Of Activated T Cells 3 (NFATC3)* - are increased in SCZ patients. Moreover, plasma levels of *dickkopf 1* and *sclerostin* were lower in SCZ patients (Hoseth *et al.*, 2018). A *synapse-Associated Protein of 97-kDa molecular weight (SAP97)* is located within the region of the 3q29 microdeletion syndrome that was linked to autism, ID and risk of SCZ (Mulle *et al.*, 2010; Quintero-Rivera *et al.*, 2010). Boccitto and collaborators (2016) discovered that *SAP97* can inhibit transcriptional activity of Wnt-activated β -catenin. They showed that increased expression of another SCZ-associated protein (*DISC1*) can stabilize *SAP97* by preventing its degradation by the proteasome (Boccitto *et al.*, 2016). O’Roak *et al.* (2012) performed exome sequencing of 209 trios with probands diagnosed with ASD. By analysis of *de novo* mutations the group found that many of these *de novo* variants are connected with the β -catenin/chromatin remodeling pathway (O’Roak *et al.*, 2012). Another group that sequenced 1116 individuals diagnosed with ASD and ID identified some genes linked to Wnt, SWI/SNF, NCOR, and Notch complexes. Mutations found in these genes were predicted to have more severe impact than the mutations in genes that were not associated with previously mentioned complexes (Hormozdiari *et al.*, 2015). Also, an ASD candidate gene prediction approach led to the identification of genes connected with the Wnt and β -catenin pathways confirming their potential association with ASD (Krishnan *et al.*, 2016). Apart from mutations observed in many neurodevelopmental disorders, some

components of the Wnt pathways are targets in their pharmacological treatments. For instance, trials in Fragile X mouse and fruit flies model with inhibitor of Wnt pathway member Glycogen Synthase Kinase 3 (GSK3) – lithium resulted in correction of multiple disease features (reviewed in Liu and Smith, 2014; McCamphill *et al.*, 2020).

➤ Cadherin signaling

The cadherin signaling pathway that involves more than 110 cell adhesion molecules plays a role in CNS development. Cadherins participate in neural circuit formation, signal transduction, synapse development, differentiation of grey matter, neuronal migration, and spine morphology (reviewed in Hawi *et al.*, 2018; Redies *et al.*, 2012). Cadherins belong to transmembrane proteins that are dependent on calcium homeostasis. They create adherent junctions between cells and tissues (Hawi *et al.*, 2018). Cadherins that are present in the brain show time-specific expression. GWAS studies revealed many cadherin related genes associated with different neuropsychiatric diseases such as ASD, SCZ, epilepsy, BD and ID. *Cadherin 15 (CDH15)* and *PCDH19* were linked to ID; *CDH5*, *CDH8*, *CDH9*, *CDH10*, *CDH13*, *CDH15*, *PCDH10*, *PCDH19*, and *PCDHb4* were associated with ASD; *CDH7*, *CDH12*, *CDH18*, *PCDH12*, and *FAT* have been related to SCZ and BD (reviewed in Redies *et al.*, 2012). Out of so many cadherins, *CDH13* was studied in particular. For instance, Sanders *et al.* (2011) during analysis of ASD families found a *de novo* deletion in the 16q23.3 locus that encompasses *CDH13* (Sanders *et al.*, 2011). Lasky-Su and collaborators (2008) identified an SNP rs6565113 in *CDH13* that was associated with the ADHD phenotype (Lasky-Su *et al.*, 2008). Another SNP rs11646411 in *CDH13* was identified in a German cohort of ADHD individuals (Lesch *et al.*,

2008). Interestingly, *CDH13* was suggested to influence the excitatory/inhibitory balance by acting as negative regulator of inhibitory synapses. *Cdh13*-KO mice display growth in basal inhibitory transmission. Moreover, these mice demonstrate learning and memory impairments (Rivero *et al.*, 2015). Cadherins negatively regulate Wnt signaling by sequestering β -catenin and play an important role in the regulation of β -catenin-dependent transcription (Howard *et al.*, 2011).

➤ Cholecystokinin receptor signaling pathway

Cholecystokinin receptor (CCKR) signaling plays an important role in the intracellular signaling apparatus. Cholecystokinin activates the CCK(1) receptor that belongs to the G-protein-coupled receptors family. The CCK(1) receptor is responsible for regulation of serotonin neuron activity and maintenance of proper dopamine levels in raphe nuclei (cluster of cell groups localized in the brain stem). It also influences hormones and neurotransmitter regulation in hypothalamus (reviewed in Ballaz, 2017). Cholecystokinin can activate some other pathways such as ERK and c-Jun N-terminal kinase (JNK) signaling. Through the PI3K-PKB-mTOR cascade, CCK can influence the initiation of translation (reviewed in Williams *et al.*, 2002). Due to its functions, CCK was suggested to be a target in the treatment of SCZ, mood disorders and drug addiction (Ballaz, 2017). Especially, the relation between CCK and the dopaminergic circuit was proposed to play a role in the development of SCZ. Concentration of CCK was showed to be lowered in some brain regions of untreated patients. CCK can display antipsychotic effects in some SCZ patients. Antipsychotic drug treatment can boost CCK and CCKR levels (Wang *et al.*, 1984).

➤ ERK mitogen-activated protein-kinase signaling pathway

Activation of the ERK pathway results from response of multiple effectors to various stimuli. These stimuli comprise Insulin-like Growth Factor (IGF1), Epidermal Growth Factor (EGF), neuregulins, neurotrophins, neurotransmitters, and increase in calcium concentration. They can affect receptor tyrosine kinases, glutamatergic NMDA receptors, voltage-gated L-type calcium channels, and G-protein coupled receptors. For instance, increased levels of calcium will lead to activation of calcium-Calmodulin Kinases (CaMKK, CaMKIV and CaMKII) and, subsequently, to activation of Rat Sarcoma Virus (RAS) G-protein that triggers Extracellular signal-Regulated Kinases (ERKs) signaling consisting of subsequent activation of three kinases: Rapidly Accelerated Fibrosarcoma (RAF), MAP-ERK kinase (MEK) and ERKs. The ERK signaling pathway was suggested to be involved in autism pathogenesis. Many mutations in ligand-gated and voltage-gated channels and some NMDA-related genes have been linked to autism. Some mutations in genes associated with RAS have been also connected to autism (reviewed in Kalkman, 2012). The ERK pathway has been also associated with other neurodevelopmental disorders, such as BD and SCZ. Yuan and collaborators (2010) measured the levels of different proteins of the ERK pathway in the postmortem cortex of patients with BD, SCZ, major depressive disorder, and healthy controls. The results showed that the levels of the majority of ERK-associated proteins were lower in patients with neurodevelopmental disorders than in the control group (Yuan *et al.*, 2010). Another analysis of postmortem prefrontal cortex showed that GNA13-ERK1-eIF4G2 signaling is downregulated in schizophrenic patients (Hirayama-Kurogi *et al.*, 2017). Ono *et al.* (2010) demonstrated the plausible neuroprotective role of Akt/ERK signaling in response to SCZ-like neuronal damage

in neural stem cells (Ono *et al.*, 2010). Investigation of the *DISC1* gene that is linked to SCZ revealed that DISC1 participates in astrogenesis in the embryonic brain by modulating RAS/MEK/ERK signaling via RASSF7 (Wang *et al.*, 2016).

➤ Muscarinic receptor pathway

The role of many neurotransmitters is under the scope of SCZ research. The most investigated is dopamine, but from some time other neurotransmitters, such as glutamate, γ -aminobutyric acid, serotonin, and Acetylcholine (ACh) started to be evaluated as well in this context. In this paragraph, I will concentrate on muscarinic ACh receptors that have been associated with SCZ.

ACh is a neurotransmitter that plays an important role in the CNS and in the peripheral nervous system. ACh is involved in memory processing, cognition, psychosis, mood regulation, and neuroplasticity. ACh can bind to pre- and postsynaptic receptors. We can distinguish five muscarinic receptors subtypes (M1 – M5), acting in the human CNS, that occur in different region-dependent concentrations. It is known that M1, M2 and M4 receptors are the most abundant in the CNS. Due to their role in CNS and multiple preclinical, clinical and *post-mortem* data suggesting their role in the pathogenesis of SCZ, it was proposed that they play a crucial role in its progression. This postulate is known as the muscarinic hypothesis of SCZ (Raedler, 2008).

It was demonstrated that muscarinic and dopamine receptors can regulate one another. Indeed, some agonist of muscarinic receptors have been reported to be able to

increase the level of dopamine. This effect is similar to the one observed with the use of some atypical antipsychotics. Moreover, M4 receptor KO mouse display hyperresponsiveness to dopamine D1 agonist (Bymaster *et al.*, 2002). There exist some molecules targeting the M4 receptor that have been promisingly evaluated as potential antipsychotic drugs (Chan *et al.*, 2008).

Vakalopoulos (2014) reviewed multiple genetic variations of small effect, which can influence functioning of muscarinic receptor pathway, such as polymorphisms and mutations in dysbindin and *DISC1* that can affect the capacity of muscarinic receptors signaling. Moreover, he described abnormalities in muscarinic receptors that have been related to frequently observed biochemical changes in SCZ, such as reduction in GABAergic markers or alterations in the level of interneuron calcium binding proteins, such as calbindin or parvalbumin. In addition, some pathways linked to mood regulation and addiction, such as ERK1/2 and PI3K/Akt, were proposed to be affected by muscarinic receptors aberrations. For instance, it was shown that the agonist of muscarinic receptors carbachol is able to activate ERK1/2 through MAPK (Vakalopoulos, 2014).

Interestingly, changes in the muscarinic receptor pathway were reported in the context of other neurodevelopmental disorders, such as ASD and FXS. For example, it was shown that the level of the M2 receptor is decreased in ASD patients (Perry *et al.*, 2001). Overactivity of muscarinic signaling has been reported in Fragile X KO mouse. Treatment with M4 agonist resulted in modulation of some behavioral traits of Fragile X KO mouse (Veeraragavan *et al.*, 2011).

c. Calcium dysregulations in neurodevelopmental disorders

Calcium plays a crucial role in neural and brain function by regulating neurotransmitter synthesis and secretion, coordinating action potential, participating in synaptogenesis, dendrite development, synaptic transmission and cell membrane excitability (reviewed in Rajagopal and Ponnusamy, 2017). It is also involved in generating brain rhythms, cognition, perception, learning, memory, and information processing. Alterations in calcium concentration can influence excitation/inhibition balance that governs brain rhythms essential for brain cognition and information processing (reviewed in Berridge, 2014). Dysregulations in calcium concentration that lead to hyperexcitability can cause neural cells damage due to excitotoxicity. Changes in calcium concentration associated with neuronal activity can impact neuronal gene expression. Many neuronal genes code transcription factors activating genes implicated in synaptic development and function, dendritic and axonal growth, neuronal plasticity, and cell survival. Calcium-dependent gene expression is initiated by elevation of intracellular calcium levels through calcium release from intracellular stores, or its extracellular entry via Voltage-Gated Calcium Channels (VGCCs), ligand-gated ion channels such as α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate type (AMPA) or the N-methyl-D-aspartate type (NMDA) glutamate receptors. Many channel characteristics such as duration of opening, conductance potential, association of channels with signaling agents determine if calcium influx triggers or not gene expression. For instance, L-type Voltage-Sensitive Calcium Channels (VSCCs) have high conductance for calcium and are localized in proximity of the somatodendric compartment. Such localization permits them to increase calcium levels in the nucleus neighborhood. On the other hand, many signaling molecules can

respond to activation of glutamate receptors such as the NMDA receptor. These signaling molecules are frequently related to the governance of activity-dependent transcription and include, among others, CaMKII, calmodulin, Tiam1, calcineurin, and proteins involved in Ras/MAP kinase signaling, comprising H-Ras and Erk1 and -2. Activation of some of the above molecules can influence the development and maturation of synapses (reviewed in Greer and Greenberg, 2008).

Due to the role of calcium in such a plethora of neuronal processes, alterations in calcium homeostasis have been related to pathogenesis of many neurodevelopmental disorders such as SCZ, BD, autism, epilepsy, Alzheimer's disease, amyotrophic lateral sclerosis, FXS, Parkinson's disease, and Huntington's disease (Berridge, 2014; Gargus, 2009; Rajagopal and Ponnusamy, 2017). Interestingly, there are mounting evidences suggesting the link between VSCCs and many neurological disorders (reviewed in Andrade *et al.*, 2019). Some SNPs in *CACNA1C* and *CACNB2* – subunits of L-type voltage-gated calcium-channels – were associated with SCZ and BD (Consortium, 2013). Moreover, some *de novo* mutations in *CaMKII* were found in patients with ID. These *de novo* mutations were shown to alter auto-phosphorylation and neuronal migration (Küry *et al.*, 2017). It was also shown that aberrations in MAPK signaling impact the severity of idiopathic autism (Rosina *et al.*, 2019). Dysfunction in calcium homeostasis in SCZ was meticulously reviewed by Lidow (2003). He described the numerous findings indicating the aberrations in multiple proteins associated with calcium signaling, such as Regulator of G protein signaling-4 (RGS4), Growth-associated protein 43 (GAP 43), S100 β , B-cell lymphoma/leukaemia-2 gene (Bcl-2), Wnt and glycogen synthase kinase-3 β (GSK-3 β), Reelin, Protein Kinase C (PKC), Parvalbumin and calbindin, Phospholipase A2 (PLA2), Gq, Gi,

and Go proteins, Dopamine, cAMP-regulated phosphoprotein of M 32 kDa (DARPP-32), and Synapsin I and II. Interestingly, the majority of calcium-related proteins and calcium-dependent processes play a role in multiple pathways and hypothesis previously linked to SCZ origins. Those pathways include dopamine signaling, GABAergic signaling, oxidative stress hypothesis, altered mitochondrial and cytosolic metabolism, and growth factor deficiency hypothesis (Lidow, 2003). If it comes to the dopamine pathway, a member of Wnt signaling - glycogen synthase kinase-3 β - is frequently a direct or indirect target of common antipsychotic drugs targeting dopamine signaling (*e.g.*, clozapine). It was described that activation of dopamine 2 receptors indirectly renders GSK-3 β more active. Also lithium, which is commonly used in BD treatments, is targeting GSK-3 β (reviewed in Singh, 2013). Souza *et al.* (2008) analyzed *GSK-3 β* markers in SCZ patients, clozapine-treated SCZ patients and control group. They found three SNPs that were associated with the SCZ phenotype. However, the results didn't indicate any association with clozapine treatment (Souza *et al.*, 2008). Decreased numbers of parvalbumin-immunoreactive neurons were found in the prefrontal cortex of individuals with SCZ (Beasley and Reynolds, 1997). Parvalbumin and calbindin were reduced in hippocampus area of isolated rats that exhibit behavioral and neurochemical deficits resembling the schizophrenic phenotype (Harte *et al.*, 2007). Also, in a mouse model of the 16p11.2 duplication that is linked to SCZ, parvalbumin and calbindin levels are decreased, and cognition and social deficits were observed (Bristow *et al.*, 2020). Decrease in parvalbumin-positive interneurons was also observed in the prefrontal cortex of individuals with autism (Hashemi *et al.*, 2017). The other group observed that the level of parvalbumin mRNA in Purkinje neurons of cerebellum was reduced in slices derived from ASD patients

(Soghomonian *et al.*, 2017). The above examples of various proteins and gene dysfunctions in calcium signaling show how multifactorial can be the origin of these impairments.

d. Overlap in Copy Number Variations

CNVs comprise DNA fragments duplications, deletions and insertions that are bigger than 1 kilobase and show different copy number when assembled to the reference sequence (Redon *et al.*, 2006). Most of CNVs encompass numerous different genes that make it extremely challenging to decipher the impact of CNVs on the phenotype (Rutkowski *et al.*, 2017). CNVs play important roles in the pathogenesis of many neurological disorders. CNVs occur more frequently in individuals with ASD, SCZ, ID, and epilepsy (reviewed in Betancur, 2011). Array-comparative genomic hybridization (aCGH), SNP-based microarray, fluorescence *in situ* hybridization (FISH), and next generation whole-genome sequencing are the most common methods of CNVs detection. FISH can detect changes between 5 -10 Mbp and aCGH between 10-25 kbp, which make them techniques of low resolution. Thanks to next generation sequencing methods even CNVs smaller than 10 kbp can be detected (Duan *et al.*, 2013; Merikangas *et al.*, 2009).

Duplications and deletions within the 1q21.1 chromosome region were identified in patients diagnosed with ID, autism, cardiac abnormalities, microcephaly, and congenital abnormalities (Kaminsky *et al.*, 2011; Mefford *et al.*, 2008). Microdeletions of different sizes in the same chromosomal locus were also reported in the study of SCZ patients and were suggested to correlate with this phenotype (Stefansson *et al.*, 2008).

Deletions within the 3q29 chromosome region were found during analysis of 245 SCZ patients and 490 controls individuals. In particular, 6 deletions within SCZ patients and one in control group were identified which suggested significant association with the SCZ phenotype (Mulle *et al.*, 2010). Also, Levinson *et al.* (2011) described microdeletions in SCZ patients that provided more evidence for the 3q29 region involvement in SCZ risk (Levinson *et al.*, 2011). Quintero-Rivera *et al.* (2010) reported two patients with a 3q29 microdeletion. The first patient was diagnosed with BD with psychotic features/SCZ and the second patient suffered from autism (Quintero-Rivera *et al.*, 2010). Deletion in the 3q29 region was also reported in a young patient with autism and early onset psychosis (Sagar *et al.*, 2013). Biamino *et al.* (2016) found a novel familial deletion in the 3q29 region. This deletion co-segregated in six members of a family. They were diagnosed with different psychiatric disorders comprising ID, developmental delay, autism, major depression, anxiety disorder, SCZ, and personality disorder. Interestingly, all patients with a detected 3q29 deletion were overweight and three patients presented optic atrophy. The authors suggested implication of three genes that are localized within the deleted region: *Optic Atrophy 1 (OPA1)*, *ATPase type 13A4 (ATP13A4)* and *Hes Family BHLH Transcription Factor 1 (HES1)*. *OPA1* is a gene linked to optic atrophy, while *ATP13A4* and *HES1* were proposed to be connected with a neuropsychiatric phenotype. In addition to the neuropsychiatric phenotype, *HES1* was associated with overweight (Biamino *et al.*, 2016). Rutkowski *et al.* (2019) generated a mouse model for the 3q29 deletion in heterozygous state. Mice heterozygous for the 3q29 deletion showed dysfunctions in social interactions, acoustic functions and cognition and had reduced body weight (Rutkowski *et al.*, 2019). In the same year, another group also engineered a mouse model for the 3q29 deletion. The phenotypes were similar to the one described by Rutkowski *et al.*. Mice with a deletion

presented reduced brain volume and body weight, impaired sensory-motor gating, dysfunction in social interactions, increase in self-grooming behavior, and impaired fear-related learning (Baba *et al.*, 2019).

Deletions within the chromosomal locus 15q11.2 together with deletions in the loci 1q21.1 and 15q13.3 were associated with SCZ during analysis of 3285 SCZ patients and 7,951 controls of European origin (Stefansson *et al.*, 2008). Zhao *et al.* (2013) analyzed CNVs at the 15q11.2 locus, encompassing the *NIPA1*, *NIPA2*, *CYFIP1*, and *TUBGCP5* genes, in a Chinese Han population. By comparing 2058 SCZ individuals with 3275 controls subjects, they found three times more deletions in the chromosomal region of interest in SCZ patients than in the control group. Moreover, they found association between two SNPs rs4778334 and rs1009153 in *CYFIP1* and the SCZ phenotype (Zhao *et al.*, 2013). Gudmundsson *et al.* (2019) were aiming at deciphering a common genetic background, in the context of CNVs, between ADHD and autism/SCZ. They found common CNVs shared among these three disorders like deletions at 1q21.1, 15q11.2, 15q13.3, and 16p11.2 (Gudmundsson *et al.*, 2019).

Isles *et al.* (2016) investigated the impact of parental origin of duplications at the 15q11.2-q13.3 locus on the risk of SCZ. They found that duplications inherited by the mother are more frequent in SCZ patients than the ones inherited from the father. Maternally inherited duplications also turned out to have higher penetrance. Interestingly, paternal inheritance of duplications was found to increase the risk of ASD, developmental delay and multiple genital anomalies. Nevertheless, penetrance of maternally inherited duplication in these neurological disorders was higher than penetrance of paternal origin, which emphasizes the impact of maternally inherited CNVs in neurodevelopmental disorders (Isles *et al.*, 2016).

The impact of parental provenance of duplication in this region was first described in 1997. Cook *et al.* associated maternally inherited duplication with autistic phenotype, whereas paternally inherited duplications did not lead to an abnormal phenotype (Cook *et al.*, 1997).

22q11.2 deletion syndrome (22q11.2DS) was demonstrated to be common among SCZ patients (Bassett and Chow, 2008; Bassett *et al.*, 2005). Monks *et al.* confirmed that 22q11.2 deletion syndrome is associated with higher rates of psychotic disorder, in particular, with SCZ (Monks *et al.*, 2014). The deletions in 22q11.2 were also suggested to be higher in Childhood Onset Schizophrenia (COS; Usiskin *et al.*, 1999). On the other hand, large duplications at 22q11.2 were suggested to lower the risk of SCZ. It was shown, that duplications at this locus are more common in the general population than in SCZ patients (Rees *et al.*, 2014). Patients with the 22q11.2 deletion syndrome are also frequently diagnosed with ADHD, anxiety disorders and ASD (Serur *et al.*, 2019).

Some rare CNVs (7q11.22 and 7q35) identified in epileptic patients were shown to overlap genes that were previously linked to autism and ID (*AUTS2* and *CNTNAP2*). In cohort with idiopathic non-lesional epilepsies analyzed by Mefford *et al.*, 2.9% of patients possessed deletions in genomic hotspots that were formerly related to SCZ, ID or autism (Mefford *et al.*, 2010).

The above examples describe only some CNVs co-occurring in various neurodevelopmental disorders. This co-occurrence of the same CNVs in different neurodevelopmental disorders confirms the genetic overlap. Multitude of studies showed that genetic variations and phenotypic features between neurodevelopmental disorders often

intertwine with each other making the elucidation of genetic causes an extremely laborious and challenging task.

Childhood Onset Schizophrenia

COS is an extremely rare mental disorder that is a form of SCZ manifesting in children before the age of 13. In the general population COS occurs in 1 out of 10,000 individuals (Burd and Kerbeshian, 1987; Gochman *et al.*, 2011). The diagnosis of COS is based on the same criteria as for adolescents and adults diagnosed with SCZ. SCZ with onset between 13 and 18 years old is referred as Early Onset SCZ (EOS) (Grover and Avasthi, 2019). According to the Diagnostic and Statistical Manual of Mental Disorders V (DSM-V), SCZ patients have to exhibit at least one of the following features: hallucinations (perception without external stimulus that seems to be real), delusions (firm convictions based on fake information that cannot be changed when confronting the reality) or disorganized speech (*e.g.*, changing from one subject to another). SCZ patients can also present abnormalities in motor behavior and negative symptoms. Negative symptoms include lack of interest in social activities and interactions, reduction in showing feelings, lower self-motivation, poverty of speech content, and anhedonia (Association, 2013). The diagnosis of COS is very challenging due to the fact that around 30% and up to 50% of individuals suffering from affective or other atypical psychotic symptoms were misdiagnosed as COS (Driver *et al.*, 2013). Before introduction of DSM-III, the diagnostic criteria for COS had not been established (Asarnow and Forsyth, 2013). Moreover in the National Institute of Mental Health (NIMH) studies of COS cohort, 90% of patients initially diagnosed with COS received alternative diagnosis in the follow up studies (Driver *et*

al., 2013). Additionally, only one third of articles relate to COS full phenotype of patients and describe its association with mutations (Fernandez *et al.*, 2019). The occurrence of COS is usually preceded by a period of normal development. Sometimes, atypical interests and beliefs can be confused with social deficits typical for ASD patients (Association, 2013). Around 27% of SCZ patients before manifestation of the first psychotic symptoms meet criteria for ASD (Driver *et al.*, 2013). Hallucinations and delusions are traits that can help to distinguish COS patients from ASD ones (Association, 2013).

Diagnosis of COS is challenging not only due to its co-occurrence with other psychiatric disorders, but also because of its genetic heterogeneity (Fernandez *et al.*, 2019). Genetic studies of COS patients suggest that rare copy number variations are more common in COS than in Adult Onset Schizophrenia (AOS). Some of these rare CNVs were also associated with ASD and ID (Addington and Rapoport, 2009). COS is thought to have higher familial transmission and higher prevalence of rare allelic variants than AOS. Risk for Schizophrenia Spectrum Disorder (SSD) in parents of COS patients is higher than in parents of AOS. SSD encompasses schizoaffective disorder, schizopreniform and atypical psychosis, schizotypal personality disorder, and paranoid personality disorder (reviewed in Asarnow and Forsyth, 2013). Moreover, Rasic *et al.* demonstrated that the progeny of parents suffering from SCZ, major depressive disorder or BD were having more chances of developing psychiatric disorders. Children of parents with SCZ had a higher risk of mood disorder. Parental BD predisposed children to higher risk of SCZ. On the other hand, offspring of parents suffering from depression did not have an increased risk of SCZ (Rasic *et al.*, 2014). COS was associated with common polymorphisms in *Glutamate decarboxylase 1 (GAD1)*, *dysbindin-1 (DTNBP1)*,

Neuregulin 1 (NRG1), *G72/G30*, and *Brain-Derived Neurotrophic Factor (BDNF)*. A GWAS study of AOS involving more than 16,161 individuals did not indicate significant changes. For this reason, and due to a low prevalence of COS, GWAS studies for COS have not been performed yet (reviewed in Asarnow and Forsyth, 2013).

Autism spectrum disorder

ASD is a neurodevelopmental disorder that is characterized by social communication and interaction deficits, repetitive behavior, and restricted interests and activities. Patients have also problems with nonverbal communication and with understanding and developing relationships. The first symptoms appear between one and two years of age. Around 70% of ASD patients display one comorbid mental disorder, and 40% can have two or more comorbid mental disorders. ASD often co-occurs with ID, language impairments and ADHD. Many patients present as well motor deficits, self-injury and disruptive behaviors. ASD patients are also more likely to suffer from depression and anxiety (Association, 2013). Data from the Danish population suggested a correlation between ASD and IQ, educational attainment, self-reported tiredness, neuroticism, SCZ, major depression, depressive symptoms, and ADHD (Grove *et al.*, 2019). Prevalence of autism in the United States is approximately 1% of the population (Association, 2013). This disorder occurs three times more often in males than in females (Loomes *et al.*, 2017). In some cases, other neurodevelopmental disorders can be confused with ASD, which can lead to misdiagnose. This confusion includes Rett syndrome, selective mutism, some forms of language disorders, ID without ASD, ADHD, and SCZ.

Heritability is estimated to range from 37% up to 90%, according to twin studies. The genetic background of ASD is barely known. Only 15% of ASD cases are linked to a known mutation, *de novo* CNVs or *de novo* variants specific for particular families. Most of these associations seem not to be fully penetrant. There exists high possibility that most of the cases are polygenic, which means that multiple genetic *loci* can be implicated and have small contribution to the general phenotype (Association, 2013). The majority of risk factors for ASD are also present in healthy individuals. Some of these risk factors can be linked to specific features, *e.g.*, around one quarter of SNPs linked to ASD overlap with social and communication disorders. *De novo* loss of function heterozygous missense variants that are not present in the Exome Aggregation Consortium (ExAC) database are more frequent in ASD patients compared to the control group (Robinson *et al.*, 2016). On the other hand, analysis of ASD patients from the Danish population indicated that *de novo* variants are more frequent when ASD is accompanied with ID (Grove *et al.*, 2019).

Epilepsy of infancy with migrating focal seizures

Epilepsy of Infancy with Migrating Focal Seizures (EIMFS), known as well as “Migrating partial seizures of infancy”, belongs to a group of rare epileptic syndromes. It is characterized by the onset of seizures before 6 months of age, decline of psychomotor development, high death rate and electroencephalograms presenting a specific ictal pattern with migrating multifocal discharges. Most patients display poor response to multiple antiepileptic drugs (Caraballo *et al.*, 2015; McTague *et al.*, 2013; Shang *et al.*, 2016). Some patients display infantile spasms, microcephaly, scoliosis, and hypsarrhythmia (Barcia *et al.*, 2019; Burgess *et*

al., 2019). For the time being, the occurrence of EIMFS has been associated with mutations found in certain genes like: *Potassium Sodium-Activated Channel Subfamily T Member 1 (KCNT1)*, *Sodium Voltage-Gated Channel Alpha Subunit 1 (SCN1A)*, *Sodium Voltage-Gated Channel Alpha Subunit 2 (SCN2A)*, *Sodium Voltage-Gated Channel Alpha Subunit 3 (SCN3A)*, *Sodium Voltage-Gated Channel Alpha Subunit 9 (SCN9A)*, and *Gamma-Aminobutyric Acid Type A Receptor Subunit Beta 3 (GABRB3)*, but the genetic background of the disease still remains unclear (Burgess *et al.*, 2019; Fry *et al.*, 2016; Howell *et al.*, 2015; Ohba *et al.*, 2015; Štěřbová *et al.*, 2018). *KCNT1* is considered the most common causative gene followed by *SCN2A*. In the past recent years, thanks to the expansion of Whole Exome Sequencing (WES) techniques new variants associated with EIMFS were revealed in the *SLC12A5* gene that codes the neuronal potassium chloride co-transporter KCC2 (Stödberg *et al.*, 2015; Saitsu *et al.*, 2016).

Fragile X-associated tremor/ataxia syndrome

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder characterized by gait ataxia, tremor, Parkinsonism, memory deficits, and cognitive decline (Berman *et al.*, 2014; Hessler and Grigsby, 2016). Males have more chances of developing this disorder (Berman *et al.*, 2014). FXTAS is linked to the presence of a premutation in the *Fragile X Mental Retardation (FMR1)* gene. This gene is located on the X chromosome and in normal conditions has from 5 up to 40 CGG repeats in its 5' untranslated region (5' UTR). The expansion of this trinucleotide repeat element to more than 200 CGG repeats is considered as a full mutation and leads to disclosure of FXS that is the most common form of ID and ASD. Presence of a full mutation causes inactivation of the *FMR1* gene due to its hypermethylation

at the promoter region (Bardoni *et al.*, 2000; Hagerman, 2013). The premutation state in the *FMR1* gene can differ between 55 and 200 CGG repeats. At earlier stage of life, carriers of a premutation can present various neurodevelopmental phenotypes, such as ADHD, ID, ASD, higher frequency of seizures in childhood, and attention deficits. At a later stage of life they are more prone to develop FXTAS or Fragile X-associated Primary Ovarian Insufficiency (EXPOI). The two last phenotypes are considered specific to the premutation range (Berman *et al.*, 2014; Hagerman, 2013). FXTAS is thought to have its origin in an earlier period of development. Sequestration of some nuclear proteins participating in the biogenesis of microRNA and mitochondrial dysregulation have been proposed to be the main processes underlying the development of FXTAS (Hagerman, 2013). Development of a mouse model of FXTAS enabled to get a deeper insight into its pathogenesis. This CGG knockin model displays elevated levels of mRNA, normal or decreased levels of Fragile X Mental Retardation Protein (FMRP) and intranuclear inclusions (Berman *et al.*, 2014). Elevated mRNA levels of *FMR1* are also increased in FXTAS patients (Tassone *et al.*, 2000). Moreover, dendritic spines show abnormal morphology, which can affect calcium homeostasis and electrical network activity (Berman *et al.*, 2014). The other process that is associated with FXTAS pathogenesis is Repeat-associated non-AUG (RAN) translation. Translation stimulated by CGG repeats leads to creation of FMRP containing poly-glycine or poly-alanine that participate in the creation of intraneuronal inclusions. This mechanism is thought to underlie other neurodevelopmental diseases, such as Huntington's disease or myotonic dystrophy (Kearse *et al.*, 2016).

Serine/threonine protein kinase 33

STK33 localization and expression

STK33 was first described as a gene that encodes a serine/threonine protein kinase. It is located on human chromosome 11p15.3 and its mouse orthologue is found on chromosome 7. This gene has been assigned to the calcium/calmodulin-dependent protein kinase group (Mujica *et al.*, 2001). Concerning its expression, *STK33* was shown to be expressed in heart, intestine, brain, retina, kidney, ovary, testis and lungs of adult mouse. In mouse embryos, particularly strong expression of *STK33* was demonstrated in head regions like pons, medulla oblongata and intermediate zones of mesencephalon and medulla. Apart from this, a strong signal was observed in the heart (Mujica *et al.*, 2005). Presence of *STK33* was also shown in the brains of rats and hamsters. It is the most abundant in cerebral ventricles, central canal of the spinal cord and hypothalamus (Brauksiepe *et al.*, 2014). Moreover, in 2017 its expression was also demonstrated in the human brain (Reuss *et al.*, 2017).

STK33 functions

STK33 is involved in many different pathways and functions in cells (Fig. 1). Initially, due to its high expression in testis, *STK33/Stk33* was suggested to play a role in spermatogenesis and organ ontogenesis (Mujica *et al.*, 2005). Later, *STK33* started to be associated with different types of cancers. In 2009 Scholl *et al.*, suggested that *STK33* boosts viability and proliferation of mutant KRAS-dependent cells (Scholl *et al.*, 2009). *STK33* was shown to participate in tumorigenesis of Hypopharyngeal Squamous Cell Carcinoma (HSCC).

In the context of this type of cancer, it was demonstrated that STK33 has impact on Ca²⁺ homeostasis in Fadu cells. *STK33* expression can be increased by ionomycin treatment. The highest increase was observed after 2 and 4 hours of treatment (Chen *et al.*, 2016). In Fadu cells, inhibition of ERK1/ERK2, apart from decreasing *ERK1/ERK2* expression, caused reduction of STK33. On the other hand, silencing of *STK33* didn't impact the protein level of ERK1/ERK2 (Huang *et al.*, 2015). It is known that aberration in Ras-RAF-ERK signaling can have positive impact on glioma progression. For instance, the activity of upstream player of Ras-RAF-ERK axis EGFR is increased in the majority of gliomas (Lo, 2010). Elevated expression of *STK33* has been associated with pancreatic and colorectal carcinomas at advanced stages (Liu *et al.*, 2017). *STK33* can bind to c-Myc and enhance its transcriptional activity (Zou *et al.*, 2016). Downstream signaling of this pathway can influence Mitochondrial Outer Membrane Permeabilization (MOMP). MOMP can be triggered through activation of BAX by p53 (Zhang *et al.*, 2016). On the other hand, *STK33* can stimulate viability and proliferation of cancer cells in mutant KRAS-dependent cells by suppressing apoptosis of mitochondria through activation of S6K1, which in turns inactivates the death activator BAD (Scholl *et al.*, 2009).

STK33 participates in promoting cell migration and invasion of lung cancer cells. *STK33* has impact on activation of transcription factors such as Twist, SNAIL and SLUG that regulate the Epithelial-Mesenchymal Transition (EMT) (Wang *et al.*, 2015; Yu *et al.*, 2010). These three transcription factors can regulate the expression of E-cadherin, which is a tumor suppressor (Yu *et al.*, 2010). It was demonstrated that reduction of *STK33* expression by RNAi caused increase in E-cadherin expression in Fadu cells (Huang *et al.*, 2015).

Chaperone complex of HSP90/CDC37 can bind to STK33 and stabilize it in human cancer cells. Inhibition of HSP90 causes degradation of STK33 by the proteasome (Azoitei *et al.*, 2012). In hypoxic tumors, degradation of STK33 caused decrease in HIF-1 α accumulation and VEGF secretion, essential processes during tumor blood vessel formation (Liu *et al.*, 2017).

In 2008, it was demonstrated that STK33 can phosphorylate vimentin and by this interaction it can indirectly influence the dynamics of the cytoskeleton. Through experiments showing interactions between STK33 and vimentin, it was revealed that autophosphorylation of STK33 is thought to be crucial for its kinase activity (Brauksiepe *et al.*, 2008). In 2014, STK33 and vimentin were demonstrated to colocalize. STK33 turned out to participate in polymerization and depolymerization of vimentin (Brauksiepe *et al.*, 2014). Vimentin is participating in the transport of cholesterol. By impacting vimentin, STK33 can potentially influence steroid hormone synthesis (Sarria *et al.*, 1992; Zhang *et al.*, 2018). Apart from this, it was suggested that Stk33 has impact on photoperiodic regulation of the endocrine system (Brauksiepe *et al.*, 2014). In 2017, Reuss *et al.* showed that Stk33 is partially colocalized with oxytocin, neuronal nitric oxide-synthase and arginin-vasopresin in hypothalamic neurons. They demonstrated that axons of Stk33-immunoreactive neurons can be involved in projections between hypothalamus and neurohypophysis. They suggested that those projections can be implicated in the passage from neuronal to humoral regulation of the endocrine system (Reuss *et al.*, 2017).

STK33 is involved in the TTC36-STK33-PELI1 signaling pathway in liver. Aberrations in functioning of this axis can cause learning and memory deficiency in *Ttc36*^{-/-} mice. In these mice, the lack of TTC36 caused impairments in phosphorylation of 4-hydroxyphenylpyruvic

acid dioxygenase (HPD) by STK33 (Xie *et al.*, 2019). This observation proves that STK33 can be associated with neurological disorders.

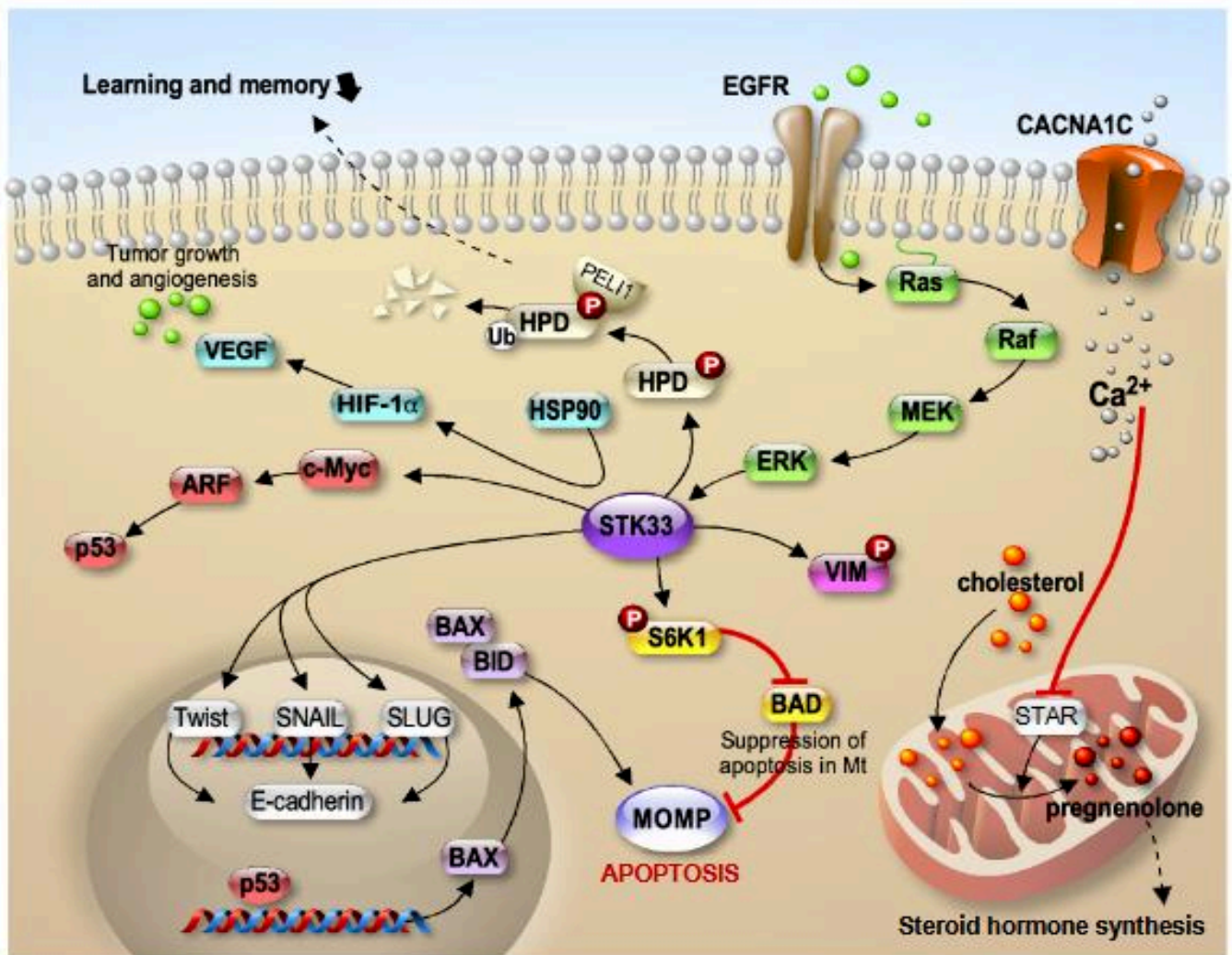


Figure 1. STK33 involvement in different pathways in cells.

STK33 genetics

So far, only few articles have related *STK33* with neurological disorders. *STK33* lies within the 11p15.4 microduplication region that was previously linked to ID, dysmorphic features and obesity (Sofos *et al.*, 2012). In 2015, *STK33* was reported to be present within the

CNV region in one SCZ patient from a Canadian cohort (Warnica *et al.*, 2015). One year after, a variant in *STK33* was detected during GWASs of patients with psychiatric diseases. This variant was found in three individuals from the same family that were diagnosed with SCZ or schizoaffective disorders. This variant was a 15 base pair deletion in the 5'UTR of the gene and was characterized as a potentially disruptive splice variant. Due to the fact that it was localized before the translated part of the protein, upon further analysis it was classified as a variant of dubious validity. However, from almost ninety individuals from nine families, it was the only non-SNP variant that was present in the candidate gene set (Homann *et al.*, 2016). In 2017, Utine *et al.* described a patient with ID who had a missense variant in *STK33* (c.1172C > T; p.Pro391Leu). Poly-Phen and MutationTester analysis indicated this variant as possibly damaging and disease-causing, respectively, whereas SIFT analysis considered this variant as a tolerated one. This variant was not associated with disease in this patient due to presence of a variant in *HERC1*, which was previously linked to ID and seemed to be a better candidate (Utile *et al.*, 2017). Leonenko *et al.* performed the largest analysis of the exomic variation in SCZ patients. *STK33* showed up in the set of 12 genes that were statistically closest to reach significant association (Leonenko *et al.*, 2017). Recently, *STK33* has been associated with suicide attempt behavior in a Mexican population. The majority of variants found in this study were intronic ones, just one of them was found in coding sequences (González-Castro *et al.*, 2019). Interestingly, individuals with SCZ and autism seem to have higher risk for developing suicide behavior (Chen *et al.*, 2017; Fuller-Thomson and Hollister, 2016; Kato *et al.*, 2013; Mayes *et al.*, 2013).

Cation Chloride Cotransporter family

The Cation Chloride Cotransporters (CCCs) family consists of nine members: four Na⁺-independent K⁺-coupled Cl⁻ co-transporters KCC1 (SLC12A4), KCC2 (SLC12A5), KCC3 (SLC12A6), and KCC4 (SLC12A7); two Na⁺-dependent Cl⁻ transporters NKCC1 (SLC12A2) and NKCC2 (SLC12A1); Na⁺-Cl⁻ co-transporter (NCC; SLC12A3), CCC8 (SLC12A9) and CCC9 (SLC12A8). The roles of the two last co-transporters have not been fully elucidated yet. CCCs are glycosylated proteins that have similarly predicted membrane topology, predicting 12 transmembrane domains. They participate in neuronal signaling, cell volume and blood pressure regulation, ion transport and neuroendocrine signaling (reviewed in Kaila *et al.*, 2014 and Marcoux *et al.*, 2017).

NKCC1

NKCC1 is located on chromosomal locus 5 q23.3 and is 1212 amino acids long (omim.org; UNIPROT). *NKCC1* is involved in chloride secretion, maintenance of potassium and chloride homeostasis in the brain, and cell volume regulation (Randall *et al.*, 1997). During mouse development, it is widely expressed in the CNS and other organs and tissues, such as gut, bladder, bones, trachea, kidney, and adrenal gland (Hübner *et al.*, 2001a).

Due to its role in the maintenance of excitation/inhibition balance, *NKCC1*, together with *KCC2*, was associated with epilepsy. *NKCC1* level was found raised in patients with hippocampal sclerosis and focal cortical dysplasia. These two disorders are linked to pharmaco-resistant epilepsy (Sen *et al.*, 2007). Altered expression of *NKCC1* and *KCC2* was

found in malformations of cortical development in patients with intractable epilepsy. These malformations are known to underlay intractable epilepsy (Aronica *et al.*, 2007). Some antiepileptic drugs were shown to act on CCCs. For instance, bumetanide is a selective inhibitor of *NKCC1* and furosemide is a non-selective inhibitor of CCCs. In 2005, furosemide was shown to block epileptic activity in brains of patients with temporal lobe epilepsy (Haglund and Hochman, 2005). A few years after, it was demonstrated that bumetanide can decrease seizure frequency in patients suffering from temporal lobe epilepsy (Eftekhari *et al.*, 2013).

For the time being, there are not many mutations described in the *NKCC1* gene. A *de novo* mutation in *NKCC1*, causing truncation of the protein, was identified in a girl suffering from multisystemic dysfunctions including exercise intolerance, seizure-like episodes, obstructive apnea, cardiomyopathy and episodes of vomiting and dehydration. The expression of mutant cDNA in oocytes of *Xenopus laevis* revealed that the mutated version of the *NKCC1* co-transporter is non-functional. When the mutated version was co-expressed with wild-type version of *NKCC1*, it did not lead to dominant-negative effects. The analysis of patient's fibroblasts showed that almost half of potassium influx was ouabain- or bumetanide-sensitive and treatment with these two agents led to an 86% decrease in potassium flux. These data demonstrate that the allele containing the mutation is not functional, but does not dominate over the wild-type allele (Delpire *et al.*, 2016). In 2016, Merner and collaborators found a heterozygous missense mutation in *NKCC1* (p.Y199C) among the SCZ patients. Further analysis of this mutation showed that it increases bumetanide-sensitive and chloride-dependent activity of the *NKCC1* co-transporter (Merner *et al.*, 2016). Previously, alterations

in mRNA expression of *NKCC1* were associated with increased risk for SCZ (Morita *et al.*, 2014). Recent studies described *NKCC1* mutations in patients suffering from gastrointestinal deficits and non-syndromic hearing loss (Koumangoye *et al.*, 2020; Morgan *et al.*, 2020).

KCC3

KCC3 was predicted to have 1083 aa. The human cDNA sequences of *KCC3* and *KCC2* are 65% identical. *KCC3* and *KCC4* share 66% identity between their sequences (Mount *et al.*, 1999). *KCC3* is localized on chromosome 15 (Race *et al.*, 1999). In humans, the *KCC3* transcript is broadly distributed in testis, salivary gland, adipose tissue, thyroid, spleen, brain, and kidney (reviewed in Garneau *et al.*, 2017). RNA *in situ* hybridization of mouse embryos indicated a robust presence of *Kcc3* mRNA in the nervous system (Boettger *et al.*, 2003).

Mutations in *KCC3* were linked to Anderman syndrome (Uyanik *et al.*, 2006). Disruption of *Kcc3* in mice leads to aberrant cell volume regulation and slightly increases interneuronal chloride levels. These mice display motor defects, neurodegeneration in peripheral and CNS, slowly progressive deafness, reduced seizure threshold, and arterial hypertension (Boettger *et al.*, 2003).

Impairments in KCC3 activity are linked to Hereditary Motor and Sensory Neuropathy with Agenesis of the Corpus Callosum (HMSN/ACC). Patients with HMSN/ACC present dysmorphic features, ID, hallucinations, and developing sensorimotor neuropathy. Howard *et al.* identified four mutations in *KCC3* in patients suffering from HMSN/ACC. They further evaluated the impact of a predominant mutation at position 2436delG causing a change

Thr813fsX813. Analysis of *Xenopus leavis* oocytes injected with mutated *KCC3* didn't reveal changes neither in glycosylation nor in localization at the membrane level. However, functional analysis demonstrated dysfunctions of the co-transporter. The authors engineered the *Kcc3*-KO mouse that showed deficits in sensorimotor gating, locomotion and central nervous system functions (Howard *et al.*, 2002). In the context of HMSN/ACC, it was shown that inhibition of *KCC3* in the peripheral nervous system may diminish the diffusion of the action potential. Analysis of *KCC3*^{-/-} peripheral nerves revealed that axonal excitability of these nerves was decreased and paranodal conductance was increased (Sun *et al.*, 2016). Kahle *et al.* (2016) reported a mutation in an individual with HMSN/ACC that affects a phosphorylation site in *KCC3* (c.2971A>G; Thr991Ala). The phenotype of mice with this mutation reflects the phenotypic features of the patient (Kahle *et al.*, 2016).

KCC4

KCC4 has about 1150 amino acids and is localized on chromosome 5 (Mercado *et al.*, 2000; Mount *et al.*, 1999). In humans, it is mainly expressed in kidney, placenta and brain (Mount *et al.*, 1999). In mouse, it is highly expressed in brainstem and midbrain, whereas in the hippocampus, cerebellum and cortex regions it is poorly expressed (Karadsheh *et al.*, 2004). In adulthood, *KCC4* has lower expression than its family members *KCC1*, *KCC2* and *KCC3* (Marcoux *et al.*, 2017). *KCC4* shares high homology with *KCC1*, *KCC2* and *KCC3* (Mount *et al.*, 1999). *KCC4*, as previously mentioned, is a glycosylated protein. Disruption of *KCC4* N-linked glycosylation sites prevents membrane localization and causes accumulation in the endoplasmic reticulum. Moreover, it leads to impairments of transporter activity (Weng *et al.*,

2013). Disruption of *KCC4* in mice causes loss of hearing and renal tubular acidosis, potentially due to chloride recycling defects (Boettger *et al.*, 2002).

KCC2 functions

KCC2 is a neuron-specific K-Cl co-transporter that is responsible for extrusion of Cl⁻ from cells. In order to pump out the chloride ions from cells, the K-Cl co-transporter makes use of K⁺ gradient kept by Na⁺, K⁺-ATPase. Contrary to Na⁺, the K⁺-ATPase K-Cl co-transporter can transport ions in and out of the cells. KCC2 has high affinity for external potassium ions. In conditions in which external K⁺ is elevated (>5 mM), KCC2 can have an impact on the net K-Cl co-transport and change the direction of K⁺ flux from efflux to influx (Williams *et al.*, 1999). This protein participates in the maintenance of chloride and other ions homeostasis in neurons, water balance, protection of neuronal networks against excitotoxicity, dendrite morphogenesis, and creation of synaptic connections. It is also involved in postsynaptic inhibition by creating the chloride ion gradient via interaction with GABA neurotransmitter and glycine receptors (Li *et al.*, 2007; Pellegrino *et al.*, 2011; Williams *et al.*, 1999). In the normal condition, when the chloride level is low, activation of the GABA_A receptor causes elevated conductance of the membrane and chloride influx into the neuron, which results in hyperpolarization of the postsynaptic membrane. This process is not prone to trigger an action potential and due to this it is defined as a GABAergic inhibition. Activation of the GABA_A receptor leads to opening of the channels permeable to chloride and bicarbonate. GABA_A receptor-mediated inhibition efficacy depends on low levels of intracellular chloride ions. The level of chloride ions is controlled by KCC2 and NKCC1 activity (Fig. 3). Contrary to KCC2, NKCC1

is responsible for chloride influx into the cells. As mentioned before, chloride influx into the cells triggers a postsynaptic hyperpolarization. Deregulations in the level of intracellular chloride ions can impact GABAergic inhibition (Hamidi and Avoli, 2015; Schulte *et al.*, 2018). For these reasons, KCC2 is crucial for maintenance of the excitation/inhibition balance within cells. The perturbation in this balance is thought to underlie many central nervous system pathologies, including epilepsy, SCZ and autism (Fukuda and Watanabe, 2019).

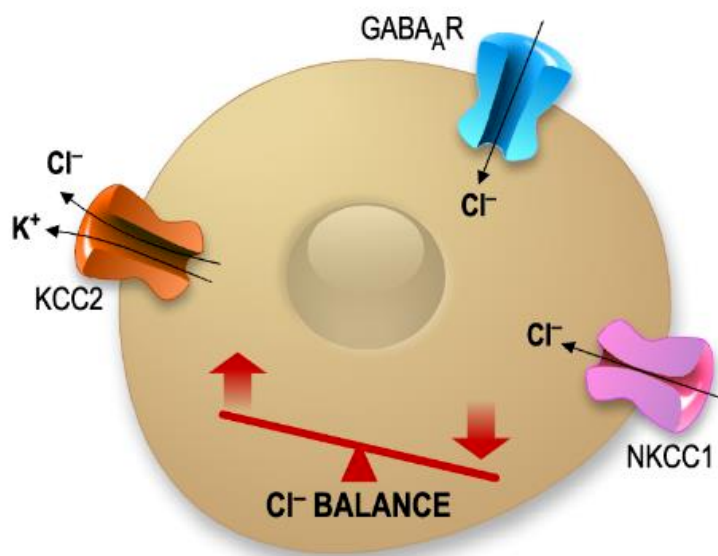


Figure 3. KCC2 and NKCC1 co-transporters anchored at the plasma membrane. GABA_A receptor-mediated currents depend on the chloride balance maintained by KCC2 and NKCC1 activities.

KCC2 localization and expression

The *Kcc2* gene consists of 24 coding exons and together with its intronic part it occupies more than 30kb of genomic DNA (Song *et al.*, 2002). Prediction of the KCC2 protein suggests

that it is composed of 12 transmembrane domains that are surrounded by N- and C-terminal cytoplasmic regions (Payne *et al.*, 1996).

Williams *et al.* developed a KCC2 antibody. They described KCC2 as a glycoprotein with molecular weight of 140-kDa that is uniquely detectable within the nervous system. Immunolocalization studies in rats showed that KCC2 is abundant in dendrites and in the soma of neurons suggesting a specific postsynaptic distribution of the protein. KCC2 was not detected in axons and glia (Williams *et al.*, 1999). KCC2 has two neuron-specific isoforms: KCC2a and KCC2b. In the N-terminus, KCC2b is 40 amino acids longer than KCC2a. This part of KCC2b encompasses a potential Ste20-related proline alanine-rich kinase/oxidative stress response-1 (SPAK/OSR1) binding motif. KCC2a is mainly present during the neonatal period of mouse development whereas the KCC2b isoform starts to dominate in mature mouse. During the neonatal period, both isoforms colocalize in the same brain regions. Co-immunoprecipitation analysis revealed that these two isoforms of Kcc2 can create endogenous heteromers. The ability to form a heteromeric complex was also demonstrated during co-expression of Kcc2a and Kcc2b in the HEK293 cell line (Uvarov *et al.*, 2009). Mice without the first exon of KCC2b can survive up to two weeks. However, KCC2b is essential for the functioning of neurons in cortex (Uvarov *et al.*, 2007). *Kcc2* is fully expressed in the brain by the end of post-natal day (PND) 14 (Khirug *et al.*, 2005).

KCC2 transcription can be triggered by the transcription factor *Early growth response 4 (Egr4)* that is a downstream member in the BDNF-TrkB signaling pathway. This factor was shown to have an expression pattern similar to *KCC2* during development. *Upstream stimulating factors 1 (USF1)* and *2 (USF2)* can also regulate the expression of *KCC2* through

binding to a E-box control element that is in the promoter region of the *KCC2* gene (Markkanen *et al.*, 2008). *In vitro* studies showed that overexpression of *Neuron Restrictive Silencer Element (NRSE)* can diminish *KCC2* promoter activity in N2a cells. On the other hand, in transgenic mouse lacking NRSE, *KCC2* expression was still restricted to the CNS, which indicates that this element is not indispensable for neuron-specific localization (Uvarov *et al.*, 2005).

KCC2 – post-translational modifications

a. Phosphorylation

There is a growing amount of evidence showing that (de)-phosphorylation of KCC2 plays an important role in Cl⁻ extrusion from the cells by impacting the speed of transport and KCC2 affinity for ionic substrates. Moreover the changes in (de)-phosphorylation can influence the amount of co-transporters at the membrane surface through its effect on proteomic cleavage and endocytosis (Kahle *et al.*, 2013).

Recent evidence suggests that the intrinsic ion transport rate, cell surface stability, and plasmalemmal trafficking of KCC2 are rapidly and reversibly modulated by the (de)-phosphorylation of critical serine, threonine and tyrosine residues in the C-terminus of this protein.

Oxidative stress impacts on phosphorylation of KCC2. The level of phosphorylated form of KCC2 decreases upon exposure to H₂O₂ (6h treatment). It was also shown that, upon a 12h treatment with H₂O₂, the KCC2 level at the membrane of hippocampal neurons decreases.

Interestingly, when incubated with the specific inhibitor of tyrosine phosphatases sodium vanadate (Na_3VO_4) and with H_2O_2 , the membrane level of KCC2 was restored. These findings suggest that the tyrosine phosphorylation level of KCC2 can influence membrane localization (Wake *et al.*, 2007).

BDNF was proved to provoke diminution of KCC2 tyrosine phosphorylation levels in rat hippocampal slices by affecting tyrosine receptor kinase B (TrkB). In addition, focal electrical stimulation of the brain led to a decline in the level of KCC2 and increase of BDNF and TrkB (Rivera *et al.*, 2002). Lack of Mg^+ can trigger interictal-like activity in hippocampal rat slices (Rivera *et al.*, 2004). Exposure to BDNF and lack of Mg^+ caused reduction in KCC2 at the membrane level. Moreover, they decreased the level of phosphorylation of KCC2 (Wake *et al.*, 2007).

Lee *et al.* (2010) demonstrated that the C-terminal intracellular tyrosine residues 903 and 1087 are the principal tyrosine phosphorylation sites of KCC2 in HEK-293 cells. They showed that phosphorylation of these sites decreases the level of KCC2 at the membrane. They suggested that this phosphorylation boosts the lysosomal degradation of KCC2 through activation of the muscarinic acetylcholine receptors (mAChRs) due to the fact that these receptors are having a strong potential to activate tyrosine phosphorylation signaling pathways. To prove it, they performed an experiment showing that stimulation of mAChRs with their agonist – pilocarpine - induces KCC2 tyrosine phosphorylation and lysosomal degradation in cultured hippocampal mice neurons (Lee *et al.*, 2010). Interestingly, earlier studies performed by Lee *et al.* indicated that protein kinase C-dependent phosphorylation of tyrosine residue 940 of KCC2 can increase its stability at the membrane level by reducing the

level of its endocytosis (Lee *et al.*, 2007). In 2015, it was shown that in the cultured hippocampal neurons the serine-threonine kinase WNK1 (with no lysine) can create a complex with KCC2. WNK1 pathway activation was related to increased phosphorylation at threonine residues 906 and 1007 in immature neurons. As a consequence, it caused elevated KCC2-dependent Cl⁻ extrusion followed by hyperpolarization of GABA receptors. These results indicate the implication of WNK1-dependent phosphorylation of KCC2 during the inhibitory/excitatory GABA switch (Friedel *et al.*, 2015).

b. Glycosylation

Initially, KCC2 has been demonstrated to be a glycoprotein through deglycosylation experiments on KCC2 isolated from HEK-293 (Payne, 1997). In 1999, Williams *et al.* confirmed that native KCC2 from rat brain undergoes glycosylation as well (Williams *et al.*, 1999). Bioinformatic analysis performed on the rat KCC2 protein sequence suggests that KCC2 has eleven N-glycosylation sites. Four of them lie on an extracellular intermembrane loop between the fifth and sixth transmembrane domains (Payne *et al.*, 1996). To obtain further insight into glycosylation of mouse KCC2, I performed a new bioinformatic analysis that revealed the potential existence of eight N-glycosylation sites (see Results section). Concerning glycosylation and transport capacity of KCC2, it was shown that there is no difference in the glycosylation pattern between transport-inactive and –active KCC2 molecules, which indicates that this process is not essential for the transport function of this protein (Blaesse *et al.*, 2006). Some mutations in KCC2 have been shown to impair glycosylation of KCC2. In particular, the three heterozygous variants Leu426Pro, Gly551Asp and Leu311His caused a decreased level

of KCC2 both in whole cell lysate and at the membrane level which suggests their impact on surface expression and post-translational modifications of KCC2 (Stödberg *et al.*, 2015).

KCC2 – genetics

The variants in *KCC2* are believed to have a strong potential to cause neurodevelopmental dysfunctions (Figure 3; Fukuda and Watanabe, 2019). Interestingly, all pathogenic variants in *SLC12A5* described so far, related the occurrence of the mutation with the defects in chloride extrusion from the cells, which has a direct impact on maintenance of the excitation/inhibition balance (Saito *et al.*, 2017; Saito *et al.*, 2016; Stödberg *et al.*, 2015). GABAergic inhibition alterations are known to be implicated in different neurodevelopmental pathologies, such as ASD, FXS, Rett Syndrome, SCZ, and multiple forms of epilepsies (Coghlan *et al.*, 2012; Duy *et al.*, 2019; Hyde *et al.*, 2011).

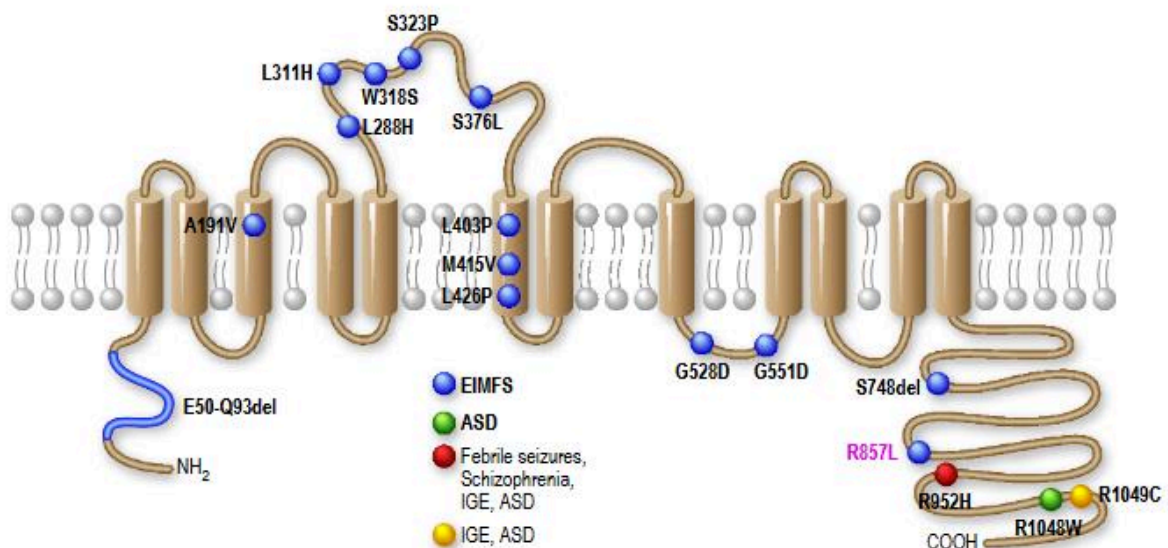


Figure 3. Distribution of mutations in the SLC12A5 and their association with different neurodevelopmental disorders. Mutations related to EIMFS are indicated in navy blue, to ASD in green, to Febrile seizures, ASD, IGE and SCZ in red, and to IGE and ASD in light red (reviewed in Fukuda et Watanabe, 2019). The pink letters indicate the variant that is located in the same position as the variant found during our analysis. R857L corresponds to R880L described by Saito et al. (2017). Difference in position results from different reference sequences.

The first variant associating *SLC12A5* with human disorders was described in 2014 by Puskarjov *et al.* who found the missense mutation p.Arg952His in an Australian patient with Early Childhood Onset of Febrile Seizures. To verify the potential impact of this mutation they heterologously expressed this variant in C17.2 cells and observed the reduction of KCC2 at the membrane level. They also showed the influence of the variant on chloride transport by performing *in utero* electroporation of cortical neurons. By consecutive measurement of GABA_A receptor-mediated current potential they observed lower levels of chloride extrusion capacity in neurons transfected with the construct possessing the mutation. Moreover, they showed that, in cultured mKcc2^{-/-} cortical neurons, the p.Arg952His mutation is unable to rescue normal spine morphology. The authors proposed this gene as a susceptibility gene to Febrile Seizures (Puskarjov *et al.*, 2014).

In the same year, another group reported two variants in *KCC2* and associated them with Idiopathic Generalized Epilepsy (IGE). This group analyzed a cohort of 380 IGE patients and compared it to a Quebec control cohort of 1214 individuals. They found a novel heterozygous *KCC2* variant p.Arg1049Cys (c.3145 C>T) in three IGE patients and in one control

sample (NM_020708.4). The other identified heterozygous variant p.Arg952His (c.2855 G>A) was found in five cases in a IGE cohort and in five healthy controls. Nevertheless, statistical analysis suggests that both variants are enriched in the IGE cohort. On the other hand, the analysis of DNA available from unaffected parents of probands revealed that these two variants were inherited. Patch clamp recordings of glycine receptors potential of N2a cells transfected with constructs with identified variants or WT-KCC2 showed that cells transfected with the mutated construct are less hyperpolarized. Recordings of the cells co-transfected with a chloride sensor revealed that cells transfected with the mutated construct have lower capacity to extrude chloride ions from the cells. The analysis of surface localization showed that the variant p.Arg1049Cys didn't have impact on surface localization whereas the variant p.Arg952His decreased surface localization of KCC2. Moreover, the authors showed that both variants affect the phosphorylation of serine 940, which is known to be implicated in chloride transport and membrane localization of KCC2. The authors concluded that those variants can be potential risk factors for IGE (Kahle *et al.*, 2014).

Saito *et al.* (2017) described a *de novo* *SLC12A5* heterozygous variant in a 12-years boy diagnosed with EIMFS. He displayed seizures before the age of 4 months, abnormal electroencephalogram and delayed intellectual development. The variant was found in position p.Arg880Leu (c.2639G>T) that lies in the intracellular C-terminus of *KCC2*. Moreover, the patient inherited the two heterozygous *SLC12A5* variants p.Arg3Cys (c.7C>T) and p.Ser399Leu (c.1196C>T) after his mother (NM_001134771.1). The two inherited variants were concluded to have a benign influence according to the bioinformatic prediction and to their presence in healthy siblings of the patient (Saito *et al.*, 2017).

Stödberg *et al.* (2015) reported three mutations in two families, one of Caucasian origin and another of Pakistani origin. In two patients from the first family who were diagnosed with EIMFS, they identified two missense mutations in *KCC2*: p.Leu426Pro (c.1277T>C) and p.Gly551Asp (c.1652G>A). In the second family, in both affected individuals diagnosed also with EIMFS, they detected the homozygous missense variant p.Leu311His (c.932T>A). The group showed that these three changes have an impact on the transporter activity of *KCC2*. The variants p.Leu426Pro and p.Gly551Asp significantly impaired chloride extrusion whereas the variant p.Leu311His caused only partial loss of extrusion capacity of *KCC2*. The authors assume that these variants can possibly trigger an intracellular accumulation of chloride ions that impede a hyperpolarization of neurons. Moreover, these variants turned out to decrease the glycosylation level of *KCC2* both at the cell surface and in whole cell lysate. Localisation of *KCC2* at the membrane was reduced in cells harboring the mutations. In addition, a *KCC2a-KCC2b* double knockout displayed atypical spasmodic movements indicating early motor deficits. The authors suggested that mutations in *SLC12A5* may diminish the performance of *KCC2* leading to disturbance in the excitation/inhibition balance (Stödberg *et al.*, 2015).

Saitu *et al.* (2016) described three compound mutations in *KCC2* in three families in which patients were diagnosed with EIMFS. In the first family, affected individuals possess the p.E50_Q93del (c.279 + 1G > C) variant inherited from the mother that generates skipping of exon 3 in the transcript and a variant p.Ala191Val (c.572 C > T) that was of paternal inheritance. In a second family, the patient had a p.Ser323Pro (c.967T > C) variant inherited from the father and a second p.Met415Val (c.1243 A > G) variant inherited from the mother. Individuals from a third family possess variant p.Trp318Ser (c.953G > C) of maternal inheritance and variant

p.Ser748del (c.2242_2244del) of paternal inheritance. Patch-clamp experiments revealed that cells harboring the mutations p.E50_Q93del and p.Met415Val strongly impaired chloride extrusion whereas mutations p.Ala191Val and p.Ser323Pro have a moderate impact on extrusion functions. The cells with mutations did not show difference in KCC2 levels at the membrane (Saito *et al.*, 2016).

Merner *et al.* (2015) reported some variants in *KCC2* in individuals suffering from ASD and SCZ. The group sequenced the C-terminus of *KCC2* in individuals from a Quebec ASD (427) and SCZ (143) cohort and from controls (1214). They found variants p.Arg952His and p.Arg1049Cys that were previously associated with IGE and EIMFS. Both variants were found in a patient with ASD and variant p.Arg952His was identified in a patient with SCZ. They identified also the *de novo* variant p.Arg1048Trp (c.3142C>T) in one ASD patient (NM_020708.4 ; NP_065759). In the SCZ cohort they found the variant p.Arg952His (Merner *et al.*, 2015). *KCC2* expression is decreased in hippocampus of adult SCZ patients. Moreover, the ratio between *NKCC1* and *KCC2* is higher in SCZ patients (Hyde *et al.*, 2011). Expression of different *KCC2* transcripts is changed in SZC and affective disorders (Tao *et al.*, 2012). Compound SNPs in the *DISC1* gene and in *NKCC1* have been shown to contribute to the risk of SCZ (Kim *et al.*, 2012).

KCC2 changes are also described in Rett syndrome. *KCC2* level was shown to be decreased in neurons derived from Rett syndrome patients with mutations in the methyl CpG binding protein 2 (*MeCP2*) gene. Decreased levels of *KCC2* in these neurons contribute to delay in the GABA switch. Overexpression of *MeCP2* or treatment with IGF-1 restores the *KCC2* level

in *MeCP2* mutated neurons (Tang *et al.*, 2016). Recently, it was demonstrated that KCC2 levels are decreased in the brain of Rett syndrome patients (Hinz *et al.*, 2019).

KCC2 as a therapeutic target

Due to KCC2 involvement in the pathogenesis of many neurological disorders, Gagnon *et al.* (2013) decided to look for compounds reducing chloride levels in cells. In order to perform a screening, they designed a fluorometric assay that permits to measure chloride ions concentration in real-time. They chose one compound and produced around 300 of its analogues for further analysis. These molecules did not affect *NKCC1*, *KCC1*, *KCC3* and *KCC4* expression. Moreover, they did not have toxic effects on cells and were easy to synthesize. After verification of chemical and physical properties, one molecule (CLP257) was chosen for subsequent characterization. Incubation of *Xenopus laevis* oocytes with CLP257 increased the activity of KCC2. Mature neurons obtained from animals with neuropathic pain treated with CLP257 restore functional transport of chloride ions. Patch-clamp recordings revealed that CLP257 can bring back chloride extrusion functions. The authors showed that CLP257 mainly influences localization and turnover of KCC2. This work shows that KCC2 can be a target in the treatment of many neurological disorders (Gagnon *et al.*, 2013).

Recently, Tang *et al.* (2019) developed High-Throughput Screening (HTS) assays in order to search for the molecules that increase the level of KCC2. These neuron-based assays enable to estimate the level of KCC2 in CRISPR-Cas9 modified reporter neurons. Thanks to this assay it was possible to identify a set of proteins that boost transcription and expression of

KCC2. Due to their properties, these molecules were called KCC2 Expression–Enhancing Compounds (KEECs). These compounds were further tested in the context of Rett Syndrome. Tests on human Rett Syndrome neurons showed that KEECs can restore the GABA reversal potential and morphological aberrations such as size of nuclei and branching. The authors suggested that amelioration of the morphological phenotype depends on Akt-mTOR pathway activation via BDNF. Apart from this, behavioral tests revealed that *Mecp2* mutant mice improve their locomotion and respiratory deficits upon treatment with KEECs (KW-2449 or piperine). Identified KEECs turned out to influence different pathways. Some KEECs inhibit fms-like tyrosine kinase 3 (FLT3) or glycogen synthase kinase 3 β (GSK3 β) kinases. The other ones activate sirtuin 1 (SIRT1) or transient receptor potential cation channel subfamily V member 1 (TRPV1) pathways (Tang *et al.*, 2019).

KCC2 animal models

a. Mus musculus

Kcc2 hypomorphic mice (insertion of a transposon into intron 3 of the *Kcc2* gene) showed increased anxiety and mice were more sensitive to tactile and thermal stimulation (Tornberg *et al.*, 2005). Knockout of *Kcc2* causes immediate death after birth due to respiratory system failure and motor deficiency (Hübner *et al.*, 2001b). In 2008, Riecki *et al.* (2008) engineered the *Kcc2*-deficient mouse model (*Kcc2*^{hy}/null) that preserves around 15-20% of KCC2 level. Patch-clamp recordings revealed that reversal potential of GABA_A receptor-mediated currents is more positive. Evaluation of field Excitatory PoStsynaptic Potentials (fEPSPs) upon

repetitious stimulation indicated impairments in synaptic dynamics (Riekki *et al.*, 2008). Another mouse model showed that specific neural over-expression of *Kcc2* in mouse embryos hinders the proper neuronal differentiation and migration (Horn *et al.*, 2010). The most recent study on *KCC2* was concerning the post-translational modification of threonine 906 and 1007 residues. Pisella *et al.* (2019) constructed heterozygous mice in which they blocked the dephosphorylation of previously mentioned sites. By doing so, they could show that *Kcc2*^{E/+} mice at P15 and P30 display aberrant GABAergic inhibition. These mice present also alteration in ultra-sonic vocalization and social behavior at P10-12 and P60, respectively. Moreover, they turned out to be more susceptible for seizures triggered by the convulsant agent flurothyl. They managed to generate the homozygous mouse for those sites, but the phenotype was lethal (Pisella *et al.*, 2019).

b. Danio rerio

Reynolds and collaborators (2008) investigated the role of overexpression of human *KCC2* in zebrafish embryos. Embryos overexpressing *KCC2* exhibit reverse chloride gradient and abnormal morphological and behavioral features. *KCC2*-expressing embryos were smaller, pigmentless and had impaired locomotion skills. They also displayed aberrations in neural development, such as abnormal axonal growth in brain and hindbrain and perturbations in neuronal differentiation in the spinal cord. These results showed that a proper chloride gradient is crucial for neurogenesis during embryonic development (Reynolds *et al.*, 2008). Reduction of zebrafish *KCC2* (*zfkcc2*) in retinal ganglion cells postponed the onset of GABA switch by one day. These findings suggested a role for *zfkcc2* during retinal network

development (Zhang *et al.*, 2010). Further investigation of retinal ganglion cells led to the discovery that reversal potential of chloride ions differs between soma and dendrites. These differences are thought to be determined by KCC2 activity due to the fact that its down-regulation with morpholino nucleotides or blocking with pharmacological agents diminished the previously mentioned differences (Zhang *et al.*, 2013).

c. Xenopus laevis

Song *et al.* (2002) introduced a construct containing *hKCC2* and *mKcc4* in oocytes of *Xenopus laevis*. In order to evaluate functional expression of these constructs after injections with cRNA or water, they performed transport activity test by measuring $^{86}\text{Rb}^+$ tracer with and without the presence of chloride ions in hypotonic and isotonic conditions. Chloride-dependent transport activity was observed in isotonic condition in oocytes transfected with *hKCC2*. The transport was not detected in oocytes with *mKcc4*, suggesting that KCC2 has higher cation affinity than KCC4 under physiological conditions. On the other hand, K-Cl co-transport activity in hypotonic conditions was higher in oocytes with *mKcc4* than with *hKCC2*. It was also shown that protein phosphatase-1 is not essential for isotonic activation of transport and inhibits transport in hypotonic conditions (Song *et al.*, 2002). Hartmann and collaborators (2010) investigated the impact of four cysteine mutations in evolutionarily conserved positions in *KCC2* and *KCC4*. All four mutations caused suppression of KCC2-dependent transport activity in *Xenopus laevis* oocytes transfected with constructs with cysteine mutations. KCC4 was not affected by these mutations (Hartmann *et al.*, 2010).

Xenopus laevis was also used for pharmacological studies of KCC2 (see section “KCC2 as a therapeutic target”).

d. Drosophila melanogaster

Young fruit flies that possess mutations in the *KCC2* orthologue *kazachoc* (*kcc*) exhibit a seizure-like phenotype and are more susceptible to seizures evoked by electroconvulsive shock. This mutation caused the reduction in *kcc* levels (Hekmat-Scafe *et al.*, 2006). A few years later, it was shown that the phenotype of these flies can be alleviated through targeted expression of *kcc* in neurons of the mushroom bodies (structures in the brain of insects controlling memory) of *Drosophila* (Hekmat-Scafe *et al.*, 2010).

Aim of the Thesis

During my thesis I was involved in research projects having in common the understanding of molecular bases of neurodevelopmental disorders in : i) patients affected by **Early Onset Schizophrenia (EOS)**, **Autism Spectrum Disorder (ASD)** and **Intellectual Disability (ID)**; ii) a mouse model for epilepsy and ID. Furthermore I participated to the definition of neurodevelopmental molecular and cellular aspects in cultured neurons derived from a mouse model of FXTAS, a disorder mainly considered as a neurodegenerative disease until a few years ago.

1. The first project aimed at the **functional characterization of new genes implicated in EOS, ASD and mild ID.**

The purpose of this part of my thesis is to better understand the link existing between ASD, schizophrenia and ID. I searched for new genes involved in patients affected by these disorders. In collaboration with the Lenval Psychiatric Hôpital in Nice, we aimed to:

- **Collect the DNAs** of a cohort of 40 families, each one including at least one young patient displaying **EOS**, which was frequently accompanied by **ASD** or **ID**;
- Perform **analysis** of **Whole Exome Sequencing** (WES) results, using different bioinformatic tools, of TRIOs (child and both parents belonging to the cohort) in order to find some very rare variants that have a putative pathological impact on the patients' phenotype;

- **Evaluate the impact of a candidate gene** by creating a cellular model mimicking the variant found in a patient in the SH-SY5Y cell line by the CRISPR-Cas9 technique and performing its characterization with different techniques (*e.g.*, calcium imaging).
2. The second project during my thesis was associated with characterization of a novel spontaneous mutation in the *Kcc2* gene in mice suffering from spontaneous tonic-clonic seizures starting at the age of four months.

During this project, I aimed to:

- **Characterize an epilepsy model** in mice due to a **spontaneous mutation** in the *Kcc2* gene by correlating: **neuronal activity, seizure appearance** and **social and cognitive behavior** with **gene and protein expression** at different times of mouse development.

To date, these functional studies have not been performed since *Kcc2*-KO mice die few days after birth and heterozygotes have never been studied in detail. The mutation I found is located at the same amino acid residue as the one found mutated in a patient (R857L corresponding to R880L in human, described by Saito *et al.*, 2017), offering a unique opportunity to study *in vivo* the aetiology of the disorder in humans and correlate the behavioral phenotype with seizure progression. Concerning the connection with **ID**, the patient exhibits deficits in intellectual development. For this reason, I evaluated the cognitive capabilities of *Kcc2-mut* mice. Apart from this, as previously mentioned in the Introduction, epilepsy is frequently accompanied by ID. Investigation of this mutation will shed new light on its impact on the phenotype. Moreover, GABAergic signaling, which is connected to inhibition/excitation balance, is thought to underlie a range of neurological disorders including

ID, ASD and epilepsy. Taking this into account, the *Kcc2-mut* mice model can not only provide us with a further insight into the pathogenesis of epilepsy, but also into its association with other neurological disorders, such as ID.

3. In addition to these two main aims, I contributed to the project during which we were investigating the impact of the reduced level of *Fmr1* mRNA on a murine model of FXTAS at the cellular and molecular levels. Hence, during this project, I aimed to identify molecular pathways underpinning some neurodevelopmental hallmarks of cultured cortical neurons obtained from a murine model of FXTAS.

Materials and Methods

Blood isolation

Blood samples were collected by the personnel of the Children's Hospitals of Nice CHU-Lenval (Nice, France). All patients and their relatives provided written informed consent for participation in the study. Inclusion criteria are described in Fernandez *et al.*, 2018 (see Annexes - Publication 1). DNA from blood was isolated with GenElute Mammalian Genomic DNA Miniprep Kit (SIGMA Cat. No. G1N70).

WES and bioinformatic analysis

WES and bioinformatic analysis of families 1-9 are described in Manuscript of publication (see Annexes – Manuscript).

Verification of WES results

After bioinformatic analysis, all potential candidate mutations were verified by SANGER sequencing. PCR fragments were purified with QIAGEN MinElute PCR Purification Kit (Cat. No. 28004).

The following primers were used for PCR and sequencing reactions:

Family 5 candidate gene primers:

STK33-primer-For	5'-GGAAGCTCTGCTGTGAAGTTAC
STK33-primer-Rev	5'-CTCATCAATCTCCAGTGAC
ZNF670-primer-For	5'-CATTAGCCCTTCATAGGC
ZNF670-primer-Rev	5'-CTCTCCAGTATGAGTTC
ORF9-new-For	5'-CCCTCATCCTAGACTTAATG
ORF9-new-Rev	5'-GTCTACACTTGTGTTTAGTG

Mouse candidate gene primers:

For-RELN	5'-GCAGTGGCTTGTACCTAC
Rev-RELN	5'-GGTATCACATCTTCACTTCAG
For-BRD7	5'-GCATGTTCAAGGTATTAAGT
Rev-BRD7	5'-GGTGACTGTATCTATTGGG
For-ITGAV	5'-CCATACAAATGAACGACTG
Rev-ITGAV	5'-GGAATCTGTCATGCAGAC
For-DNM1	5'-CCATTCTCTACACAGCAG
Rev-DNM1	5'-CCAGTCCCTTATACTGTGG
For-ABCC8	5'-TGGACCGAACCACACTAGAC
Rev-ABCC8	5'-GGCAGACTCAATCCCAGGAC
For-GABRB	5'-AGGCCAGAGAGCAACTAGGA
Rev-GABRB	5'-TCTGTGGAATCATCTTGACCAGG
For-CSMD3	5'-ACACTGTCTCTGACAGCCTT

Rev-CSMD3	5'-TTCCAATTCCGGCTGACTCC
For-CDK13	5'-ATATGCCTTTGCATGCTGCT
Rev-CDK13	5'-CCAGTCAACCTAAGTGCCAGT
For-ZFR	5'-TGTTCTCTGTTCAGTTTGGA
Rev-ZFR	5'-GCAGGTGTGACTGGTTCTGA
For-HSP90AB1	5'-AAAGCACTAAGTCTGCCCAAAGG
Rev-HSP90AB1	5'-GTCCACAGGTGACAATCTCC
For-GABRG2	5'-CCCAACCATTTCCGGCTTTGG
Rev-GABRG2	5'-AGCAGTCGCATGCAGTGAAA

Sequencing reaction was performed according to the following reaction protocol:

Step	Temperature	Time
Initial denaturation	96°C	1 min
Denaturation	96°C	10 sec
Annealing	50°C	5 sec
Extension	60°C	4 min
Hold	4°C	

} 25 cycles

Reaction products were subsequently delivered to the sequencing platform at IPMC for further analysis. Chromatograms were analyzed with SnapGene Viewer.

Zygoty verification

In order to know the zygoty of the variants, PCR fragments were cut out from the gel after electrophoresis and were purified with the QIAquick Gel Extraction Kit (Cat. No. 18704) according to manufacturer's instructions. Purified PCR fragments were subsequently cloned into the pGEM-T Easy Vector (PROMEGA Ref.A137A; T4 ligase NEB Ref. M0202S). After transformation by electroporation with electrocompetent *E. coli* (SIGMA) bacteria with plasmids were plated on dishes with LB supplemented with Amp. Single colonies were harvested, amplified and DNA was purified with the QIAprep Spin Miniprep Kit (QIAGEN Cat. No. 27106) according to manufacturer's instructions. Purified clones were sequenced with the T7 promoter primer (5'-TAATACGACTCACTATAGGG-3').

Targeted deletion with CRISPR/Cas9

The gRNA design was performed with two independent on-line platforms: tefor (<http://crispor.tefor.net/>) and Chopchop (<https://chopchop.cbu.uib.no/>). Both tools predicted the same following gRNA sequence as the best one: GCCAAAGGTAAACCTCTATTAGG. gRNA cleavage performance was verified with Guide-it sgRNA In Vitro Transcription and Screening System (Clontech Cat No. 631440). Subsequently, the guide sequence was cloned into the MLM3636 plasmid (Addgene; # 43860) with the sense and antisense primers designed with the tefor tool (respectively: 5'-ACACCGCCAAAGGTAAACCTCTATTG-3' and 5'-AAAACAATAGAGGTTTACCTTTGGCG-3'). Cells were subjected to lipofectamine transfection (Lipofectamine 2000; Invitrogen™ 11668-019) with the MLM3636 plasmid containing gRNA,

pSpCas9n(BB)-2A-GFP carrying the nuclease Cas9 and GFP that was obtained from Addgene (#48140) and the ssODNE template (5'-TTCATTGATTAAGGCTGGAAGCTCTGCTGTGAAGTTACTTGAACGAGAGGTGAACATTCTGAAAAGTGTAAAACATGAACACATCATACTCTGGAACAACCTCTATTAGGCGCTGCATTTTGAAAGTTGTTATTATTACAAAATGAATATCATTCCATTCTGAAATAATCCCTTTGGGGCACAGTTGCTCATGTCCTTG-3') containing precise 22nt deletion that was designed on the benchling platform (<https://www.benchling.com/>). Transfection was performed according to manufacturer's instructions. Cells were sorted for GFP with a cell sorter FACS Aria III (BDBiosciences) and single cells were plated on a 96-well plate. Genomic DNA from cell clones was isolated using standard phenol/chloroform protocol (see "Extraction of DNA"). Screening was performed by a specific PCR protocol (with initial denaturation at 95°C for 5 min, 30 cycles of denaturation (95°C for 30 sec), annealing (58°C for 30 sec) and extension (72°C for 30 sec), and 1 cycle of final extension (72°C for 5 min) with primers encompassing the deletion site (PCR-STK33-Forward 5'-GAGGTGAACATTCTGAAAAG-3' and PCR-STK33-Reverse 5'-CAAAATGCAGCGCCTAATAG-3'). PCR products were resolved on 2% agarose gel at 100V for 1h. The chosen clones were further evaluated by sequencing. They were amplified with the following primers: STK33-primer-For 5'-GGAAGCTCTGCTGTGAAGTTAC-3' and STK33-primer-Rev 5'-CTCATCAATCTCCAGTGAC-3' and subsequently purified with the Qiagen PCR purification kit. Primers to detect the off-targets were designed with tefor tool (Table 1).

Gene (off-target)	Primer sequence
CNTNAP5 (off-target: GCCAAA ACCT AACCTCTATT)	
For-CNTNAP5-off	5'- GTGCCTCCACTTTGAATCCAG
Rev-CNTNAP5-off	5'- CTGAGTCATGCTGCTAGAGC
FAM155A (off-target: GCAAAA AGTTAAAGCT CTATA)	
For-FAM155A-off	5'- GACCAGGTAATGAGCATTGATC
Rev-FAM155A-off	5'- GCCACACAACATCAACAAATC
TRIQK (off-target: CATAAAGGTAAACCT CTATT)	
For-TRIQK-off	5'- GAAGATGTCCGTCAGTATCCTC
Rev-TRIQK-off	5'- CTGCTAACATCATTCTCAGTTGC
NCOA2 (off-target: GCCAAAA ATAAATCTTT ATT)	
For-NCOA2-off	5'- CAGCTAGTCAGCATATTTG
Rev-NCOA2-off	5'- CAGTACACAGATACTTCAAGAGC
KCNJ3 (off-target: GCCACAGGTAA ACTTCCTTT)	
For-KCNJ3-off	5'- GCAACAGCCACATGGTCTC
Rev-KCNJ3-off	5'- GTGTTACTAGTCCAGTAAGTG
NTNG1 (off-target: CCAAAAGATAAACTT CTATT)	
For-NTNG1-off	5'- CAGGAACTCAAATAGTATCTCACG
Rev-NTNG1-off	5'- GATTAGGATCTTGCTGTCATTC
KIF3C (off-target: GCCA AGTGAAAACAT CTATT)	
For-KIF3C-off	5'- GAATCACTTGAACCCGGGAGGCA
Rev-KIF3C-off	5'- CAGCAACAATCCAGGACAC

KCNH7 (off-target: GACAAAGCTAGATCTCTATT)	
For-KCNH7-off	5'- CTACTGTGGTTTATCTGTG
Rev-KCNH7-off	5'- GTGCTCAGGTGCTCATCAG

Table 1.

Cell culture and differentiation of SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were cultured on 75cm² Nunc Easy Flasks (Thermo SCIENTIFIC) in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (SIGMA; #) and 1% penicillin-streptomycin (SIGMA # P4333). Cells were incubated at 37°C in humidified atmosphere of 95% O₂ and 5% CO₂. Five days prior to experiments the cells were washed with PBS, treated with 0,05% Trypsin-EDTA (SIGMA # T3924) and sub-cultured on 35mm culture dishes (Greiner # 627160). Two days after plating the medium was changed to differentiation medium (DMEM supplemented with 1/100 laminin; SIGMA #L2020), 1% Glutamax (Gibco), 1/1000 IGF (SIGMA; I3769; stock: 100 µg/mL) and 1% P/S (differentiation protocol was based on Dwane *et al.* (2013).

Extraction of DNA

Cells and tissues (*e. g.*, tails pieces) were incubated at 55 °C overnight in lysis buffer* with addition of proteinase K. After inactivation of proteinase K for 10 min at 96 °C, total DNA from cells or homogenized brain tissues was extracted with Ambion Acid Phenol:Chloroform:IAA (25:24:1) (Invitrogen™ AM9732). The pellet was precipitated with 0.1 sample volume of

sodium acetate (3M) and 2,5 sample volume of ethanol (100%). DNA was subsequently resuspended in desired volume of water.

*Lysis buffer

Final composition	For 50 ml
Tris pH 8 0,1M	5 ml (from stock 1M)
EDTA 10mM	1ml (from stock 0,5M)
0,1% SDS	250ul (from stock 20%)
0,5% NP40	2,5ml (from stock 10%)
H ₂ O	41,25ml (up to 50ml)

Extraction of RNA

Total RNA from cells and homogenized brain tissues was extracted using Trizol reagent (Sigma) according to manufacturer's instructions. RNAs were purified with 1000 µl of Trizol reagent and precipitated from the aqueous phase with 500 µl of Isopropanol (VWR Medicals) and 1 µg of glycogen (Invitrogen). RNAs were resuspended in 30 µl of RNase-free H₂O. After resuspension, samples were stored at -20 °C or were used immediately.

Extraction of proteins

In order to extract proteins, cells were placed on ice and washed 2 times with cold PBS. To each 100 mm plate 150ul of lysis buffer* was added. Afterwards, the cells were scraped and transferred to an Eppendorf tube. They were spun for 30 min at 4 °C to allow the cells to

homogenize. The homogenization was followed by 10min centrifugation. The supernatant was removed and the pellet was resuspended in 70 μ l of lysis buffer and freezeed.

*Lysis buffer for 10ml

	Final concentration	10ml
Hepes (1M stock)	50mM (20x)	0,5ml
NaCl (3M)	150mM (20x)	0,5ml
NP40 (10%)	0,5% (20x)	0,5ml
EDTA (0,5M)	10mM (500x)	200ul
DTT (0,1M)	0,5mM (200x)	50ul
Tablet protease inhibitor	1 tablet/10ml	1tablet
H ₂ O	-	8,25ml

Protein extraction from mouse brains:

Brain tissues were homogenized with the syringe after addition of lysis buffer. For 100 μ g of tissue, 500 μ l of lysis buffer were used. The rest of procedure was performed as described above.

Protein extraction of cytoplasmic and nuclear fractions

Cells were washed twice with PBS and treated with buffer A*. Afterwards, the cells were scraped and transferred to Eppendorf tubes. Cell lysis was verified under the microscope. 0.3%

Igepal was added to lysed cells. Cells were homogenized with the syringe and through 30 min incubation on ice. Cytoplasmic fractions were recuperated with the supernatant after centrifugation at 3000 rpm for 3 min. Pellets containing the nuclear fraction were rinsed with buffer A and spinned for 3 min at 3000 rpm. Supernatants were removed and the pellets were resuspended in 70 µl of buffer A. At the end, samples were sonicated after addition of 1% of SDS (SIGMA).

* Buffer A (pH 7.5)

	Final C°	Stock C°	Volume
Sucrose	0,32M	1M	96 ml or 33 g
Tris-HCl pH7,5	10mM	1M	3 ml
MgCl ₂	0,5 mM	1M	150 ul
H ₂ O			Up to 300 ml

RT-qPCR

Complementary DNA (cDNA) was synthesized from 1 µg of total purified RNA. RT was performed with the Superscript IV synthesis kit (Invitrogen). Initial annealing of primers to RNA template was performed by 5 min incubation at 65°C. The RT reaction was as follows: denaturation at 23°C for 10 min, primer annealing at 53°C for 10 min and primer extension at 80°C for 10 min. The reaction was stored at 4°C or used immediately.

Quantitative PCR

Quantitative PCR (qPCR) was performed on a Light Cycler 480 (Roche) with MasterMix SYBRGreen (Roche) following manufacturer's instructions. The primer sequences for qPCR are as follows:

For-STK33-qPCR-mut	5'-AAAAGGCTGGAAGCTCTGCT
Rev-STK33-qPCR-mut	5'-TCCTCACAAAGCTCCATCACA
For-STK33-qPCR1	5'-ATGCCCCGACTGTTCATCTG
Rev-STK33-qPCR1	5'-TGGAGGAACCCCTTGTTTTGCT
GAPD-qPCR-For	5'-ATTCCACCCATGGCAAATTC
GAPD-qPCR-Rev	5'-GGGATTTCCATTGATGACAAGC
For-HSP-qPCR-t1,2	5'-ACGTCTTCGGAACATGGCT
Rev-HSP-qPCR-t1,2	5'-TCCTTCAGCTGTTGGACACA
For-NCALD-qPCR	5'-GACCACTCGTTGCTGACAGT
Rev-NCALD-qPCR	5'-GCCTTCCTCGGTATCACGAC
For-CIB3-qPCR	5'-TGGAAAGCGTATCAGGACTGC
Rev-CIB3-qPCR	5'-ATCGGGCAGGTGGTATAGT
For-EFCAB2-qPCR	5'-ATGACTGAAGAAGGTGAGCCT
Rev-EFCAB2-qPCR	5'-CTGGATCAATTGCAGCAGAC
For-CABP7-qPCR	5'-ACCCTTCTGGGACCCAAACT
Rev-CABP7-qPCR	5'-TGTCGCACTTCCAGAAGACA
For-USP5-qPCR	5'-TGGCAACGAAGACGAAGACT

Rev-USP5-qPCR	5'-TGATTCATCCAGCATGGGCG
For-OCM-qPCR	5'-GATGGAGATGGGAAAATTGG
Rev-OCM-qPCR	5'-TCCAGAGACTGGGGCTTTTA
For-CIB2-qPCR	5'-CCGAGAGCTCAAGGCAAA
Rev-CIB2-qPCR	5'-CCTCCTCCTCATCCAGCTCT
For-CIB1-qPCR	5'-ACAGGCGGTTTTGTGAGC
Rev-CIB1-qPCR	5'-GCTCCTGAAGGGGTTGG
For-HPCA-qPCR	5'-GACGGCAACGGCTACATC
Rev-HPCA-qPCR	5'-CGGACGAAACCATCTTGTA
Neuronal differentiation markers:	
* β 3-tubulin-F:	5'-AGTGTGAAAAGTGCCTGCTGC
* β 3-tubulin-R:	5'-ATACTCCTCACGCACCTTGC
*NF-H-F:	5'-CAGCCAAGGTGAACACAGAC
*NF-H-R:	5'-GCTGCTGAATGGCTTCT
*MAP2-F:	5'-TGGCATTGACCTCCCTAAAGAG
*MAP2-R:	5'-TTGCTTCCGTTGGCATTTCG
*NF-M-F:	5'-GACGGCGCTGAAGGAAATC
*NF-M-R:	5'-CTTGCGGAGCGGATGGCCT
Gps1-Rev	5'-ACGGTTGCGAATCTGGGTGTA
Gps1-For	5'-AAGATGCTGGACGAGATGAAGGA
Mouse primers :	
**KCC2a-113-F	5'-CCAGAGTCCC GCCGCATTC

**KCC2a-113-R	5'-TCCGTGTCCGTGCTGTTG
**KCC2b-130-F	5'-TCAACAACCTGACGGACTG
**KCC2b-130-R	5'-ATGTCCTGCCATCGTACTC
**KCC2a/b-108-F	5'-CTGGACCAAGGATAAGTCAG
**KCC2a/b-108-R	5'-GGTTCAAGTTTTCCCACTCC
NKCC1-F2	5'-ACCTGGTACCAAGGATGTGGT
NKCC1-R2	5'-GGAGTCTTGCCATCCTCTTCC
TBP-Rev	5'-GGGTGGTGCCTGGCAA
TBP-For	5'-AGGCCAGACCCCACTC

*Designed by Ullah *et al.* (2016)

**Designed by Kursan *et al.* (2017)

Western blot

Denaturing protein gel electrophoresis was performed with NuPAGE Bis-Tris Mini Gels. Samples were combined with NuPAGE LDS Sample Buffer (4x) and NuPAGE Reducing Agent (10x) and incubated at 70°C for 10 minutes. Samples were run for about 1,5 h (150V; 1x NuPAGE MOPS SDS Running Buffer). After gel electrophoresis, proteins were transferred on a NC-membrane for 1.5 h (at 0,25A). Subsequently, membranes were saturated in 5% milk for 1 h and incubated with primary antibodies overnight: anti-GAPDH (proteintech, 1:10000); anti-STK33 (Millipore, 1:200); anti-KCC2 (GenTex, 1:500), anti-ERK1/2 (Cell Signaling, 1:1000), anti-p-ERK1/2* (Cell signaling, 1:1000). Membranes were washed 3 times in PBS-0.1 % Tween and incubated with secondary antibodies (1:5000) for 1h. After 3 washes in PBS-0.1% Tween, membranes were revealed with Immobilon Western (Millipore Ref. P90720).

*For investigating the phosphorylation, gels were stripped with Restore Western Blot Stripping Buffer (Thermo SCIENTIFIC Ref. 21059) for 10 minutes.

Immunofluorescence

SH-SY5Y cells subjected to the previously described differentiation protocol were fixed with 4% PFA for 15 minutes. After washes with PBS, they were incubated 10 minutes in 0.25% Triton X-100, washed again with PBS and blocked for 30 minutes in 2% BSA. Afterwards, cells were incubated overnight at 4°C with selected antibodies in 0.2% BSA: anti-NF (BioLegend, 1:1000), anti-MAP2 (BioLegend, 1:1000), anti- β -tubulin (BioLegend, 1:2000). Primary antibodies incubation was followed by three washes with PBS and secondary antibodies incubation for 1 hour in 0.2% BSA solution (1:5000). At the end, cells were washed again three times in PBS, stained in DAPI for 3 minutes, washed with PBS and mounted on the slides. The slides were subsequently analysed under Fluorescence Microscope (Zeiss Axioplan 2).

Calcium imaging

SH-SY5Y WT and siRNA transfected cells subjected to the previously described differentiation protocol were incubated in their differentiation medium containing 20 μ M Fluo4-AM (Invitrogen) and 2 μ l of F- Pluronic127 (20 % solution in DMSO) for 30 min at 37°C. After two washes with HEPES-buffered Tyrode's calcium solution (139 mM NaCl, 15 mM glucose, 1.25 mM Na₂HPO₄ dibasic heptahydrate, 1.8 mM MgSO₄ heptahydrate, 1.6 mM CaCl₂ dihydrate, 3 mM KCl, 10 HEPES), dishes were placed in a metal chamber on an inverted microscope

(AxioObserver, Carl Zeiss) equipped with a 300W Xenon lamp (Sutter Instruments) and a 20× W/1.0NA objective. Cells were perfused at 22°C throughout the recording with Tyrode's calcium solution. Cells were stimulated with carbachol (10 μM). At the end of every recording cells were incubated in 0 Ca²⁺ solution (129 mM NaCl, 15 mM glucose, 1.25 mM Na₂HPO₄ dibasic heptahydrate, 1.8 mM MgSO₄ heptahydrate, 0.5 mM EGTA, 3 mM KCl, 10 mM HEPES), then 0 Ca²⁺ + ionomycin (5 μM) and finally 10 Ca²⁺ + ionomycin (5 μM; 0 Ca²⁺ solution + 10 mM CaCl₂ dihydrate) solutions. These final steps enable to quantify the lowest and the highest probe fluorescence. Every recording experiment followed the same protocol:

Tyrode's—20 s; Tyrode's + Carbachol—30 s; Tyrode's—40 s; Tyrode's (0 Calcium) - 40s; Tyrode's (0 Calcium) + ionomycin—20 s; Tyrode's (10 Calcium) + ionomycin—30 s.

Fluo-4 was sequentially excited at 485 nm and the emission monitored at 520 nm. Images were acquired with a cascade 512 EMCCD camera every 2 sec using the Metafluor software (Roper Scientific). For each recorded cell, the intracellular calcium concentration [Ca²⁺]_i was estimated by calculating the $\Delta F/F = (F - F_{\text{baseline}}) / (F_{\text{baseline}} - F_{\text{background}})$, where F_{baseline} is a resting calcium level ("baseline") measured as the average fluorescence from the perfusions with Tyrode's. F reflects fluorescence intensity of Fluo-4 at any given time and $F_{\text{background}}$ is fluorescence intensity of the background. Around 50-60 ROIs were selected from each culture plate and subjected to further analysis.

Proteomics

Proteomic analysis was performed by the Proteomic facility at IPMC. Gel slices containing proteins were treated by DTT/IAA (to reduce and alkylated S-S bond) and digested by trypsin. Peptides extracted were next analyzed by nano LC-MS/MS. Data reprocessing was done using Protein Discoverer 2.2 against the human reviewed data base (Human_reviewed_180910).

Further bioinformatic analysis was performed with the following programs: Galaxy/ProteoRE (<http://www.proteore.org>), PANTHER (<http://www.pantherdb.org/>), STRING (<http://www.string-db.org/>) and DAVID (<https://david.ncifcrf.gov/>).

Proteins from WT, HOM and HET cell lines were delivered after total protein extraction and extraction from the cytoplasmic and nuclear fractions.

Proteins for KCC2 analysis were delivered after immunoprecipitation.

Statistical analysis

Specific tests were chosen according to analyzed samples. Data were expressed as mean \pm SEM, and the P values (or adjusted P values) < 0.05 were considered statistically significant. Statistical analysis was performed using Prism Software 7 and 8 version (GraphPad Software, Inc.).

Genotyping

Fragments of mouse tails were cut and incubated overnight at 55 °C in lysis buffer (for composition see "DNA extraction") with addition of proteinase K. After inactivation of

proteinase K for 10 min at 96 °C, DNA was diluted 10x and used directly for PCR reaction with following primers and protocol:

Primers:

Seq-mSlc12a5-Rev	5'-TCATCCACTGACGGCTATGG
Seq-mSlc12a5-For	5'-ACGGGACCTTTCTTTGGGA

PCR program:

Step	Temperature	Time
Initial denaturation	96°C	1 min
Denaturation	96°C	30 sec
Annealing	60°C	30 sec
Extension	72°C	30 sec
Final extension	72°C	5 min
Hold	4°C	

} 30 cycles

PCR products were purified with QIAGEN MinElute PCR Purification Kit (Cat. No. 28004) and subjected to sequencing reactions according to the previously described protocol (see “Verification of WES results”). Reaction products were subsequently delivered to the sequencing platform at IPMC for further analysis. Chromatograms were analyzed with SnapGene Viewer.

Immunoprecipitation

WT and *KCC2*-mut mice cortexes and hippocampi were homogenized into powder and resuspended in PBS containing 1% Igepal. Cleaning of samples was performed with 15 μ l of naked Dynabeads A (ThermoFisher) for 30 min at 4°C on a rotating wheel. In the meantime, 30 μ l of Dynabeads A were incubated with anti-KCC2 antibody (GeneTex) on a rotating wheel for 1 h at room temperature, with 100 μ g of tRNA, ssDNA and BSA. The “pre-clear” beads are removed from samples which are subsequently centrifuged at 14,000 rpm at 4°C for 10 min. Antibody-coated beads are added to the samples and incubated on a rotating wheel overnight at 4°C. The next day, beads were washed three times with PBS containing 0.1% Igepal and incubated for 15 min at 55°C with 100 mM dithiothreitol and 2 \times Laemmli sample buffer. Eluted proteins were then resolved on NuPAGE Bis-Tris Mini Gels using MOPS buffer (Invitrogen).

Immunohistochemistry

Mouse brain slices were washed 2 times in PBS for 5 min and permeabilized with PBS-Tween 0.3% for 10 minutes. Afterwards, slices were incubated for 5 minutes in H₂O₂ and washed subsequently with water and PBS. They were blocked with 2.5% goat serum for 2,5h. Then, they were incubated in primary antibody (anti-KCC2, Millipore, 1:500) overnight at 4°C. They were washed with PBS and incubated in 100-200 μ l of GAR-HRP solution (Dako Ref. K4003) for 30 minutes. They were washed 2 times for 5 minutes in PBS-0.1% Tween. After, brain slices were stained with Liquid DAB + Substrate Chromogen System (Dako Ref. K3467) and mounted on cover slips with Eukitt Quick-hardening mounting medium for microscopy.

Crosslinking assay

Crosslinking assay was performed in collaboration with Dr. Alessandra Folci according to the protocol previously described by Boudreau *et al.*, 2012.

Synaptoneurosomes preparation

The day before isolation, a Percoll-Sucrose Density gradient was prepared by creating 20%, 10%, 6% and 2% layers of Percoll solution (Percoll resuspended in sucrose solution: sucrose 0.32 M, Tris-HCL 10mM, pH 7.4 in desired percentage), respectively, in the Beckman centrifuge tubes. The day of isolation cortexes from mouse brains were homogenized and resuspend in sucrose buffer (7x cortex weight/volume ratio). Samples were centrifuged (1000xg, 5min) and the supernatant was collected in a new tube. The final volume of all samples was adjusted to 2 ml, which were subsequently added to the gradient creating the new layer. Beckman tubes with gradients were centrifuged at 18000 rpm for 10 min at 4 °C. We recuperated the phase between 20% and 10% and transferred it to a new tube. We adjusted the volume up to 10 ml with HEPES buffer (NaCl 140 mM, KCl 3 mM, MgSO₄ 1.2 mM, CaCl₂ 1.2 mM, NaH₂CO₃ 5 mM, Glucose 10mM, HEPES 5 mM, pH 7,4) and centrifuge at 10000g for 30 min. We removed about 8 ml of solution from the top, leaving 2 ml with the pellet. We transferred 1ml from the bottom with the pellet to the new tube and centrifuged at 10000g for 30 min. We removed the supernatant and resuspended the pellet in 500µl of Trizol reagent and moved to RNA isolation according to the previously described protocol (see “RNA extraction”).

aiFACS

aiFACS experiments were performed according to the protocol established by Castagnola *et al.* (2020). Full brains of WT and *KCC2* mice were dissected at PND17. They were washed with complete D-PBS supplemented with 0.5% bovine serum albumin, 1% pyruvate and 15 mM glucose. After the wash, brains were cut sagittally in 6 equally thick sections (2 mm) using a mouse brain matrix slicer (CellPoint Scientific) and 5 razorblades. Subsequently, mechanic (gentleMACS™ octodissociator) and enzymatic (Adult Brain Dissociation kit; Miltenyi Biotec) dissociation of brain slices was performed according to manufacturer's instructions. Magnetic selection of neuronal cells was done with the Neuron Isolation Kit (Miltenyi Biotec). Labeling of neurons was done with a combination of the Fluo-4 calcium indicator (5 µg; Invitrogen) and either Wheat Germ Agglutinin 594 (WGA 594; Invitrogen) or WGA 647 (5 µg; Invitrogen) in complete D-PBS for 30 and 10 min, respectively. Cells were sorted with a FACSAria III (BDBiosciences). The sorter is implemented with a homemade injection system. Baseline acquisition and sorting were performed using D-PBS buffer. Cells were stimulated with GABA agonist (Isoguvacine hydrochloride SIGMA G002-25MG; 10 µM) and responding cells were collected in D-PBS buffer. Data were analyzed with the BD FACSDiva v6 software (BD Biosciences).

Electrophysiology

Electrophysiological analysis or intracranial electroencephalography (iEEG) was performed in collaboration with Dr. Mantegazza. IEEG is a kind of electrophysiological monitoring that enables recording electrical activity from the cerebral cortex through electrodes positioned

directly on the exposed surface of the brain. This technique enables the recording of behavioral and electrographic seizures simultaneously. The IEEG of epileptic mice was recorded following previously described protocol (Salgueiro-Pereira *et al.*, 2019). Cortical screws linked to tungsten wires (diameter 0.125mm) were placed on frontal and parietal cortex. A further ground and reference screw was inserted in the occipital region, just above the cerebellum. All screws were connected to the recording system (PowerLab, AD Instruments) and the signal was constantly acquired over several days. The presence of interictal spikes (burst of synchronized neuronal activity common for the epileptic brain between seizures) was distinguished utilizing a custom written script on MATLAB.

Behavioral Tests

All experiments were performed between 8:00 and 12:00 each day over the 12-h light period. Male and female mice were used. Animals were maintained in line with the European Community Directive 2010/63/EU. All experiments were approved by the local ethics committee (Comité d’Ethique en Expérimentation Animale CIEPAL-AZUR N. 00 788.01) and by the French Ministry of Research.

UltraSonic Vocalizations (USVs) Test

The USVs Test permits to estimate social communication in pups by recording mother-offspring interaction via USVs. The test was performed following a previously described

protocol (Servadio *et al.*, 2016). Pups at PND14 were separated individually from their home cage and placed inside a temperature-controlled and sound-attenuating chamber. Pup's ultrasonic vocalizations (USVs) were recorded for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Germany), attached 10 cm above the chamber, able to detect frequencies between 10 and 250 kHz. Number, frequency, amplitude, and duration of calls were analyzed through Avisoft Recorder software (Version 5.1).

Homing Behavior Test

The litter was separated from the dam at the age of PND13 and placed in a new cage for 30 min. Then, each mouse pup was placed individually into a Plexiglas box whose bottom was coated for 1/3 with "old" bedding from the pup's home cage and for 2/3 with "new" clean bedding. The pup was located at the end of the box covered by "new" clean bedding, and its behavior was video recorded for 5 min. Subsequent analysis includes the following parameters: latency (s) (time needed to reach the "old" home-cage bedding area) and nest time (s) (total time spent by the pup in the "old" home-cage bedding area).

Novel Object Recognition (NOR) test

The novel object recognition test enables to evaluate learning and memory capabilities. This test consists of three phases: habituation phase, familiarization phase and test phase. During the familiarization phase, mice were placed in a cage with two identical objects. In the test

phase, one of two previous objects was replaced with a new one and the time spent on exploring those two objects was measured. At the end, the ratio between time spent on exploring the new object with time of two objects exploration was compared. This ratio is expressed as discrimination index. Mice were tested at the age of two months.

Social Interaction Test

The social Interaction test was carried out at PND29-30, four months and six months according to a previously described protocol (Jamain *et al.*, 2008; Terranova and Laviola, 2005). The test consisted of two phases. The first day, mice were undergoing habituation phase in which they were placed individually in the cage for 10 minutes. The second day, two mice were placed together in the same cage and their behaviour was recorded for 10 minutes. We evaluated the following behavior patterns: social sniffing (mouse of interest sniffs any part of the body of the second mouse), crawling (mouse of interest crawls underneath or over the other mouse), mutual circle (both mouse are sniffing mutually each other's anogenital region, creating tight circles with their reciprocal following movements), following (mouse of interest pursues the second mouse), and social grooming (mouse of interest chews and licks the fur of the other mice). Results of the test were expressed as the number of events representing average frequency of total social activities recorded within 10 minutes.

Locomotor activity test

Mice are placed in testing cage box (dimensions: 20,5 × 11cm; height 17cm; with plexiglass wall and grid floor) separately. Experiments are performed under usual day-night cycle conditions and recorded with Actimeter apparatus for 72h. During this period food and water is supplied *ad libitum*. The following conditions are analyzed: ambulations corresponding to the number of times the mouse of interest crosses squares in front and back areas of the cage and when the mouse cross its central area between two peripheric parts of the cage, as well as rearings (when the mouse stands up on its hind feet).

Results

Project 1. Functional characterization of new genes implicated in Early Onset Schizophrenia (EOS), Autism Spectrum Disorder (ASD) and Intellectual Disability (ID)

1. Analysis of WES results from Family 5

The Whole Exome Sequencing (WES) of 9 TRIO (child and the two parents belonging to a cohort of 40 families sharing a similar phenotype) was performed in order to find novel genes that could be involved in the pathogenesis of EOS (see annexes – manuscript). Probands were diagnosed with EOS, which in some cases was combined with other phenotypes such as ASDs and mild ID. Here I present the family 5 in which a very rare variant is present with a putative pathological impact on the phenotype of the patient, according to *in silico* analysis.

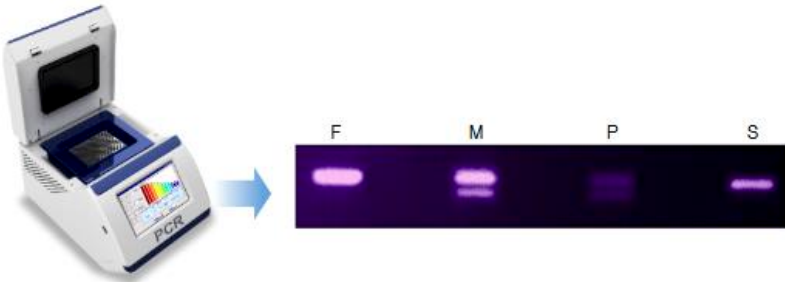
In this family (Fig 1.B) the patient was diagnosed with EOS and ASD at the age of 12 years old using the semi-structured interview Kiddie Schedule for Affective Disorders and Schizophrenia–Present and Lifetime Version (K-SADS-PL) psychosis section. His IQ (47) was estimated with Wechsler Intelligence Scale for Children (WISC-IV, abridged version). The perinatal period of the patient was normal. The subject presents language delayed (1st word at 18 months). The Magnetic Resonance Imaging (MRI) and ElectroEncephaloGraphy (EEG) analysis were normal and no dysmorphic features were observed. However, the patient was separated from his mother in his early childhood, which can be assigned as exposure to a traumatic event that can cause the disclosure of SCZ. The mother of the patient was diagnosed

with personality and psychotic disorder. Personality tests are performed only on the subjects above 16/18 years old that is why the patient could not be analyzed in that context. The mother was reported to have hallucinations, which is one of the psychotic disorder features. The sister and the father were qualified as a healthy individuals. In order to look for potential genetic cause of proband phenotype, I isolated DNA from the blood of the patient and his parents and sent it for subsequent WES analysis to the INTEGRAGEN company. Results of WES were subsequently subjected to bioinformatic analysis with different computational (*in silico*) predictive programs such as: Exome Resequencing Intelligent Sorter (ERIS), Residual Variation Intolerance Score (RVIS), Gene Damage Index (GDI), Polymorphism Phenotyping v2 (PolyPhen2), SIFT, MutationTaster and Human Splicing Finder.

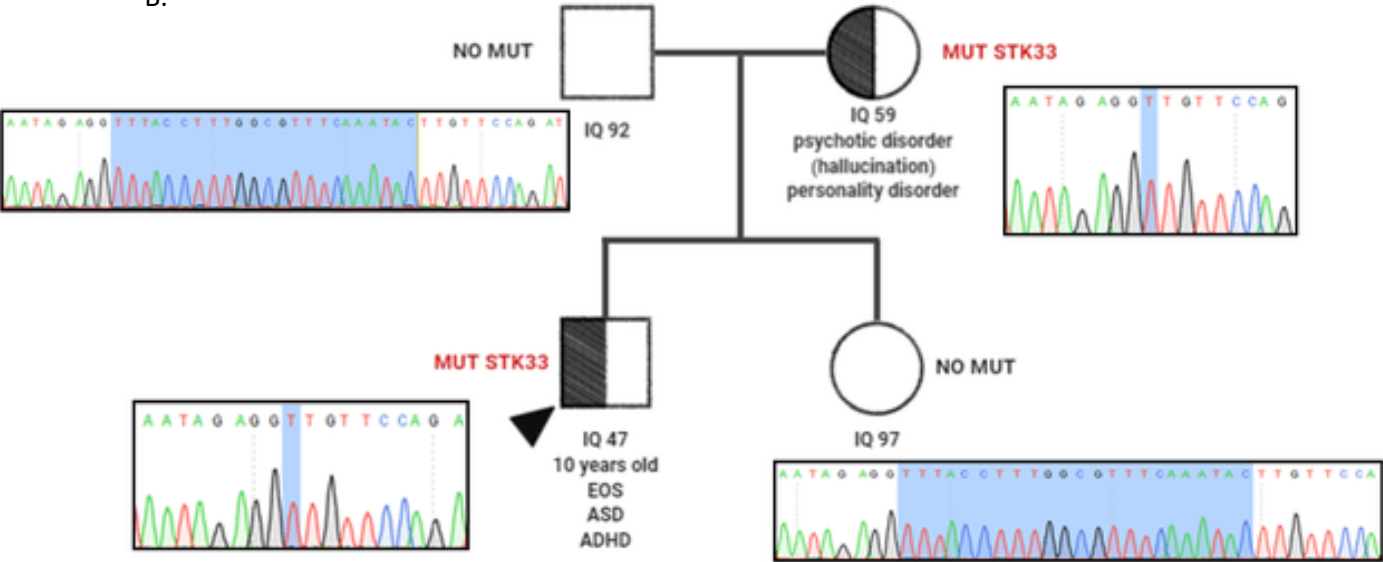
Bioinformatic analysis of WES results revealed one particularly interesting variant in the gene *Serine/Threonine Kinase 33 (STK33)* that is a distant member of the Ca^{2+} /calmodulin-dependent protein kinases (CAMKs). The members of this family contribute to the regulation of calcium homeostasis, a pathway that is known to be altered in patients affected by SCZ. This change is a deletion of 22 nucleotides within a splicing region (Chr11:g.8483346delTTTACCTTTGGCGTTTCAAATAC) in a heterozygous state and was inherited from the patient's mother (Fig 2). This variant was confirmed by SANGER sequencing. PCR products of the reaction with specific primers encompassing the mutation site showed a distinct pattern between healthy (father and sister) and affected (mother and a child) individuals (Fig 1A). I think that this variant can predispose to develop EOS due to the fact that the mother of the patient presents some features of SCZ. The major feature that links the patient and his mother is suffering from hallucinations, however, the mother has a milder

phenotype. I think that the strong phenotype of the patient could be connected with a traumatic event at his early childhood that could have triggered the disclosure of EOS. During WES, I detected also many other changes in the patient but the one in the *STK33* gene seemed to have the strongest pathological impact, according to *in silico* analysis, that is why I decided to analyze it more profoundly. It is important to emphasize that this gene is probably not the only one responsible for developing the disease but has a strong potential to be a major one.

A.



B.



LEGEND Affected individual Unaffected individual Proband

MUT STK33 → Chr11:g.8483346delTTTACCTTTGGCGTTTCAAATAC

Figure 1. A. Products of PCR reaction performed on DNA of members of the affected family. F- father, M- mother, P- proband, S- sister. B. Pedigree of the family in which the genetic variant was found. DNA chromatograms illustrating the detection of a deletion in STK33 via Sanger sequencing.



Figure 2. Localization of the 22nt deletion in the STK33 DNA sequence (<https://www.benchling.com/>).

2. Differentiation of the SH-SY5Y cell line

Due to the fact that EOS is a neurological disorder and that I was aiming to create a model of neuronal function, I performed the protocol that allows differentiation of the SH-SY5Y cells into a neuronal-like phenotype. This enabled me to progress further with characterization of the obtained cell lines in a neurological context. I performed the differentiation protocol according to Dwane *et al.* (2013) and verified the presence of neuronal markers in differentiated cell lines by immunofluorescence and RT-qPCR techniques (Fig. 3A and B, respectively). On images from immunofluorescence, I observed prolongments created by the cells during the differentiation process. RT-qPCR analysis showed an increase in the specific

neuronal markers level in reference to undifferentiated cells. These results suggest that differentiation of the cell lines (HOM and HET cell lines are described in part 4.) was successfully carried out.

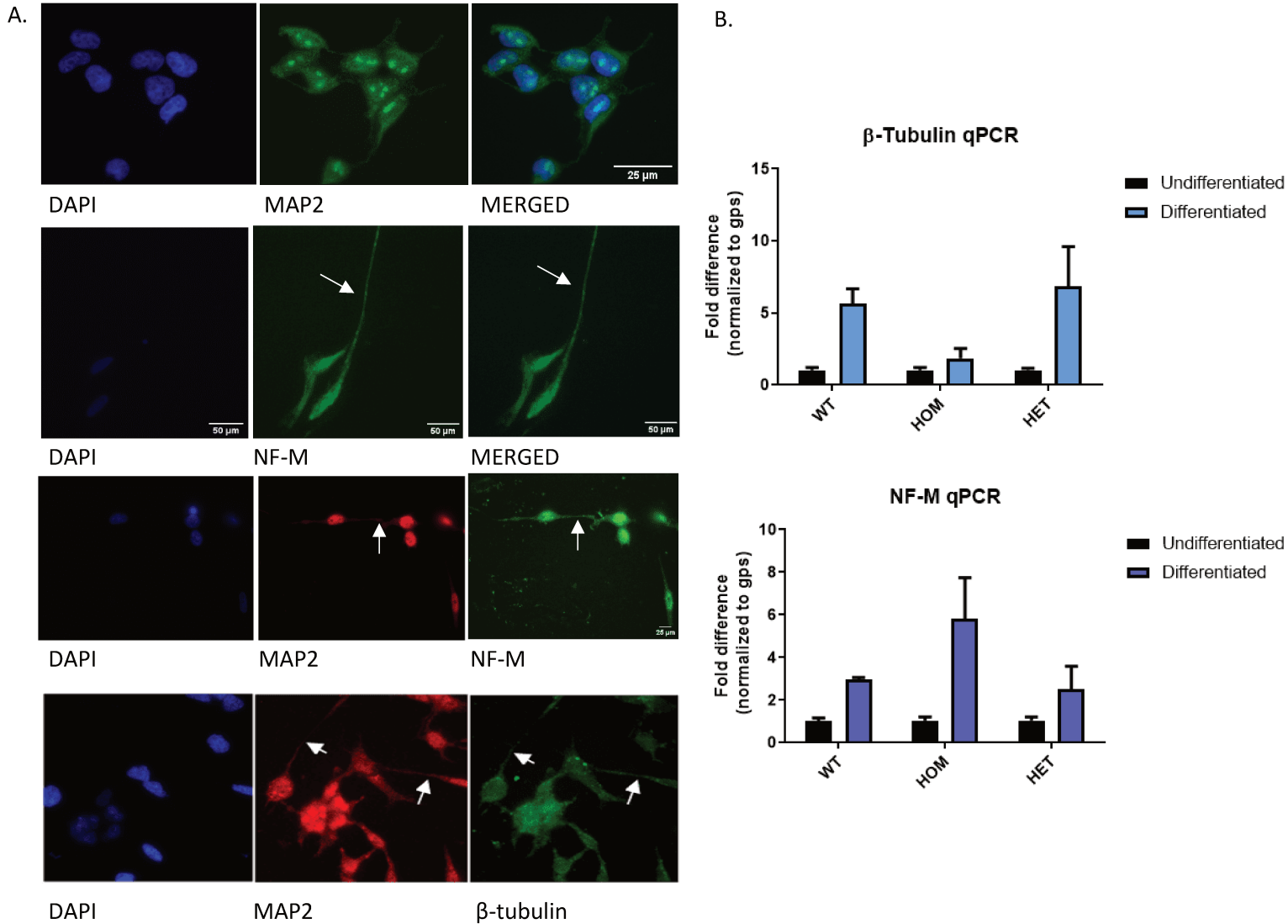


Figure 3. A. Differentiation of SH-SY5Y cells after treatment with IGF. The first upper panel shows undifferentiated cells. Second, third and fourth lower panels show differentiated cells. Nuclei are stained in blue (DAPI), in red (neuronal marker MAP2) and in green (MAP2 (first panel), neuronal markers NF-M and β -Tubulin). B. qPCR analysis of neuronal marker β -Tubulin and NF-M. The mRNA level of each marker in differentiated cells is compared to the level in undifferentiated cell lines.

3. Impact on calcium homeostasis

STK33 is a distant member of the CAM kinase family. I decided to verify the potential impact of this gene on calcium homeostasis. For this reason, I used a calcium imaging technique to measure the changes in calcium levels in cells. Knowing that in the future I am planning to perform this experiment in the clone lines to further investigate the impact on the identified mutation on calcium homeostasis, initially I decided to verify the potential of the SH-SY5Y cell line to respond to different stimuli. I also intended to verify if the approach with the *STK33* siRNA technique can have potential impact on the SH-SY5Y cell line. For these reasons, in these preliminary experiments I did not use the control siRNA that will be used in the final experiments.

I reduced the level of *STK33* by RNA interference. SH-SY5Y cells were transfected with ON-TARGETplus Human *STK33* siRNA (Horizon Discovery) one day before calcium imaging. Cells were subjected to the differentiation protocol as described in Materials and Methods. Efficacy of siRNA transfection was verified by western blot and RT- qPCR techniques. Results indicated that siRNA transfection led to 50% reduction of mRNA and protein level of *STK33* in comparison to WT cells (Figure 4. A, B and C).

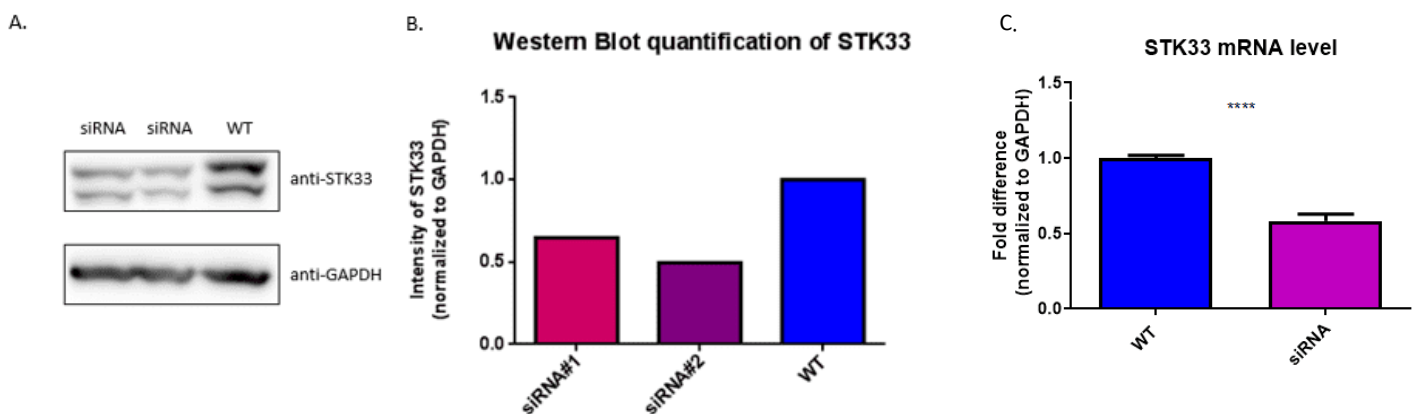


Figure 4. A. Western blot analysis of STK33 protein level in cells transfected with siRNA and in WT cells. B. Quantification of western blot of STK33 protein level. C. Unpaired t-test revealed differences in mRNA levels between WT cells and cells transfected with siRNA (P value $<0,0001$).

In order to investigate the impact on calcium levels, WT cells and cells transfected with the STK33 siRNA were loaded with membrane-permeant calcium indicator Fluo-4 whose fluorescence increases upon binding with calcium ions (Fig. 6A). Fluo-4 is frequently used for measurements of intracellular calcium level. Changes in Fluo-4 fluorescence were recorded under an inverted microscope (AxioObserver, Carl Zeiss). During recording, cells were perfused with different solutions as follows: Tyrode's—30 s; Tyrode's + Carbachol—20 s; Tyrode's—40 s; Tyrode's (0 Calcium) - 40s; Tyrode's (0 Calcium) plus ionomycin—20 s; Tyrode's (10 Calcium) plus ionomycin—30 s (Fig. 5 and 6B).

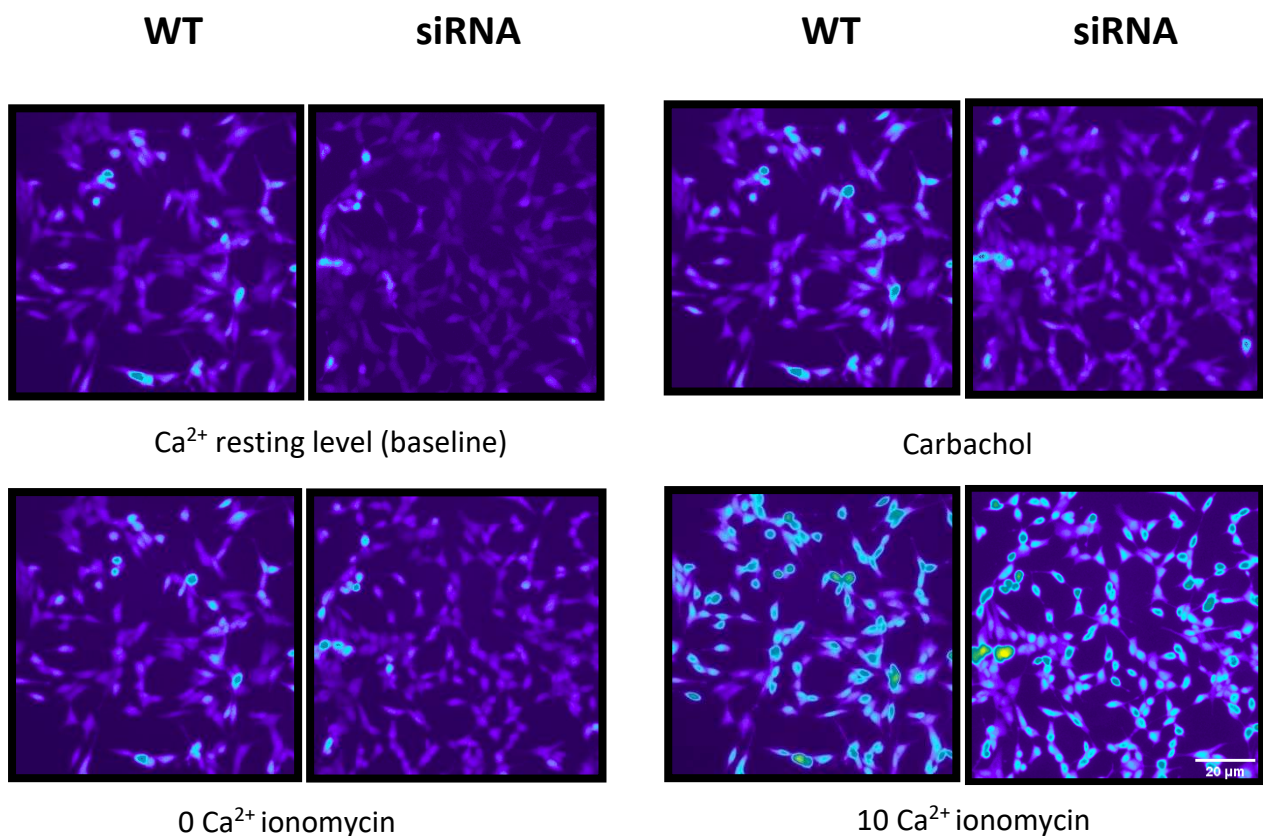


Figure 5. Profiles of the calcium imaging response. Panels show the emission of Fluo-4 upon following perfusions: 1.6 mM Ca^{2+} , Carbachol, 0 Ca^{2+} ionomycin and 10 Ca^{2+} ionomycin. Left panels show WT cells and right panels cells transfected with siRNA specific for STK33. The scale bar of each panel is 20 μm .

I investigated the impact of *STK33* decrease on carbachol-stimulated release of calcium from intracellular calcium stores. Carbachol is a well-known muscarinic receptor agonist that induces inositol-1,4,5-triphosphate (IP_3) response mainly via M1 and M3 muscarinic receptors. These receptors can couple with PhosphoLipase C (PLC) and activate the IP_3 pathway that leads to increase in intracellular calcium levels. The process of phosphoinositide hydrolysis in the SH-SY5Y cell line resembles the same process that occurs in different brain regions such as hippocampus and cortex (Larsson *et al.*, 1998). My results showed that response to carbachol is lower in cells with decreased levels of *STK33* (Fig. 6D). Upon stimulation with 0 Ca^{2+} , the level of calcium decreases more in WT cells, which indicates faster extrusion of Ca^{2+} from the cells (Fig. 6E). Subsequent stimulation with 0 Ca^{2+} + ionomycin led to calcium entry into the intracellular compartments (Fig. 6F). Ionomycin is a calcium ionophore that causes immediate increase in calcium influx into the intracellular regions of the cells. Calcium measurements at this stage showed lower level in the cells transfected with siRNA. Together with measurements from the previous step, this result suggests that entry of Ca^{2+} is slower and lower in cells with decreased *STK33* level compared to WT cells. Upon treatment with 10 Ca^{2+} plus ionomycin, the level of Ca^{2+} in WT cells is much higher than in cells transfected with siRNA (Fig. 6G). It is consistent with previous stimulation and indicates slower and lower Ca^{2+} intake in cells with decreased levels of *STK33*. I did not observe any difference concerning the

baseline between WT and siRNA-transfected cells. It can indicate that loading with Fluo-4 is equal and the basic calcium level is not changed between the different cell types. These results suggest that *STK33* does not influence the basic level of calcium, but can participate in the response to different stimuli. These results are only preliminary ones and will be confirmed in the future with the mutated clone cell lines and a proper siRNA control. Nevertheless, these results indicate that SH-SY5Y cells are able to respond to different stimuli and it is possible to detect differences in their response.

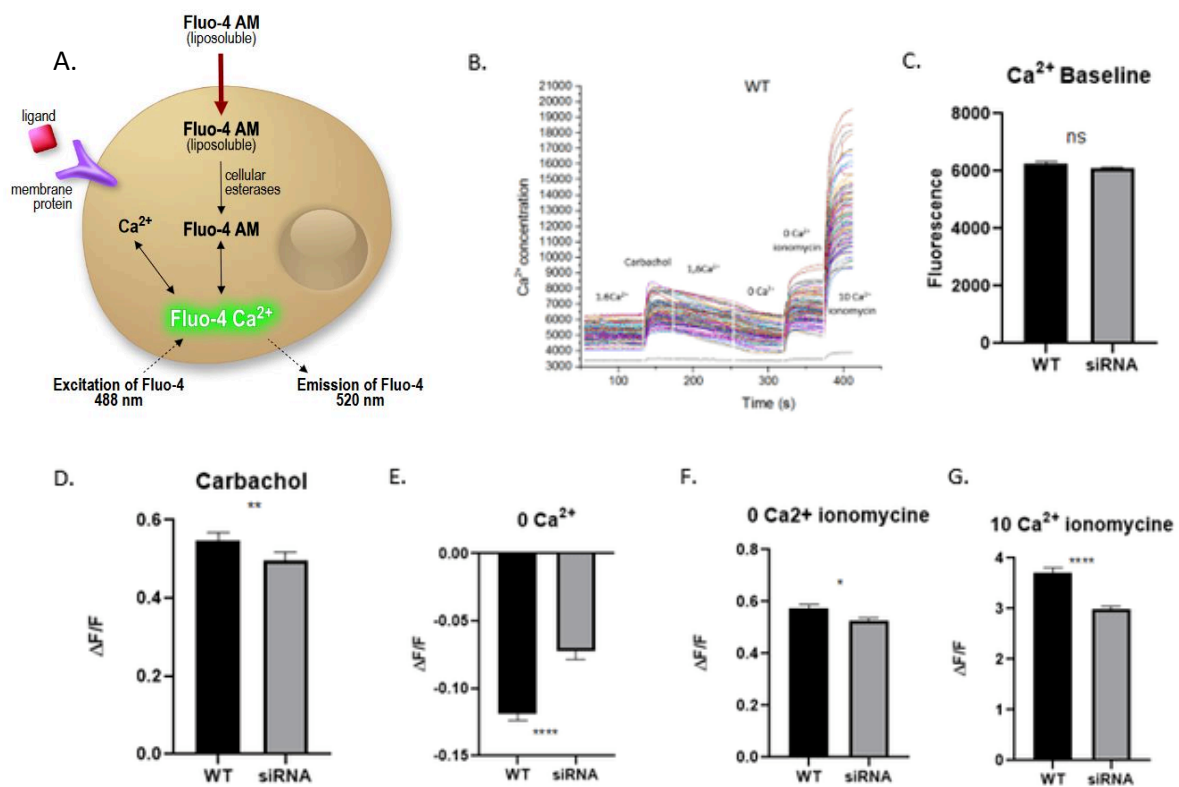


Figure 6. A. Scheme of Fluo-4 entry into the cell. B. Ca^{2+} concentration upon treatment with different perfusions. C. Fluo-4 fluorescence upon treatment with Tyrode's (mean from all baselines). D. Ca^{2+} level upon treatment with Tyrode's + carbachol. E. Ca^{2+} level upon perfusion with Tyrode's ($0 Ca^{2+}$). F. Ca^{2+} level upon perfusion with Tyrode's ($0 Ca^{2+}$) + ionomycin. G. Ca^{2+} level upon perfusion with

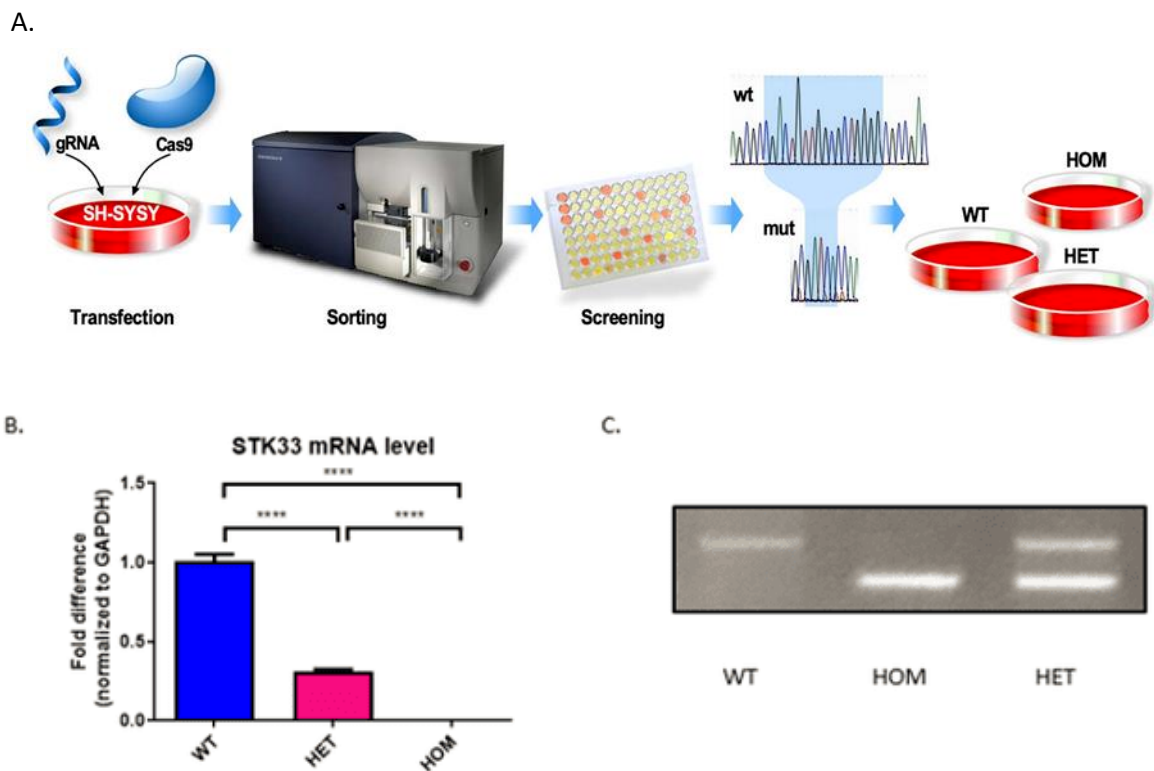
Tyrode's (10 Ca²⁺) plus ionomycin. Mann-Whitney test: ns P=0.2103; ** P =0.0058; **** P<0.0001, * P=0.0140, **** P<0.0001. WTn = 610; siRNA n = 797.

4. Cellular model mimicking the variants found in patients in the SH-SY5Y cell line by the CRISPR-Cas9 technique

In order to evaluate the pathological potential of the identified variant, I decided to create a cellular model in the SH-SY5Y cell line. This cell line is derived from *Homo sapiens* bone marrow neuroblasts and is commonly used as *in vitro* model of neuronal function. Its potential to be differentiated in neuronal like cells has been previously demonstrated (Dwane *et al.*, 2013). To attain this aim I used the CRISPR-Cas9 system, which permits to introduce precise changes in the DNA sequence. I designed a guide RNA (gRNA) targeting the fragment of the *STK33* gene in which the variant in the patient was found (Fig. 7A). I also designed a ssODN template with the same change that was identified in the patient. I introduced these two elements of the system together with Cas9 nuclease into SH-SY5Y cells and subsequently I isolated single cells containing the system. This was possible since the plasmid [pSpCas9n(BB)-2A-GFP] co-expresses Cas9 nuclease and GFP. I performed a screening in order to detect positive clones. I designed specific PCR primers encompassing the mutation site in order to detect clones carrying the deletion. All clones with a potential deletion were subjected to sequencing to verify the sequence and to establish the exact position of the deletion. Clones with double signals were subjected to cloning in order to define the zygosity of the cells. I managed to obtain heterozygous and homozygous clones with the mutation that mimic the variant found in the patient. I checked cell lines for potential off-targets with the set of

primers, as described in the Materials and Methods section. I verified off-targets that had the highest potential to affect neurological functions.

I performed RT-qPCR analysis in order to verify the potential impact of the introduced mutation on the *STK33* transcript. RT-qPCR analysis showed that the mRNA level of the WT form of *STK33* is decreased in the heterozygous clone and is not present in the homozygous clone (Fig. 7B). PCR reaction with specific primers encompassing the mutation site performed on cDNA from WT, heterozygous and homozygous cell lines revealed specific pattern for each of these lines (Fig. 7C). Sequencing of the specific bands revealed that the introduced mutation leads to deletion of exon 4 in the *STK33* transcript (Fig. 7D).



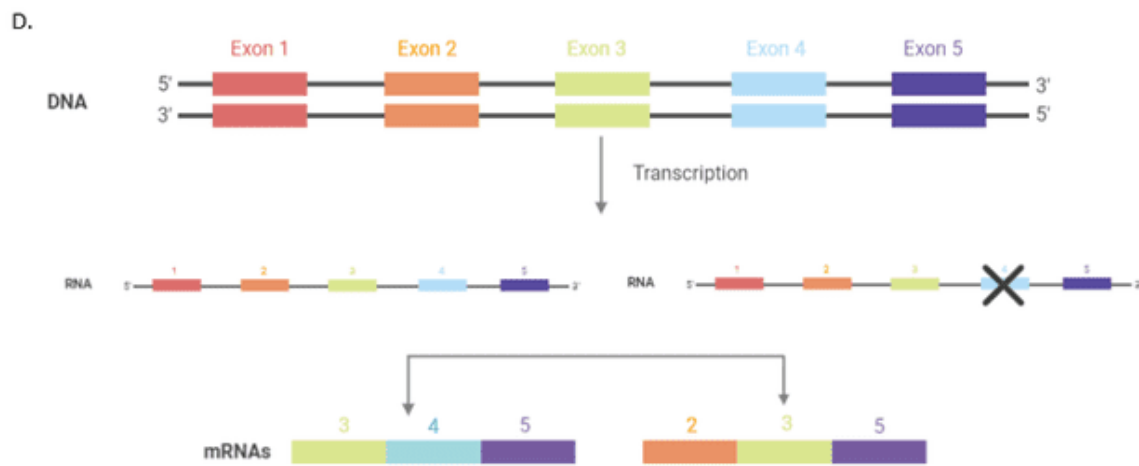
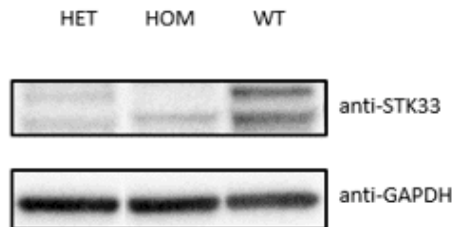


Figure 7. A. Scheme of the generation of clone lines with the CRISP-Cas9 system and subsequent single cell sorting, screening and verification of positive clone lines by sequencing. B. STK33 mRNA level in WT, heterozygous and homozygous cell lines. Ordinary one-way ANOVA test revealed statistical differences. **** <0.0001 C. PCR fragments amplified from cDNA with primers encompassing the mutation site. D. Effect of the STK33 mutation (deletion of 22nt in DNA) on the transcript. The mutation leads to deletion of exon 4 of STK33.

To evaluate the impact on the protein level, I performed western blot analysis with anti-STK33 antibody (Fig. 8A and B). This analysis revealed that the STK33 level in the heterozygous and homozygous cell lines is decreased. Moreover, in the homozygous cell line I observed the absence of one band, which is in concordance with the analysis of STK33 transcript that lacks exon 4. Probably, introduced mutation caused absence of one of STK33 isoform. This part of the results, confirmed that I managed to successfully introduce the variant found in the patient into SH-SY5Y cells and revealed the potential consequences of this mutation on RNA and protein levels.

A.



B.

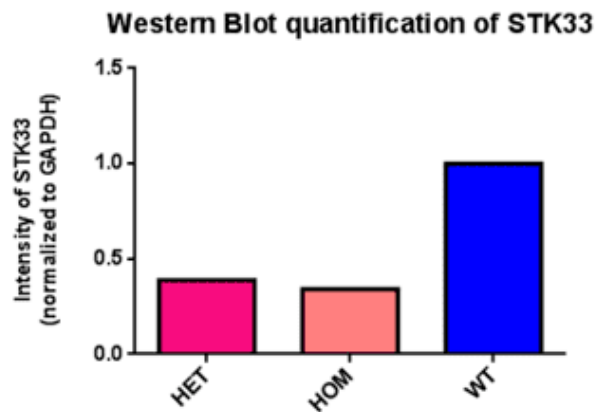


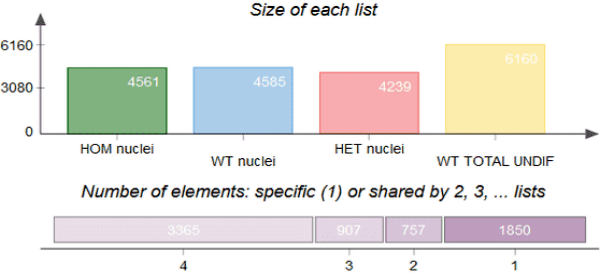
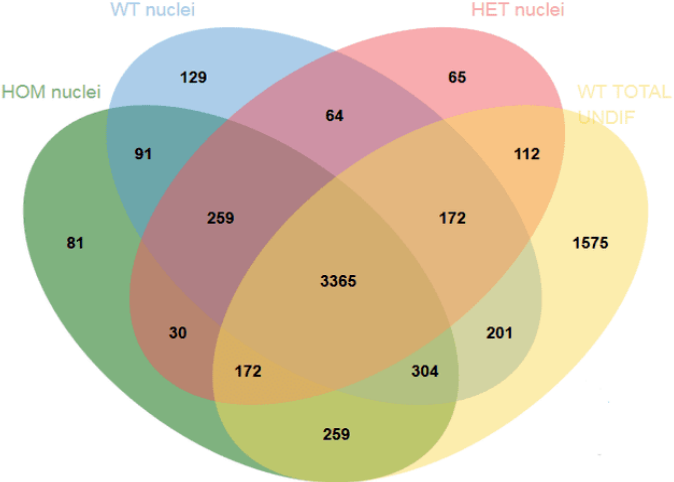
Figure 8. A. Western blot analysis of STK33 protein in heterozygous, homozygous and WT cell lines, respectively. B. Quantification of Western blot of STK33 protein level in heterozygous, homozygous and WT cell lines, respectively.

5. Proteomic analysis of clone cell lines

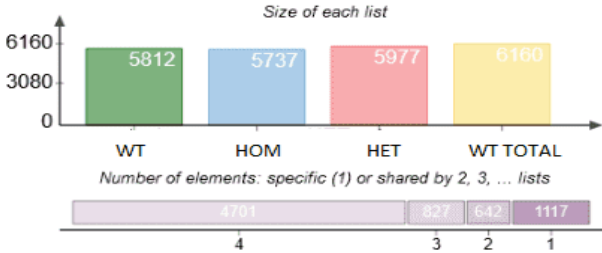
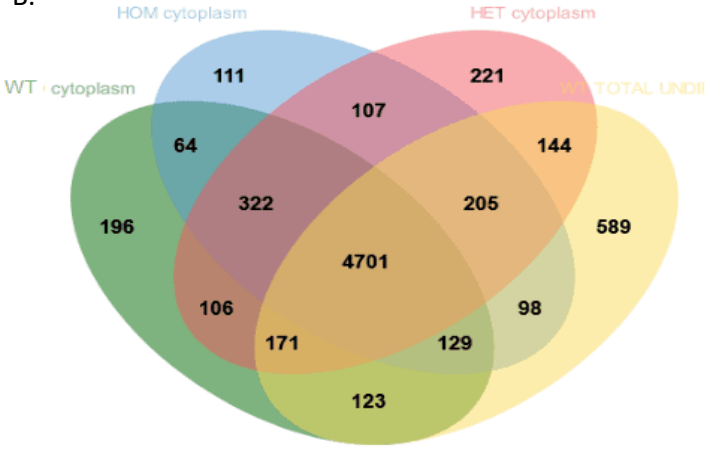
I performed isolation of proteins from total, cytoplasmic and nuclear fraction of WT, heterozygous and homozygous cell lines. I created a Venn Diagram to see common and distinct proteins identified via LC-MS/MS (Fig. 9A and B). I used total extract from WT undifferentiated cells to remove detected proteins that can be associated with differentiation process. Further on, I focused on specific proteins from nuclei and cytoplasmic fractions. Through analysis with the DAVID tool I was looking at specific/unique proteins from each cell type (WT, HOM and HET) in order to see if among those proteins are proteins associated with psychological or neurological dysfunctions (Fig. 9C and F). I identified many proteins meeting these criteria and, among them, various factors (*e.g.*, DISC1, CHGA, FSTL1, GJA1, CAMKV, ALDH1A3, CYTB, and GRIP1) were previously associated with the SCZ phenotype. Molecular function analysis with PANTHER showed that the majority of these proteins are involved in binding, catalytic activity, transcription, translation, transport and activity of molecular function (Fig. 9D and G). Pathway analysis of these proteins revealed pathways such as CCKR, Wnt and Cadherin Signaling.

Changes in these pathways have been associated with SCZ (Fig. 9E and H). Moreover, I detected proteins belonging to pathways linked to axon guidance that can have an impact on the neurological phenotype.

A.



B.



C.

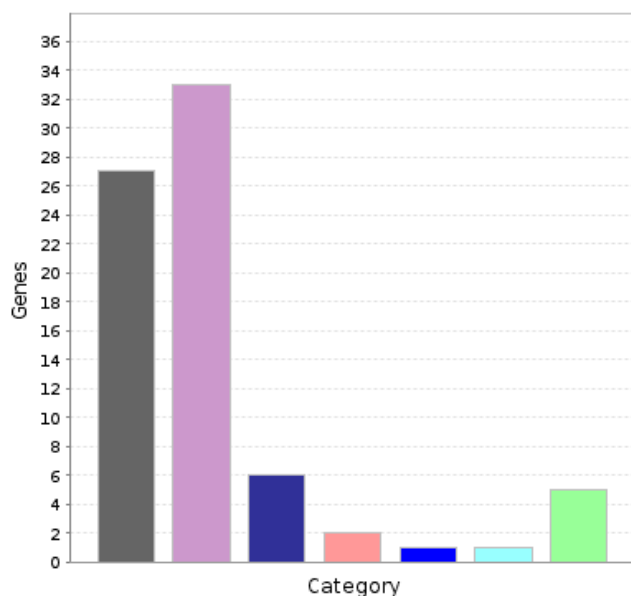
Association: PSYCH or NEUROLOGICAL

WT cytoplasm	HOM cytoplasm	HET cytoplasm
CAMKV	GNAL	ABCA13
EDIL3	MEIS2	HMGXB4
FAT1	PKNOX1	CHGA
GMIP	ARHGEF10	CNTN1
RBFOX1	CPE	FSTL1
TBC1D1	CNTNAP4	FOXP1
AP3M2	DOCK3	GJA1
ADCY9	DISC1	GPC1
ALDH1A3	MROH2A	MAN2A2
AMOTL1	HLA-A	MME
ARNTL	HLA-C	PI4K2B
CRY1	HLA-DRB1	PIK3CB
CYTB	PGPEP1	PLA2G4C
GRIP1	RGS19	PRB2
HKDC1	SDK1	BNIP2
LSAMP	HAAO	CD44
NET1	FAS	EPHB2
NRCAM	LRP1B	RBMS3
NCOA7	RIC1	TBC1D20
NR3C2	CUX1	THAP4
PIGA	DLG5	TNFAIP3
PDE4B	GPAM	ULBP2
PRNP	MICAL2	ACTA2
SYT1	OGDHL	APH1A
TFAP2B	PHYH	C9orf72
TGM2	PRKCE	CNNM2
VAT1L	SLC25A52	ENTPD7
BTBD9	TSPAN15	EIF2AK3
KIAA0586		FAM63A
MEIS3P1		FGA
NDST2		FGF2
RAP1GAP2		GSTM4
ACTN3		GPM6B
CAPN5		PSD3
CAV1		PHLDB2
FAM63B		RNF41
IRS2		STBD1
KRT82		TRPM7
LRRK1		USP40
MLK4		
MYO10		
STK11		
SORCS1		
SCSD		
TMEM55A		

D.

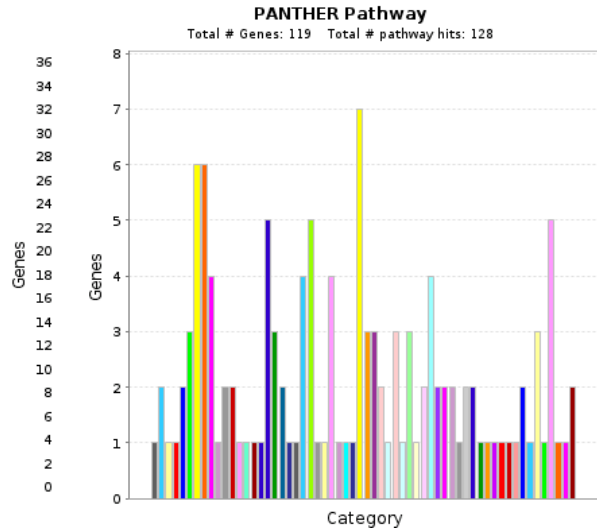
PANTHER GO-Slim Molecular Function

Total # Genes: 119 Total # function hits: 75



- [binding \(GO:0005488\)](#)
- [catalytic activity \(GO:0003824\)](#)
- [molecular function regulator \(GO:0098772\)](#)
- [molecular transducer activity \(GO:0060089\)](#)
- [transcription regulator activity \(GO:0140110\)](#)
- [translation regulator activity \(GO:0045182\)](#)
- [transporter activity \(GO:0005215\)](#)

E.



- [Gonadotropin-releasing hormone receptor pathway \(P06664\)](#)
- [Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway \(P00026\)](#)
- [Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway \(P00027\)](#)
- [Histamine H1 receptor mediated signaling pathway \(P04385\)](#)
- [Histamine H2 receptor mediated signaling pathway \(P04386\)](#)
- [Huntington disease \(P00029\)](#)
- [Hypoxia response via HIF activation \(P00030\)](#)
- [Inflammation mediated by chemokine and cytokine signaling pathway \(P00031\)](#)
- [Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade \(P00032\)](#)
- [Insulin/IGF pathway-protein kinase B signaling cascade \(P00033\)](#)
- [Integrin signalling pathway \(P00034\)](#)
- [Interleukin signaling pathway \(P00036\)](#)
- [Muscarinic acetylcholine receptor 1 and 3 signaling pathway \(P00042\)](#)
- [Nicotinic acetylcholine receptor signaling pathway \(P00044\)](#)
- [Notch signaling pathway \(P00045\)](#)
- [Oxytocin receptor mediated signaling pathway \(P04391\)](#)
- [PDGF signaling pathway \(P00047\)](#)

- [5-Hydroxytryptamine degradation \(P04372\)](#)
- [5HT2 type receptor mediated signaling pathway \(P04374\)](#)
- [5HT4 type receptor mediated signaling pathway \(P04376\)](#)
- [Adrenaline and noradrenaline biosynthesis \(P00001\)](#)
- [Alpha adrenergic receptor signaling pathway \(P00002\)](#)
- [Alzheimer disease-amyloid secretase pathway \(P00003\)](#)
- [Alzheimer disease-presenilin pathway \(P00004\)](#)
- [Angiogenesis \(P00005\)](#)
- [Apoptosis signaling pathway \(P00006\)](#)
- [Axon guidance mediated by Slit/Robo \(P00008\)](#)
- [Axon guidance mediated by netrin \(P00009\)](#)
- [B cell activation \(P00010\)](#)
- [Beta1 adrenergic receptor signaling pathway \(P04377\)](#)
- [Beta2 adrenergic receptor signaling pathway \(P04378\)](#)
- [Beta3 adrenergic receptor signaling pathway \(P04379\)](#)
- [Blood coagulation \(P00011\)](#)
- [CCKR signaling map \(P06959\)](#)
- [Cadherin signaling pathway \(P00012\)](#)
- [Circadian clock system \(P00015\)](#)
- [Corticotropin releasing factor receptor signaling pathway \(P04380\)](#)
- [Cytoskeletal regulation by Rho GTPase \(P00016\)](#)
- [EGF receptor signaling pathway \(P00018\)](#)
- [Endothelin signaling pathway \(P00019\)](#)
- [Enkephalin release \(P05913\)](#)
- [FAS signaling pathway \(P00020\)](#)
- [FGF signaling pathway \(P00021\)](#)
- [Fructose galactose metabolism \(P02744\)](#)
- [GABA-B receptor II signaling \(P05731\)](#)
- [Glycolysis \(P00024\)](#)
- [PI3 kinase pathway \(P00048\)](#)
- [Pentose phosphate pathway \(P02762\)](#)
- [Plasminogen activating cascade \(P00050\)](#)
- [Ras Pathway \(P04393\)](#)
- [Synaptic vesicle trafficking \(P05734\)](#)
- [T cell activation \(P00053\)](#)
- [Thyrotropin-releasing hormone receptor signaling pathway \(P04394\)](#)
- [Toll receptor signaling pathway \(P00054\)](#)
- [VEGF signaling pathway \(P00056\)](#)
- [Vasopressin synthesis \(P04395\)](#)
- [Wnt signaling pathway \(P00057\)](#)
- [p53 pathway by glucose deprivation \(P04397\)](#)
- [p53 pathway feedback loops 2 \(P04398\)](#)
- [p53 pathway \(P00059\)](#)

F.

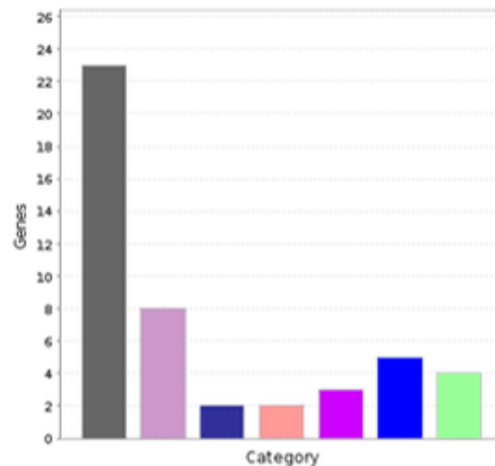
Assotiation: PSYCH or NEUROLOGICAL

WT nuclei	HOM nuclei	HET nuclei
ABAT	OGG1	JUNB
ADAMTS4	DDX53	PHF1
ABCD1	Hsp40/DNAJB2	STARD9
BCL11A	GPALPP1	EGR1
POLL	ND4	HOMER2
ELAVL4	NFKBIL1	KBTBD11
FHDC1	RBMS3	MAPT
KIAA1161	PHF12	NCEH1
PKNOX1	SEC61A2	NFIC
PKNOX2	TEAD3	PDGFRA
RIT1	TRAF4	POF1B
TAF1C	ACTBL2	SC5D
ATF2	ADIPOR1	
APBB1IP	BRWD1	
BMP1	GDF15	
CALCOCO1	LAMB2	
CNNM2	LMAN2L	
ELMO1	NET1	
FMNL2	NOS1AP	
MAB21L1	NDEL1	
NEFL	ZRSR2	
NFIB		
NCOA4		
RFWD2		

G.

PANTHER GO-Slim Molecular Function

Total # Genes: 61 Total # function hits: 47



- [binding \(GO:0005488\)](#)
- [catalytic activity \(GO:0003824\)](#)
- [molecular function regulator \(GO:0098772\)](#)
- [molecular transducer activity \(GO:0060089\)](#)
- [structural molecule activity \(GO:0005198\)](#)
- [transcription regulator activity \(GO:0140110\)](#)
- [transporter activity \(GO:0005215\)](#)

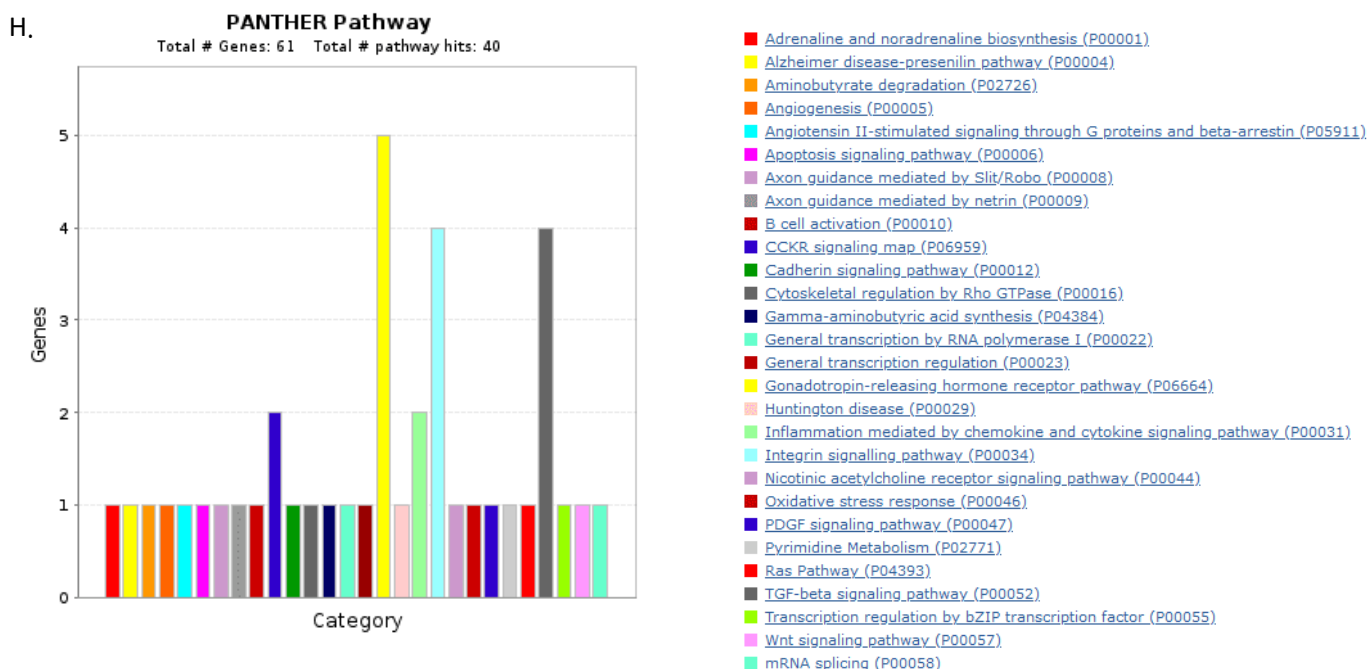


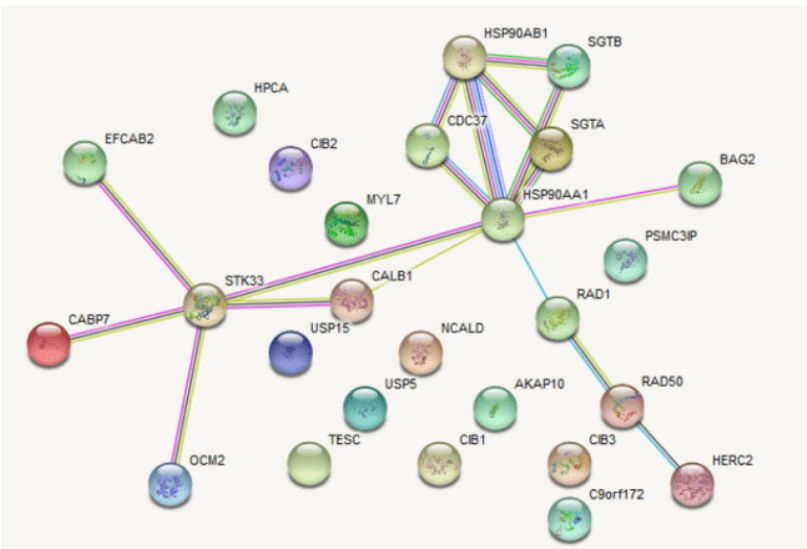
Figure 9. A and B. Venn Diagrams represent proteins identified from nuclei and cytoplasm, respectively. C and F. Unique proteins from different cell types associated with terms psychological or neurological (according to DAVID tool). D and G. Molecular functions of protein analysis associated with terms psychological or neurological (PANTHER). E and H. Pathway analysis of proteins associated with terms psychological or neurological from nuclei and cytoplasm, respectively (PANTHER).

6. STK33 interactors

I performed STRING analysis of potential *STK33* interactors in order to find some genes/proteins that can be affected by changes in *STK33* (Fig. 10A). The analysis revealed that one of the direct interactor of *STK33* is *HSP90AA1* that encodes Heat shock protein HSP 90-alpha. Interestingly, a small deletion (c.1093_1098delGAAGAA) in this gene was found in the proband from family 5. This change has been predicted as polymorphism by MutationTaster. Hsp90 is related to Akt signaling that can affect Wnt/ β -catenin signaling, which lies downstream this pathway (Halder *et al.*, 2012). It was also shown that Hsp90 can reduce phosphorylation of ERK by affecting Raf protein (Dou *et al.*, 2005). Hsp90 can as well interact

with EGFR (Ahsan *et al.*, 2012)(Fig. 10B). EGF and neuregulins, which belong to ligands of EGFR and ErbB1-4 receptors, are involved in brain development and its function (Iwakura and Nawa, 2013). This analysis unveils a potential involvement of this change in the phenotypic features of the patient. I could hypothesize that this polymorphic change that is not present in the mother can deteriorate the phenotype of the patient.

A.



B.

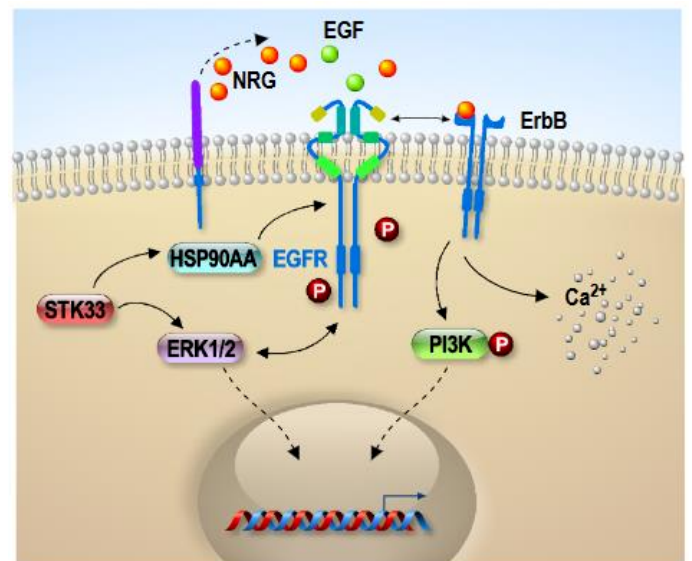


Figure 10. A. *STK33* interactors according to STRING database. B. Interaction between *STK33* and *HSP90AA* and their link to EGFR and ERK-signalling.

The previously demonstrated impact of *STK33* on calcium homeostasis encouraged me to verify the level of mRNA of *STK33* calcium-associated interactors. RT-qPCR analysis indicates decrease in mRNA level of *CABP7* (Calcium Binding Protein 7) and *HPCA* (Neuron-Specific Calcium-Binding Protein Hippocalcin) in homozygous cell line.

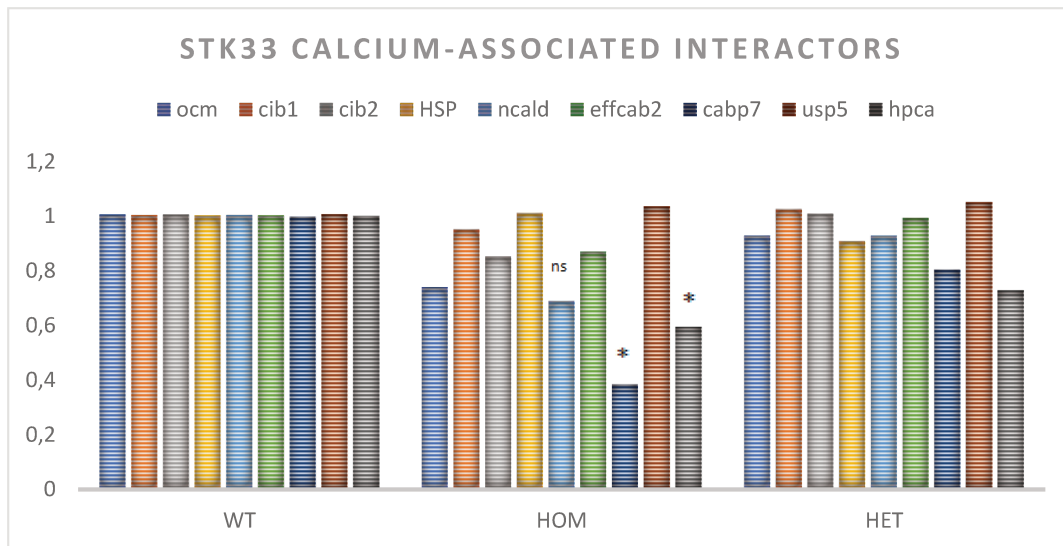


Figure 11. RT-qPCR analysis of mRNA level of STK33 calcium-associated interactors. Ordinary one-way ANOVA test (*cabp7* * $P=0,0163$) and (*hpca* * $P=0,0215$).

Due to the association of ERK-signalling with SCZ, I decided to perform western blot analysis of the ERK1/2 phosphorylation level. My results indicate decreased phosphorylation in homozygous and heterozygous cell lines.

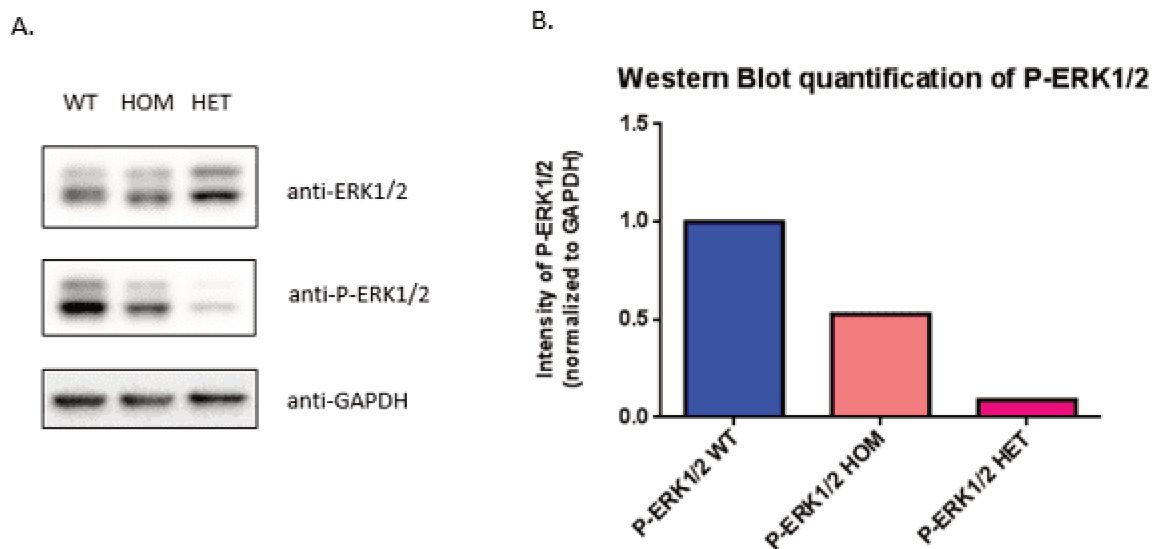


Figure 12. A. Western blot analysis of ERK1/2 protein level in WT, homozygous and heterozygous cell lines. B. Quantification of western blot of ERK1/2 protein level.

Project 2. Characterization of a novel spontaneous mutation in the *Kcc2* gene in mice suffering from spontaneous tonic-clonic seizures starting at the age of four months

1. Identification of a novel *Kcc2* mutation

The project emerged from the observation of a cohort of *Fmr1-KO* mice (Fragile X model mice) suffering from spontaneous tonic-clonic seizures at adult age. While young mice never displayed this phenotype, starting from four months, seizures could be provoked just by the sharp movement of the cage. After the observation that the phenotype was inherited as an autosomal dominant character (Fig. 1), I decided to analyze two affected and one non-affected mice (an affected father and two offspring with different phenotypes) by whole exome sequencing.

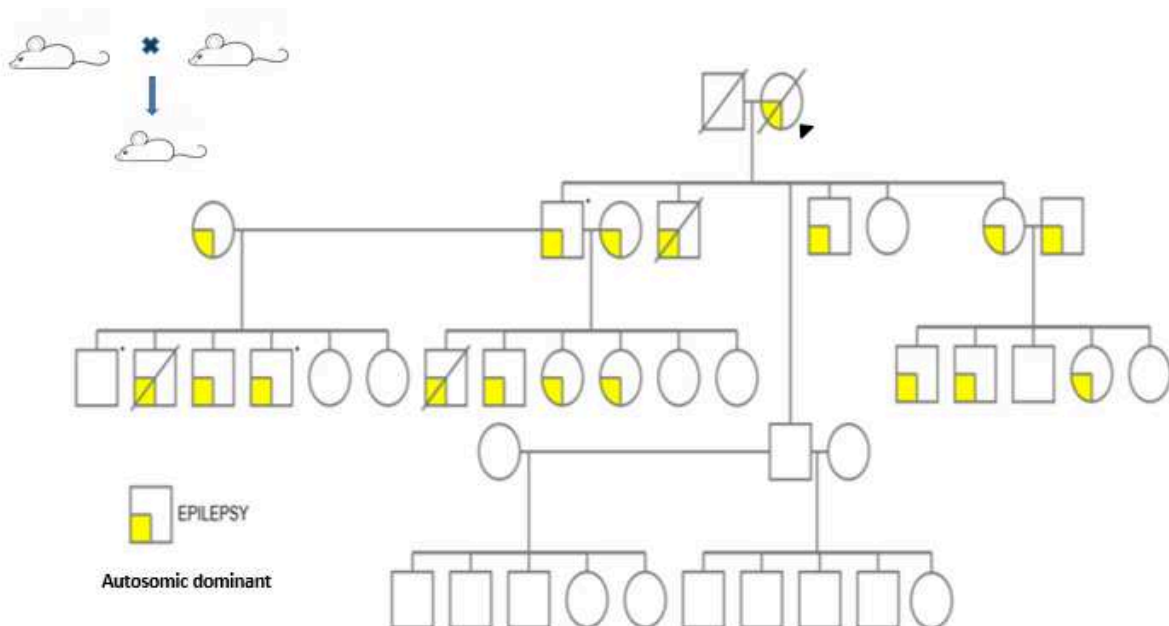


Figure 1. Part of the pedigree of a large mouse cohort that were subjected to sequencing in order to evaluate the presence of the *Kcc2* c.2569C>G (R857G) mutation. Mice possessing the mutation suffering from epileptic crisis are indicated in yellow. A black arrowhead indicates the proband.

From the WES results, I only extracted the variants in common between the two affected individuals, discarding the variants shared with the healthy one (Fig. 2A). By doing so, I obtained 2443 variants that were further evaluated with Variant Effect Predictor (Fig. 2B, C and D) and Mouse Genome Informatics (MGI). During this evaluation I concentrated on moderate and high impact variants (according to VEP) and their correlation with human disease phenotypes (according to MGI). Through this analysis, I managed to find an interesting variant (c.2569C>G; R857G) in the *Potassium-chloride transporter member 5* (aka: *KCC2* and *SLC12A5*) gene which is located in the homologous position as the already known mutation connected with EIMFS in a human patient (Fig. 3). This patient was diagnosed with EIMFS and displayed seizures before the age of four months, abnormal electroencephalogram and delayed intellectual development (Saito *et al.*, 2017). I also performed prediction tests for the identified variant with tools such as Provean and SIFT to predict its potential consequences. Indeed, both tools described the c.2569C>G variant as potentially deleterious (Provean score -6.51) and damaging (SIFT score 0). Moreover, the region in which the mutation is positioned is highly conserved among multiple species (Fig. 3).

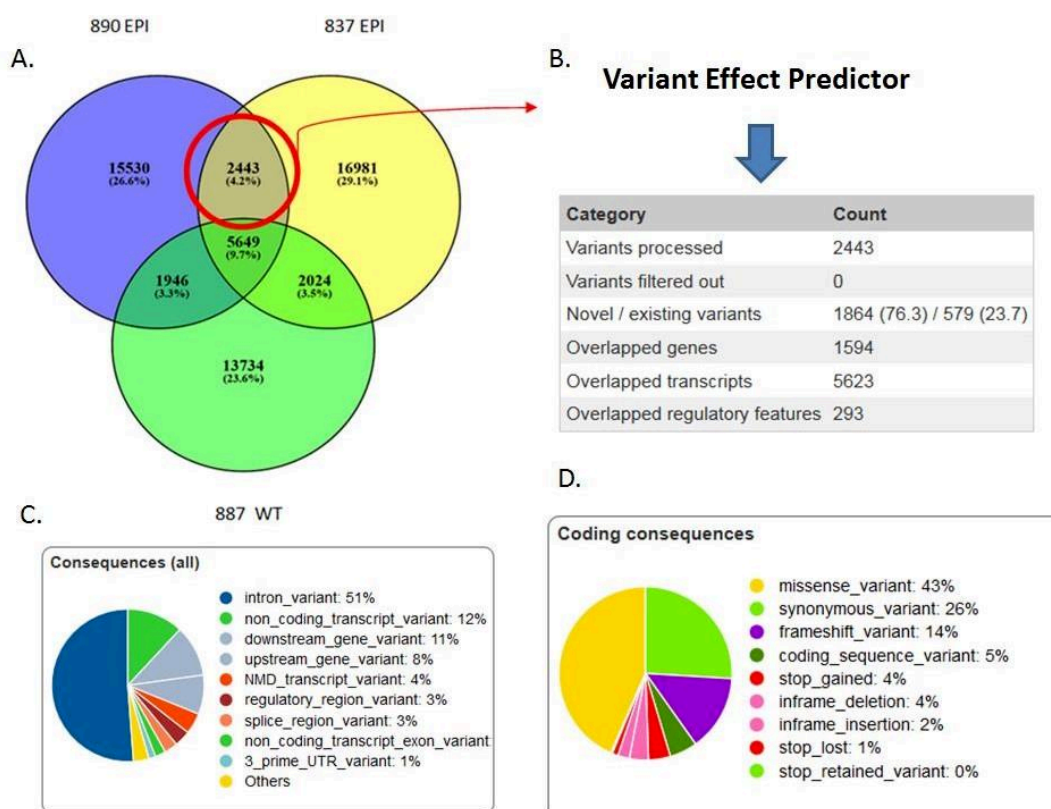
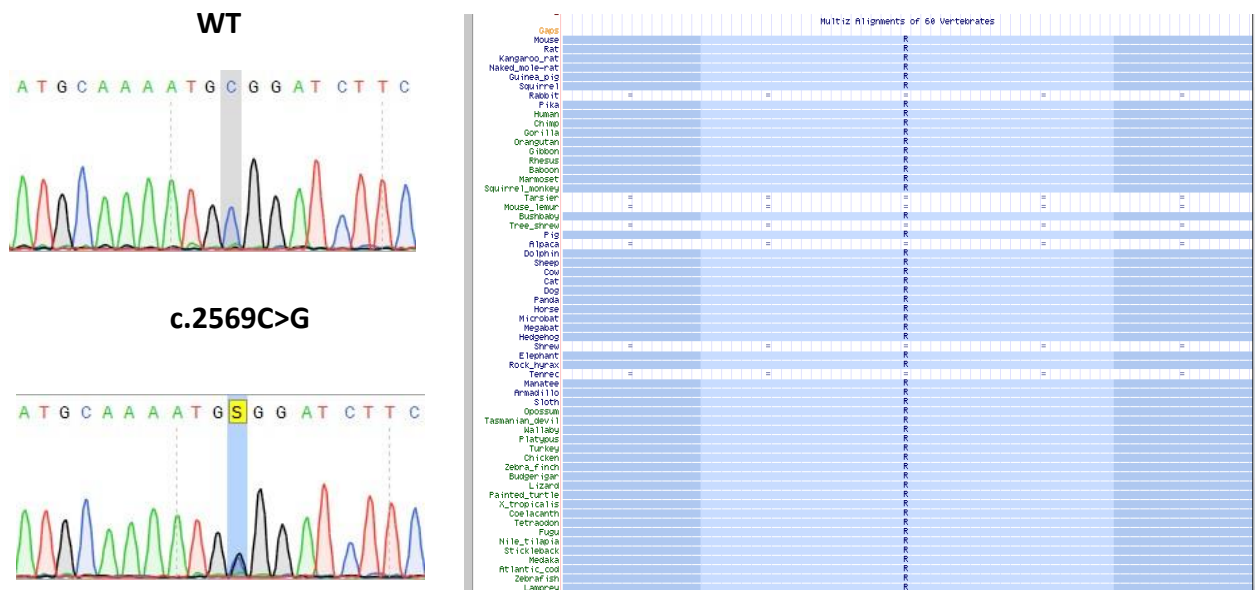


Figure 2. A. Venn diagram representing the overlap between variants from the DNA of three mice (animals #890 and #837 suffering from epileptic attacks and animal #887 used as a healthy control) subjected to analysis by Whole Exome Sequencing; B. Summary of Variant Effect Predictor (VEP) analysis performed on the overlapping variants between two epileptic mice; C. Predicted consequences of 2443 variants subjected to VEP analysis; D. Predicted coding consequences of 2443 variants subjected to VEP analysis.



de novo p.Arg857Gly in SLC12A5 (KCC2)



Figure 3. A. DNA chromatogram illustrating the detection of c.2569C>G (R857G) by Sanger sequencing. B. Conservation of a reference amino acid (R) among 60 vertebrates evaluated by USCS Genome Browser. C. Position of the mutation in the DNA and amino acid sequence.

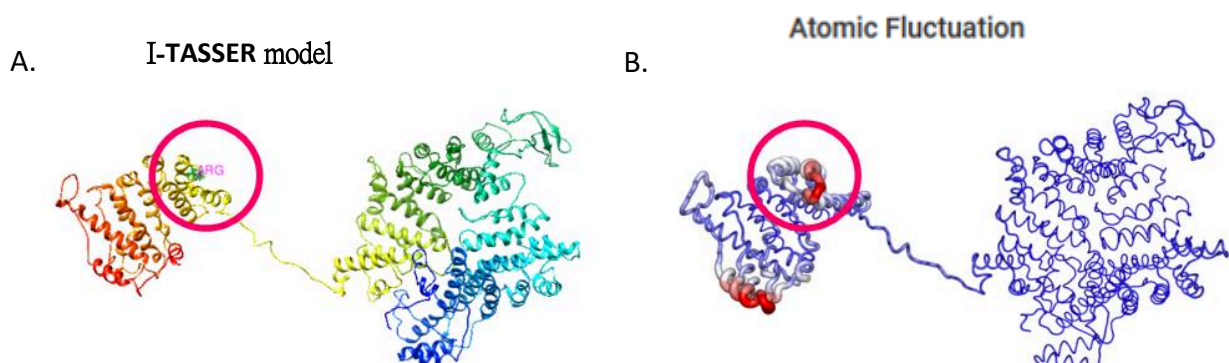
Due to the absence of a KCC2 structure in the Protein Data Bank (PDB) archive, I decided to create a model of the KCC2 protein using the I-TASSER tool, which designs protein

pdb structures based on their amino acid sequence. It enabled me to make predictions based on KCC2 structure. I further used the MUpro software that enables prediction of protein stability changes for single site mutations from sequences and pdb structures. By submitting the information about the KCC2 sequence, the mutation that I identified and the model obtained with I-TASSER, I obtained following predictions:

1. Predicted both value and sign of energy change using SVM and sequence information only:	
Method 1	delta delta G = -1.5856284 (DECREASE stability)
2. Prediction of the sign (direction) of energy change using SVM and neural network with a smaller sequence window:	
Method 1	Support Vector Machine, use sequence information only. Effect: DECREASE the stability of protein structure. Confidence Score: -0.82874206
Method 2	Neural Network, use sequence information only. Effect: DECREASE the stability of protein structure. Confidence Score: -0.957728548882489 Structure predictions based on model (Kcc2 model from I-TASSER).
Method 3	Support Vector Machine, use structure information. Effect: DECREASE the stability of protein structure. Confidence Score: -0.5791299
Method 4	Neural Network, use structure information (Kcc2 model from I-TASSER). Effect: DECREASE the stability of protein structure. Confidence Score: -0.840923018966198

Table 1. Predictions for Kcc2 mutation with MUpro software.

All different predictions performed with MUpro indicated that the identified mutation decreases the stability of protein structure. In addition to MUpro, I also used the Dyna-mut tool that utilizes structure-based approaches to assess the impact of mutations on protein structure. By using this tool, I was able to predict that the identified mutation can affect flexibility and fluctuations of KCC2 structure (Fig. 4A-D).



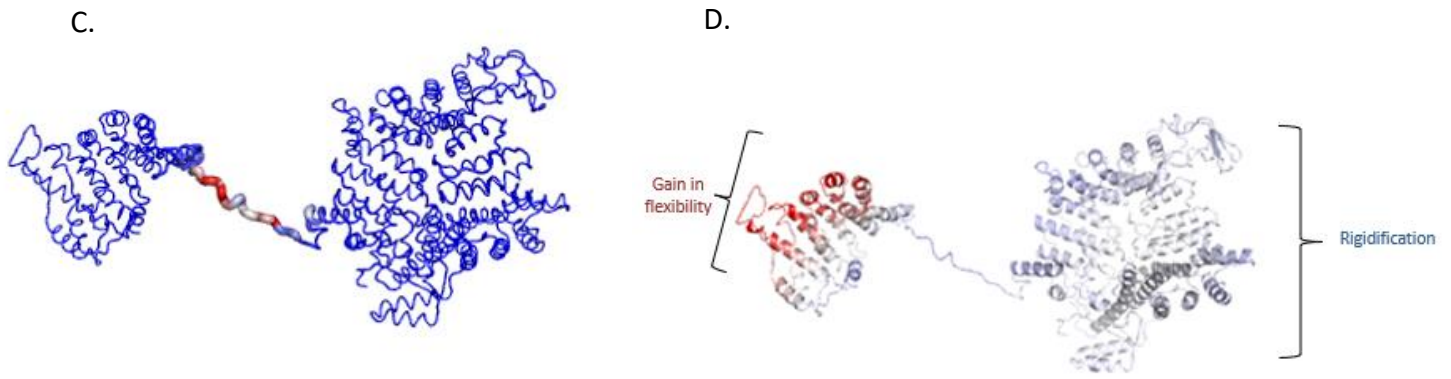


Figure 4. A. KCC2 I-TASSER model with indicated mutation in circle. B and C. The magnitude of the fluctuations (B) and deformations (C) represented by thin to thick tube colored blue (low), white (moderate) and red (high). Atomic fluctuations provide the amplitude of the absolute atomic motions, whereas deformation energy provides a measure for the amount of local flexibility in the protein. D. Amino acids colored according to the vibration entropy change upon mutation. Blue represents a rigidification of the structure and red a gain in flexibility.

N-glycosylation defects have been associated with many multisystem diseases, predominantly connected with the nervous system. Interestingly, common features characterize these disorders involved ataxia and seizures (Patterson, 2005). Due to the lack of information about N-glycosylation sites in the mouse KCC2 sequence, I decided to perform bioinformatic analysis in order to find out potential N-glycosylation sites and to verify if the identified mutation can have an impact on the glycosylation process. This analysis revealed eight potential N-glycosylation sites (Fig. 5). None of them lies in the proximity of identified mutation.

Name: Sequence	Length: 1133	
MSRRFTVTSLPPAASAASADPESSRRHSVADPRRLPREDVKGDNPKESSPFINSTDETEKGREYDGRNMALFEEEMDTSPM		80
VSSLLSGLAN Y TNLPQGSREHEEAENNEGGKKKPVQAPRMGTFMGVYLPCLNIFGVILFLRLTWVVGIAGIMESFCMVF		160
ICCSCTMLTAISMSAIATNGVVPAGGSYYMISRSLGPEFGGAVGLCFYLGTTFAGAMYILGTIEILLAYLFPAMAIKAE		240
DASGEAAAMLNNMRVYGTCVLTCMATVVFVGVKYNKFAVFLGCVILSILAIYAGVIKSAFDPPNFPICLLGNRTLSRH		320
GFDVCAKLAWE NET VTTTRLWGLFCSSRLLNATCDEYFTRNNVTEIQGIPGAASGLIKENLWSSYLTKGVIVERRGMP		400
GLADGTPVDMDDHPYVFSDMTSYFTLLVGIYFSPVGTGIMAGSNRSGDLRDAQKSIPTGTILAIATTSAVYISSVVLFGACI		480
EGVVLDRDFGEAVNGNLVVGTLAWPSPWVIVIGSFFSTCGAGLQSLTGAPRLLQAI SRDGIVPFLQVFGHGKANGAPTWA		560
LLLTACICEIGILIASLDEVAPILSMFFLMCYMFVNLACAVQTLRLTPNWRPRFRYYHWTLSFLGMSLCLALMFICSWYY		640
ALVAMLIAGLIYKYIEYRGAEKWGDGIRGLSLSAARYALLRLEEGPPHTKNWRPQLLVLRVDQDQNVVHPQLLSLTSQ		720
LKAGKGLTIVGSVLEGTFLDNHPQAQRAEESIRRLMEAEKVGFCQVVISSNLRDGVSHLIQSGGLGGLQHNTVLVGWPR		800
NWRQKEDHQ T WRNFIELVRETTAGHLALLVTKN VS MFPGNPERFSEGSIDVWVIVHDGGMLMLLPFLLRHHK V WRCKMR		880
IPTVAQMDDNSIQMKDLTTFLYHLRITAEVEVEMHESDISAYTYE K TLVMEQRSQILKQ M HLTKNEREREIQSITDES		960
RGSIRRNK N PANRRLRLNVPEETACDNEEKPEEEVQLIHDQSAPSCSPSSPSPGEEPEGERETDPEVHLTWTKDKSVAEKN		1040
KGSPVSVSEGIK DF SMKPNQSNVRRMHTAVRLNEVIVNKS R DAKLVLLNMPGPPRRNRNGDENYMEFLEVLTEQLDRVML		1120
VRGGGREVITIIYS		

Figure 5. Amino acid sequence of the mouse *KCC2* protein with marked N-glycosylation sites. Asn-Xaa-Ser/Thr sequence (including Asn-Pro-Ser/Thr) is shown in blue. Asparagines predicted to be N-glycosylated are shown in red.

To make sure that the *Kcc2* R857G variant is responsible for the mouse phenotype, I analyzed the pedigree of a large cohort of mice bearing the mutation or not (Fig. 1). I performed Sanger sequencing of DNA from 188 mice to evaluate the presence of the c.2569C>G (R857G) mutation. This analysis enabled me to follow the inheritance of the mutation. I performed multiple crosses of mice to confirm that the identified variant occurs only within the descendants of the mice that presented the epileptic phenotype. In all analyzed mice, the observed epileptic phenotype was always accompanied by the presence of the c.2569C>G variant, while it was absent in mice that did not display the phenotype. This represents strong evidence in favor of a causal role of the *Kcc2* R857G variant in determining the epileptic phenotype in mice. The pattern of inheritance indicates the dominant character of this variant. This mutation is located in the C-terminal region of the protein where variants have been associated with neurodevelopmental phenotypes in patients.

I decided to continue the characterization of the *Kcc2*-mut mouse in a wild-type background (not carrying the mutation in the *Fmr1* gene) by various analyses that are described further on.

2. Electrophysiological analysis

In collaboration with the team of Dr. Mantegazza at the IPMC, a preliminary electrophysiological analysis through intracranial electroencephalography (iEEG) was performed. iEEG enables to record electrical activity from the cerebral cortex. The presence of interictal spikes (burst of synchronized neuronal activity) was detected at the age of two months using a custom written script on MATLAB. Analysis of the two months old *Kcc2*-mut mice showed the shape of the typical spike for epileptic mice (Fig. 6).

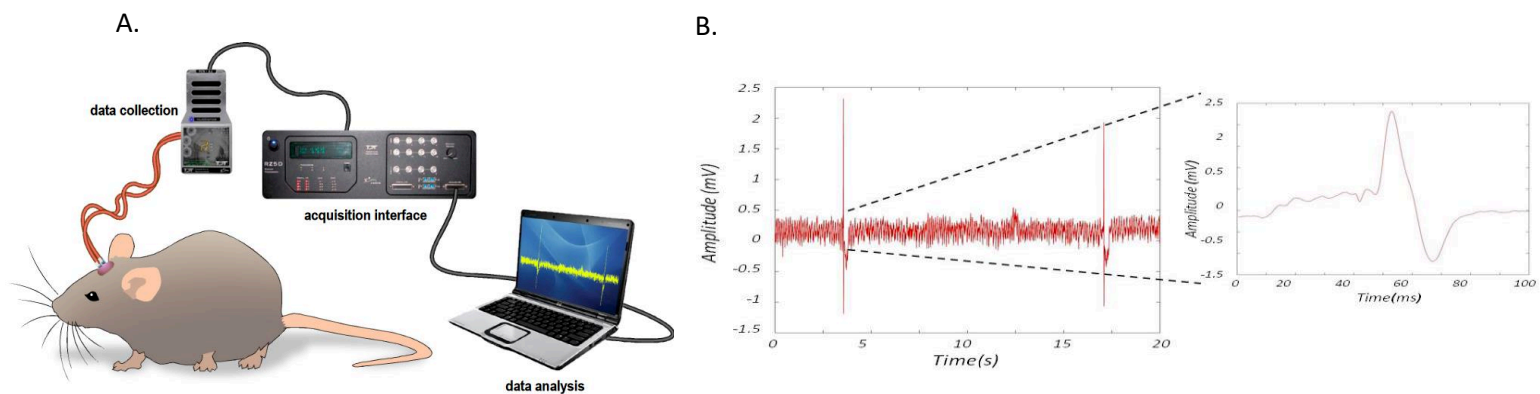
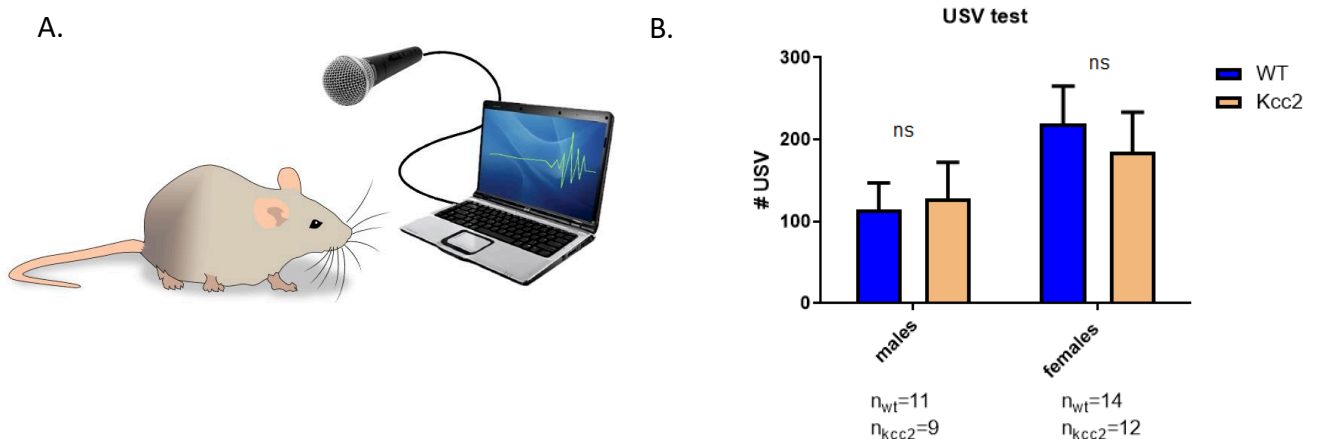


Figure 6. ***Kcc2* mice display an EEG pattern typical for epileptic mouse.** A. Scheme representing iEEG recordings. B. iEEG of *Kcc2* mutant mice revealing the presence of interictal spikes. On the left, two well characterized spikes showed on 20 seconds of iEEG recording of a *Kcc2*-mut mouse. On the right side, a graph zoom is shown encompassing 100 ms displaying the spike shape typical of epileptic mouse.

3. Behavioral studies

The patient bearing the *KCC2* mutation exhibits deficits in intellectual development (Saito *et al.*, 2017). Moreover, *KCC2* is involved in the maintenance of GABAergic signaling (Saito *et al.*, 2016), which is connected to inhibition-excitation balance that is thought to underlie a range of neurological disorders including ID, ASD and epilepsy. Due to these reasons, I decided to evaluate the potential impact of this mutation on the behavioral phenotype of the *Kcc2-mut* mouse. In order to investigate the plausible aberrations in social communication and discrimination I performed **ultrasonic vocalisation and homing tests** (Fig. 7 A and C, respectively). The tests were performed at PND12 and PND13, respectively. Importantly, *Kcc2* reaches its high expression in the brain by the end of PND14. Testing USVs allowed estimating social communication in pups by recording and measuring the number, frequency, amplitude and duration of calls. I analyzed the number of ultrasonic vocalization in WT and *Kcc2-mut* mice at PND12 and I did not observe significant difference between the WT and *Kcc2-mut* mice (Fig. 7 B). Concerning social discrimination evaluated by the homing test, after analysis of the latency(s) to reach the home-cage bedding area and total time (s) spent by each pup in the nest bedding area, I did not observe any differences between WT and *Kcc2-mut* mice (Fig. 7 D and E).



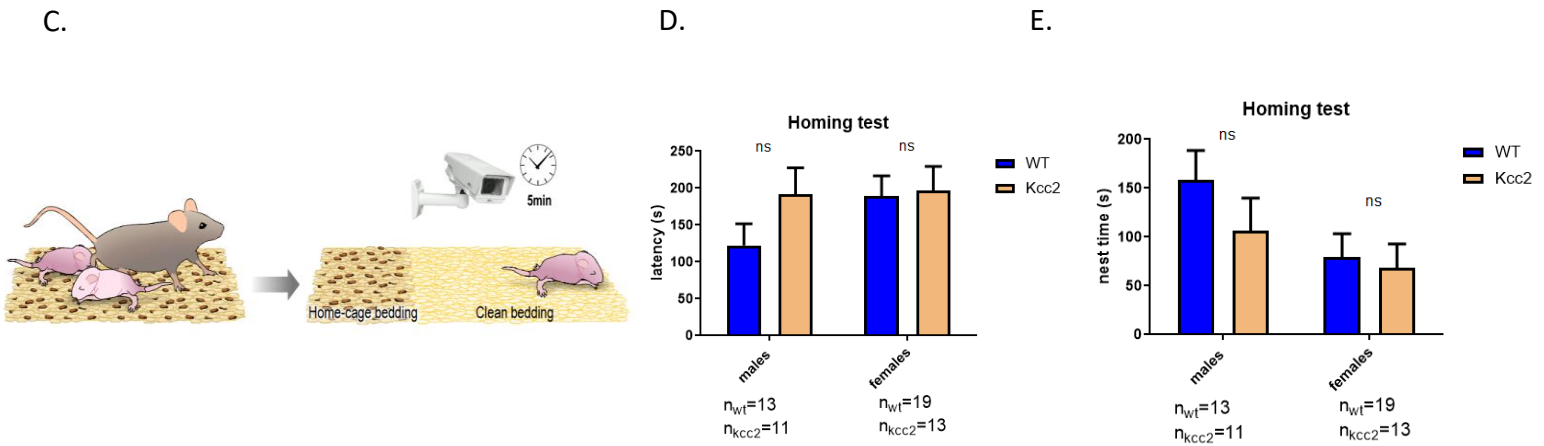


Figure 7. *Kcc2*-mut mice do not exhibit early deficits in social communication and social discrimination A. Schematic representation of ultrasonic vocalisation test. B. USV test performed on WT and *Kcc2* mice at PND12. C. Schematic representation of Homing test. D and E. (latency and nest time, respectively). Homing test performed on WT and *Kcc2*-mut mice at PND13. Data represent mean \pm SEM. *t*-test analysis for USV and homing tests did not reveal any statistical difference between WT and *Kcc2*-mut mice.

To further evaluate potential autism-related behavioral deficits, a social interaction test was performed at different mouse ages: one month (females and males), four months (males) and six months (males). Tests were performed at four and six months in order to evaluate if epileptic crises that usually appear at the age of four months can affect social behavior. During the test, I analyzed the following behavioral patterns: social sniffing, crawling, mutual circle, following, and social grooming. No differences in social interaction were observed between WT and *Kcc2*-mut mice at any investigated age (Fig. 8 A, B and C). Social interactions decreased at the age of four and six months, both for WT and *Kcc2*-mut mice (Fig. 8 D). This observation can be associated with the process of aging.

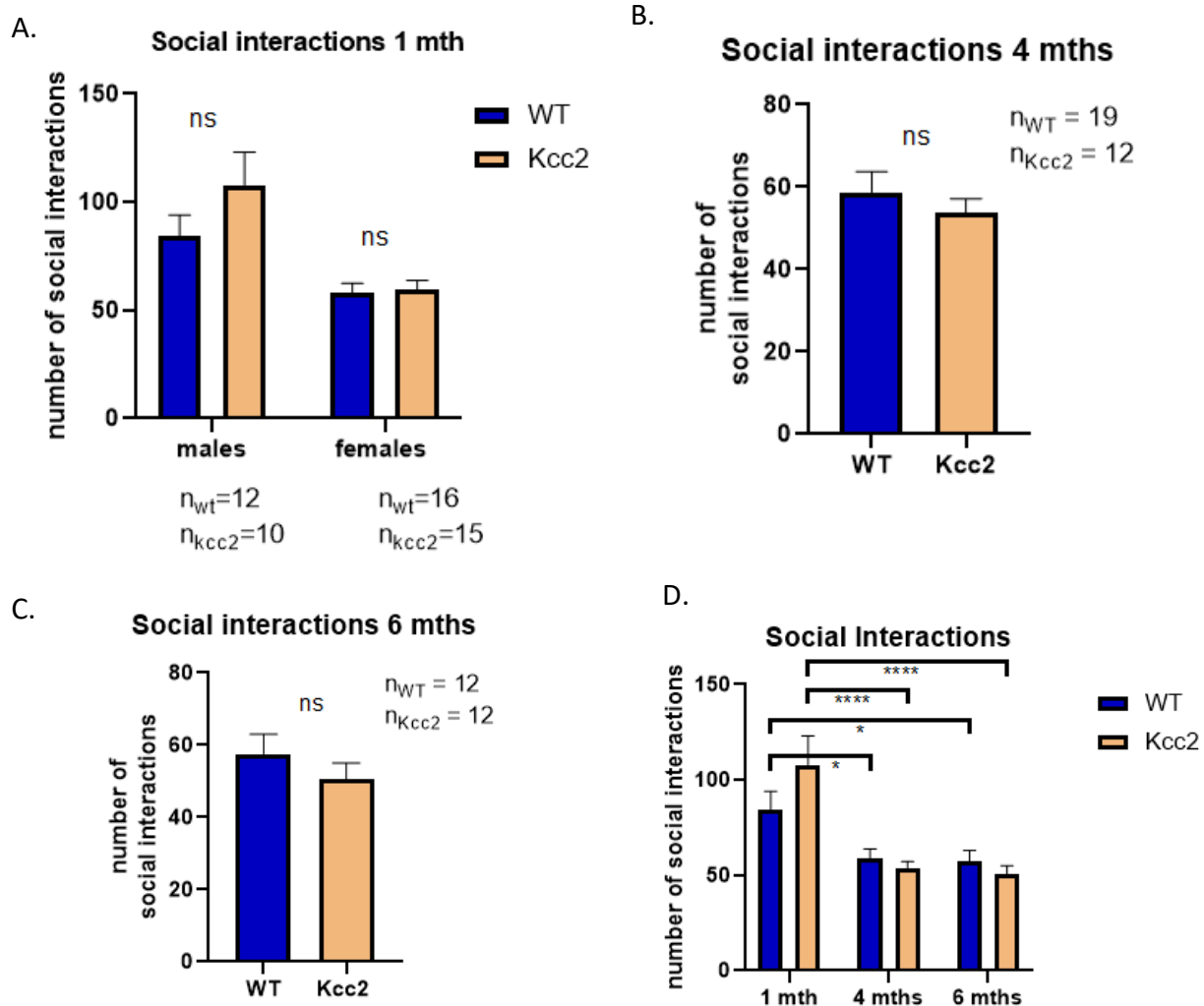


Figure 8. *Kcc2*-mut mice do not exhibit deficits in social interactions. A. Social Interaction test performed at the age of one month for males and females. B. Social Interaction test performed at the age of four months for male mice. C. Social Interaction test performed at the age of six months for male mice. D. Comparison of social Interaction tests performed at the age of one month, four months and six months, respectively. Data represent mean \pm SEM. t-test analysis of social Interaction did not reveal any statistically significant difference between WT and *Kcc2*-mut mice. Tukey's multiple comparisons test revealed differences between social interactions at one month compared to four months or six months. *comparison 1 month vs. 4 months $P = 0.0293$, * comparison 1 month vs. 6 months $P = 0.0422$, **** $P < 0.0001$.

In order to evaluate learning and memory capabilities of *Kcc2*-mut mice, I performed the novel object recognition test at the age of two months (Fig. 9A). This

test revealed changes in cognition between WT and *Kcc2-mut* male mice (Fig. 9B). Here I would like to mention that no difference was present in females (data not shown).

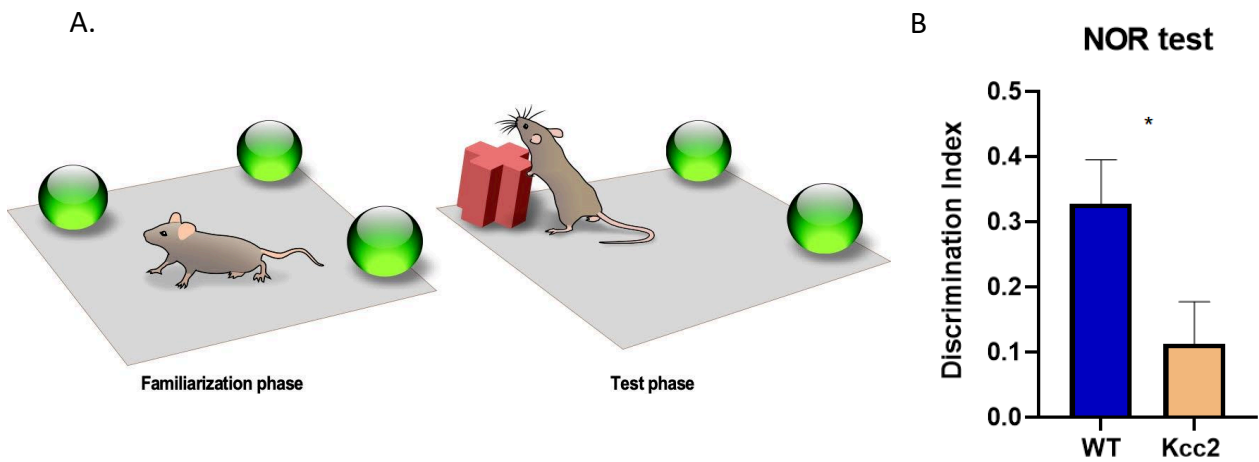
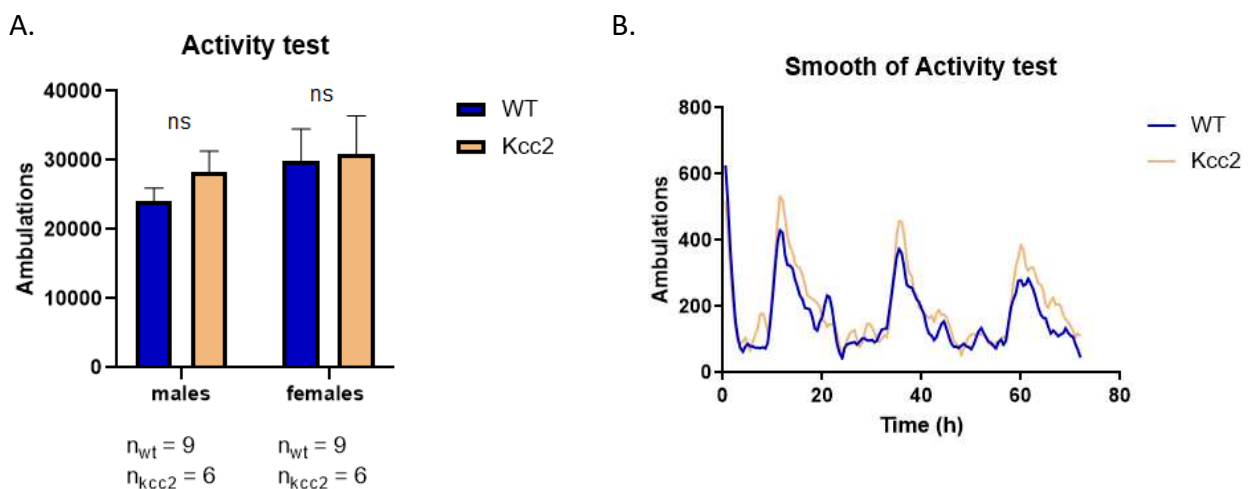


Figure 9. *Kcc2-mut* male mice exhibit learning and memory deficits (adult mice). A. Scheme representing the NOR test. B. Novel Object Recognition test performed at the age of two months. Data represent mean \pm SEM. t-test analysis of NOR test results showed a statistically significant difference between WT and *Kcc2-mut* male mice. * P value = 0.0401.

Subsequently, at the age of two and four months, *Kcc2-mut* and WT mice were subjected to locomotor activity test. I analyzed ambulations recorded during 72 h that encompass locomotor activity, activity in the front, activity in the back and rearings. The results did not reveal any difference between *Kcc2-mut* and WT mice (Fig. 10 A and B).



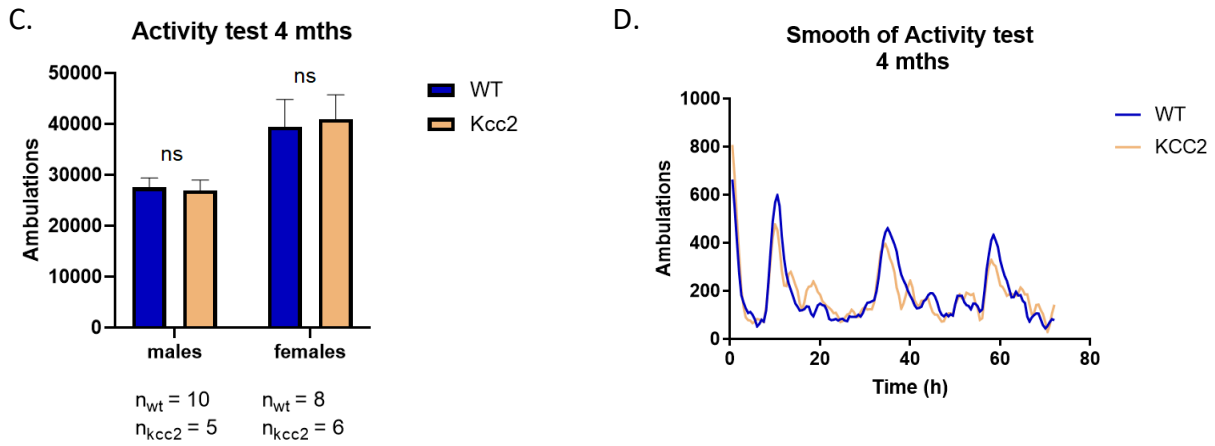
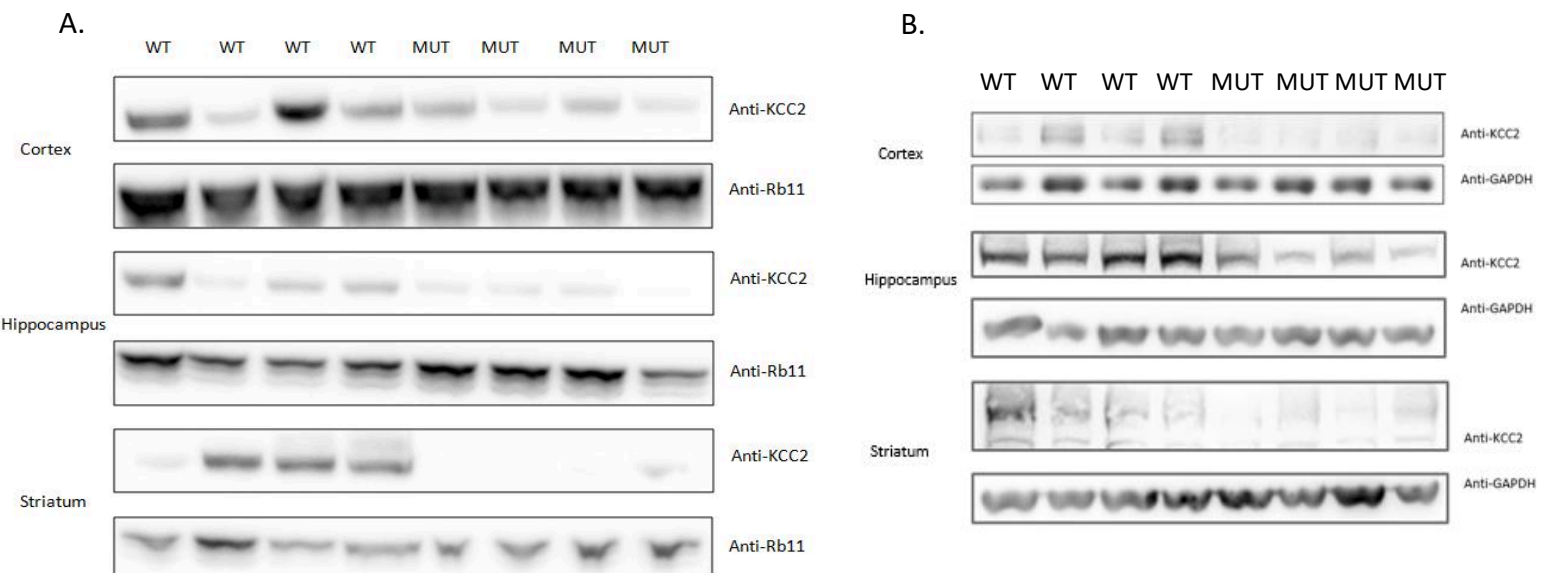


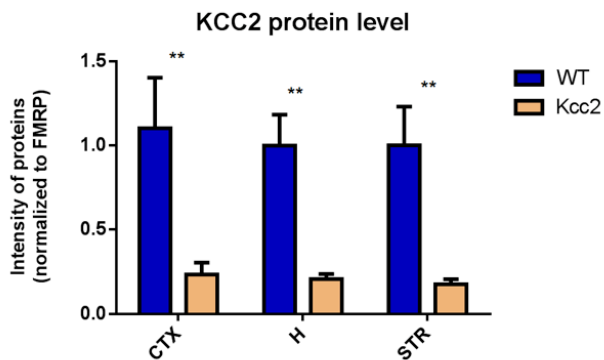
Figure 10. *Kcc2*-mut mice do not exhibit deficits in locomotor activity. A. Locomotor activity test performed at the age of two months. B. Smooth representation of locomotor activity test results representing WT and *Kcc2*-mut male mice over 72 h. Data represent mean \pm SEM. Sidak's multiple comparisons test did not reveal any statistically significant differences between *Kcc2*-mut and WT mice. *ns* males adjusted $P = 0.6991$, *ns* females adjusted $P = 0.9813$. C. Locomotor activity test performed at the age of four months. D. Smooth representation of Locomotor Activity test results representing WT and *Kcc2*-mut male mice over 72 h. Data represent mean \pm SEM. Sidak's multiple comparisons test did not reveal any statistically significant differences between *Kcc2*-mut and WT mice. *ns* males adjusted $P = 0.9936$, *ns* females adjusted $P = 0.9562$.

4. Biochemical studies

In order to investigate KCC2 protein level alterations in *Kcc2*-mut mice, I performed western blot analysis that demonstrated that protein production is strongly decreased in two months old male *Kcc2*-mut mice (Fig. 11A and C). At the age of four months, western blot analysis of proteins also indicated the decrease of KCC2 (Fig. 11B and D).



C.



D.

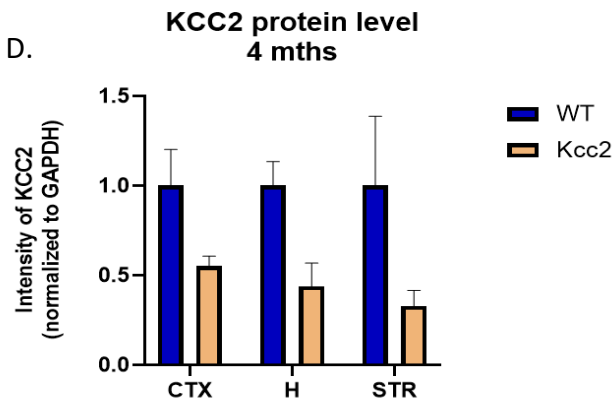
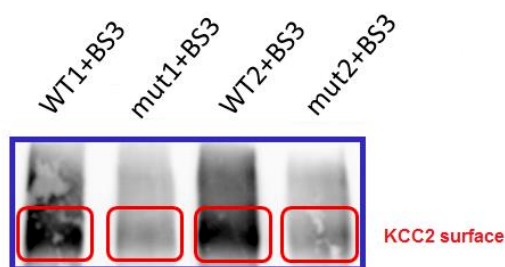


Figure 11. Significant changes in the protein levels of KCC2 between WT and Kcc2-mut mice in cortex, striatum and hippocampus. A. Western blot analysis of KCC2 protein at the age of 2 months. B. Western blot analysis of KCC2 protein at the age of four months C. Quantification of Western blot analysis of KCC2 protein at the age of 2 months. D. Quantification of Western blot analysis of KCC2 protein at the age of 4 months.

Due to the fact that some previously described mutations in *Kcc2* led to changes in the localization at the membrane level, I decided to study the localization of KCC2 with cross-linking assay and immunohistochemical analysis. Cross-linking assay performed in cooperation with Dr. Alessandra Folci and immunohistochemical analysis revealed the decrease of KCC2 at the membrane level at two and twelve months of age (Fig. 12A and B, respectively).

A.



B.

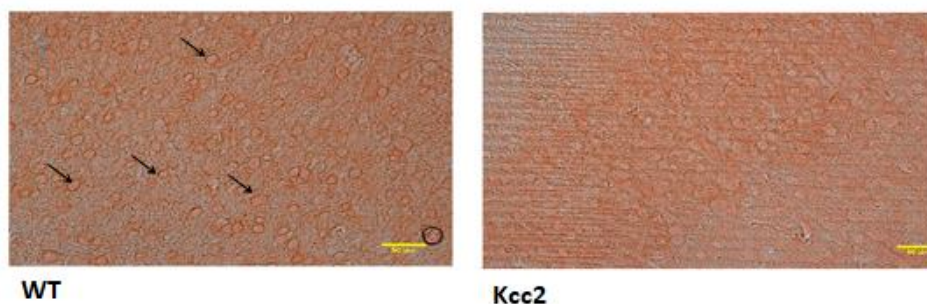


Figure 12. Decrease of KCC2 at the membrane level. A. Cross-linking assay of KCC2 WT and its mutated form. B. Immunohistochemistry analysis of WT and Kcc2-mut mice cortex slices. The scale bar of each panel is 50 μm .

To get further insight on what is happening at the transcriptomic level, I performed RT-qPCR. In 2-months *Kcc2-mut* mice RT-qPCR experiments showed that the level of *Kcc2* mRNA is not changed neither in cortex nor in the striatum (Fig. 13 A and B). I did not observed differences in the levels of two isoforms of *Kcc2* (a and b) between WT and *Kcc2-mut* mice in the cortex. I also analyzed *Nkcc1* mRNA whose protein product participates in the maintenance of the excitation/inhibition balance together with KCC2. The level of *Nkcc1* mRNA was unchanged (Fig. 13 A and B).

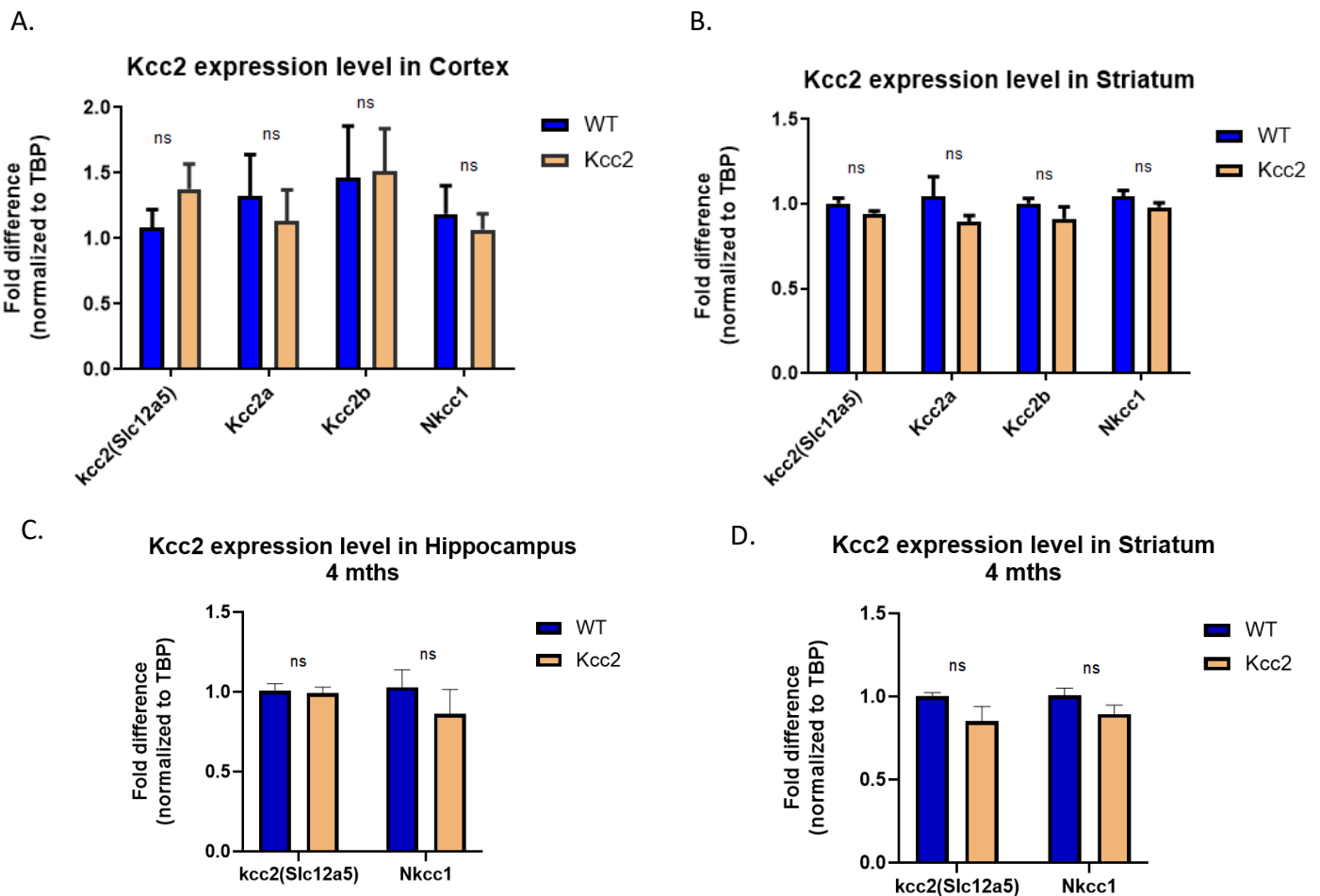


Figure 13. *Kcc2* mRNA level remains unchanged in cortex, striatum and hippocampus. A and B. RT-qPCR analysis of *Kcc2* isoforms and *Nkcc1* level in striatum and cortex at the age of two months, respectively. C and D. RT-qPCR analysis of *Kcc2* isoforms and *Nkcc1* level in hippocampus and striatum

at the age of 4 months, respectively. Data represent mean \pm SEM. Two-way ANOVA analysis did not reveal any statistical difference between WT and *Kcc2-mut* mice.

5. Proteomic analysis

To get deeper insight into the molecular background of *Kcc2-mut* mice, I performed a proteomic analysis of cortex and hippocampal proteins that were co-immunoprecipitated with the anti-KCC2 antibody (Fig. 14A). In order to obtain a general overview at the hippocampal and cortex levels together, my first approach was to combine WT samples from hippocampus and cortex (two WT replicates from cortex plus two WT replicates from hippocampus). I performed the same procedure for *Kcc2-mut* samples. Afterwards, I removed proteins detected in negative samples co-IPd with IgG to get rid of non-specific proteins from WT and *Kcc2-mut* samples. A Venn diagram analysis revealed that only three proteins are detectable in all WT sample replicates and are not present in all mutated samples replicates (Fig. 14B). Among them was KCC2 (SLC12A5). This result confirms that KCC2 is decreased in *Kcc2-mut* mice because it was not detected in all *Kcc2-mut* sample replicates, although, it was present on western blot. Two other proteins – RAB3A and TUBA1B – turned out to belong to Synaptic vesicle trafficking and Gonadotropin-releasing hormone receptor pathways, respectively (Fig. 14 C). TUBA1B is known to participate in the cytoskeleton-dependent intracellular transport and translocation of SLC2A4 (GLUT4) to the plasma membrane. RAB3A is involved in the intracellular protein transport, synaptic vesicle transport, GABA synthesis, release, reuptake and degradation, serotonin neurotransmitter release cycle, and protein localization to plasma membrane (neXtProt; Zahn-Zabal *et al.*, 2020). Its role can suggest potential implications in KCC2 localisation to the plasma membrane.

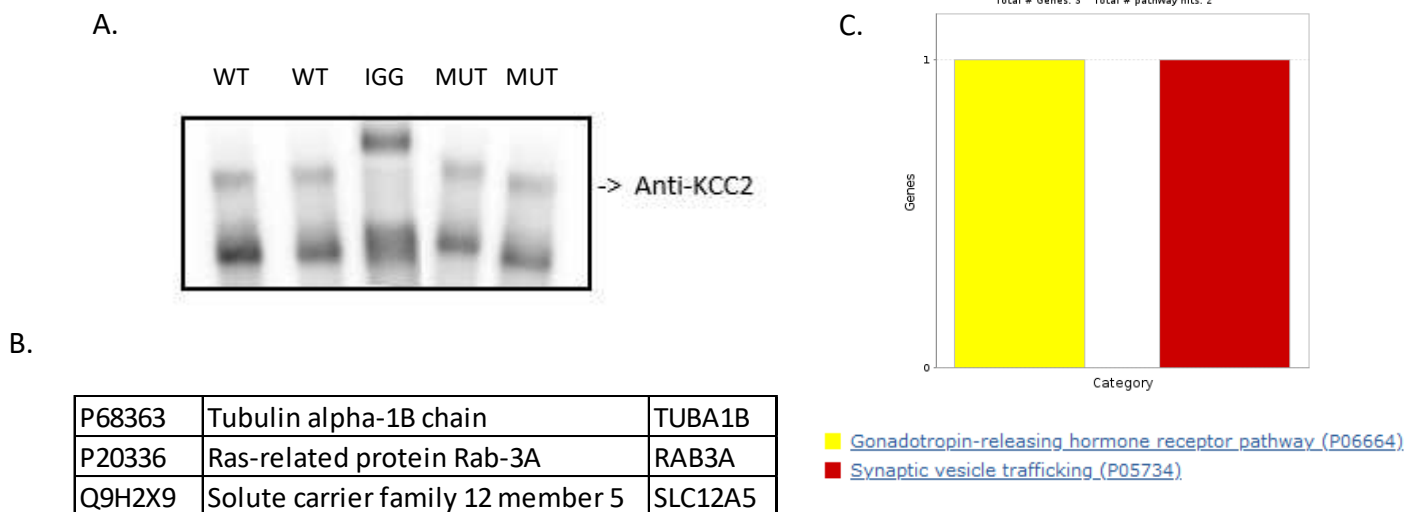


Figure 14. A. Western blot of co-immunoprecipitated proteins with anti-KCC2 antibody from cortex. B. ID's of three unique proteins from WT samples. C. Pathway analysis with PANTHER tool based on three unique proteins from WT samples.

In addition, I studied hippocampus and cortex unique proteins (specific for each fraction) separately two WT and two mutated sample replicates from cortex and two WT and two mutated samples replicates from hippocampus. I performed enrichment analysis of KEGG and Reactome pathways with the STRING database for 4 subsets of proteins: *Kcc2-mut* proteins from cortex, WT proteins from cortex, *Kcc2-mut* proteins from hippocampus, and WT proteins from hippocampus (Fig. 15 A-D; Supplementary Table 1). Results show that protein samples from *Kcc2-mut* and WT are differently enriched. In the *Kcc2-mut* sample from cortex and hippocampus I detected pathways such as axon guidance, dopaminergic synapse and long-term depression that are known to be dysregulated in many neurodevelopmental disorders (Kullmann *et al.*, 2000; Starr, 1996; Van Battum *et al.*, 2015). Axon guidance and dopaminergic synapse pathways were common for hippocampus and cortex proteins, which indicates that those subsets (cortex and hippocampus from mutated samples) are more similar in

comparison to WT samples. In WT samples from cortex and hippocampus I detected pathways that have wide occurrence and belong to basic metabolic pathways such as glycolysis or gluconeogenesis. This suggests that those subsets are more similar than *Kcc2-mut* samples.

A. *Kcc2* mice cortex:

KEGG Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
hsa04730	Long-term depression	11 of 60	2.79e-08
hsa04728	Dopaminergic synapse	14 of 128	2.79e-08
hsa04071	Sphingolipid signaling pathway	13 of 116	4.74e-08
hsa04144	Endocytosis	17 of 242	6.77e-08
hsa01230	Biosynthesis of amino acids	10 of 72	4.37e-07

Reactome Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
HSA-422475	Axon guidance	37 of 541	1.45e-16
HSA-1430728	Metabolism	65 of 2032	1.32e-14
HSA-376176	Signaling by ROBO receptors	23 of 210	5.18e-14
HSA-195253	Degradation of beta-catenin by the destruction complex	16 of 79	3.42e-13
HSA-5683057	MAPK family signaling cascades	23 of 273	5.64e-12

B. WT mice cortex:

KEGG Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
hsa05034	Alcoholism	10 of 142	2.05e-07
hsa05322	Systemic lupus erythematosus	8 of 94	1.25e-06
hsa04217	Necroptosis	8 of 155	3.16e-05
hsa00010	Glycolysis / Gluconeogenesis	6 of 68	3.45e-05
hsa05230	Central carbon metabolism in cancer	5 of 65	0.00044

Reactome Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
HSA-3214858	RMTs methylate histone arginines	7 of 49	2.13e-06
HSA-2029481	FCGR activation	5 of 11	2.13e-06
HSA-1266738	Developmental Biology	20 of 1023	2.93e-06
HSA-912446	Meiotic recombination	6 of 55	3.03e-05
HSA-210990	PECAM1 interactions	4 of 11	3.96e-05

C. *Kcc2* mice hippocampus:

KEGG Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
hsa03010	Ribosome	16 of 130	7.23e-11
hsa04728	Dopaminergic synapse	14 of 128	4.80e-09
hsa04360	Axon guidance	11 of 173	6.88e-05
hsa04921	Oxytocin signaling pathway	10 of 149	0.00010
hsa04713	Circadian entrainment	8 of 93	0.00016

Reactome Pathways			
<i>pathway</i>	<i>description</i>	<i>count in gene set</i>	<i>false discovery rate</i>
HSA-156842	Eukaryotic Translation Elongation	18 of 90	3.15e-15
HSA-156902	Peptide chain elongation	17 of 86	1.62e-14
HSA-975957	Nonsense Mediated Decay (NMD) enhanced by the Exon J...	18 of 112	2.93e-14
HSA-975956	Nonsense Mediated Decay (NMD) independent of the Exon ...	17 of 92	2.93e-14
HSA-927802	Nonsense-Mediated Decay (NMD)	18 of 112	2.93e-14

D. WT mice hippocampus:

KEGG Pathways			
pathway	description	count in gene set	false discovery rate
hsa01200	Carbon metabolism	11 of 116	9.88e-12
hsa01230	Biosynthesis of amino acids	6 of 72	7.11e-06
hsa00220	Arginine biosynthesis	4 of 20	3.19e-05
hsa04964	Proximal tubule bicarbonate reclamation	4 of 23	3.93e-05
hsa00471	D-Glutamine and D-glutamate metabolism	3 of 5	3.93e-05

Reactome Pathways			
pathway	description	count in gene set	false discovery rate
HSA-373760	L1CAM interactions	7 of 116	1.85e-05
HSA-1430728	Metabolism	21 of 2032	1.85e-05
HSA-70614	Amino acid synthesis and interconversion (transamination)	4 of 31	0.00031
HSA-70326	Glucose metabolism	5 of 85	0.00031
HSA-70263	Gluconeogenesis	4 of 33	0.00031

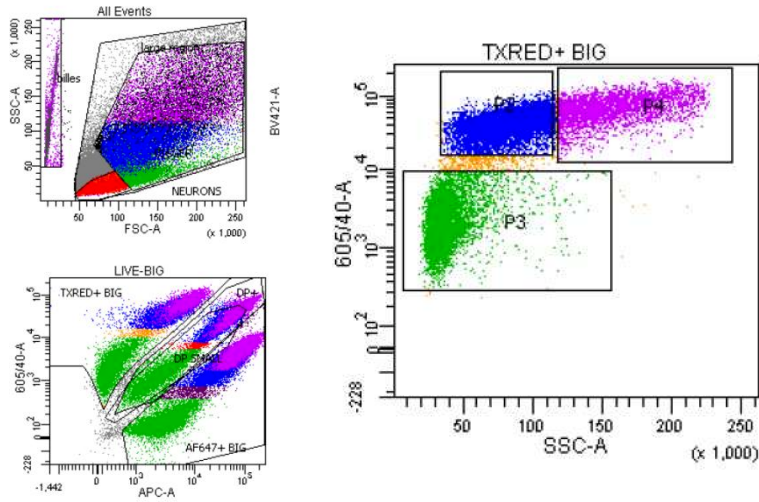
Figure 15. A. Enrichment analysis among proteins detected in *Kcc2-mut* samples from cortex. B. Enrichment analysis among proteins detected in WT samples from cortex. C. Enrichment analysis among proteins detected in *Kcc2-mut* samples from hippocampus. D. Enrichment analysis among proteins detected in WT samples from hippocampus. STRING database.

6. Studying the GABAergic switch via the aiFACS technique

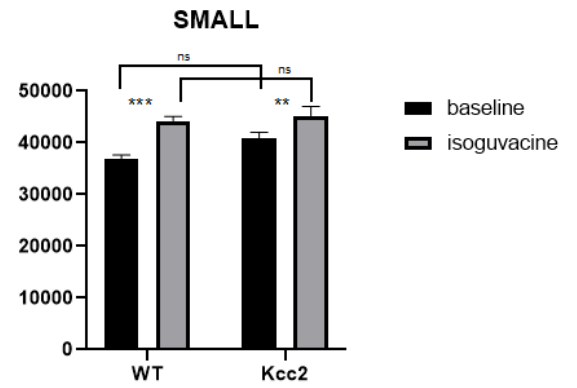
In order to get further insight on the inhibitory-excitatory balance, I decided to use a novel approach that was developed recently in our laboratory: agonist-induced Functional Analysis and Cell Sorting (aiFACS). This technique enables simultaneous stimulation, recording and sorting of cells upon a specific pharmacological stimulation (Castagnola *et al.*, 2020). In this part of the work, I tried to evaluate if it is possible to use this technique to estimate the response of neurons, derived from WT and *Kcc2-mut* mice upon stimulation with a GABA agonist. I was aiming to assess whether this tool allows me to detect changes in the GABA switch that are frequently present upon dysregulation of KCC2 levels.

For the time being, I was able to perform preliminary trials with neurons collected at PND17. For aiFACS results, I used samples from males and females. I used neuronal populations derived from WT and *Kcc2-mut* mice and labelled them with the Fluo-4 fluorescent calcium indicator. Subsequently, they were injected in the FACS supplying the cell flow with a PBS neutral calcium recording solution (baseline condition). After three minutes of recording, cells were stimulated with the GABA agonist isoguvacine. It enabled me to observe an increase in Fluo-4 fluorescence. The cells that displayed increased Fluo-4 fluorescence in the presence of isoguvacine were sorted. Moreover, I selected BIG and SMALL cells according to their size/structure. Due to their heterogeneity, BIG cells were divided into P2, P3 and P4 sub-populations (Fig. 16A). The results showed that it is possible to detect a response of the cells to GABA agonist both in SMALL and BIG cells (Fig. 16B and D, respectively). I was able to see that BIG cells sub-populations respond differently. Sub-populations P2 and P4 did not show any significant response to the GABA agonist. Also, the baseline fluorescence level was different between sub-populations (Fig. 16F, G and H). When I analyzed the difference between isoguvacine and baseline I observed smaller response to isoguvacine in SMALL and BIG *Kcc2-mut* cells (Fig. 16C and E, respectively). RT-qPCR analysis showed that SMALL WT cells are enriched in *Neurod1* that is a marker for excitatory neurons, whereas the population of SMALL *Kcc2-mut* cells is enriched in inhibitory interneurons (Fig. 16I and J, respectively).

A.

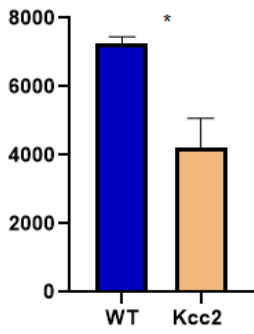


B.



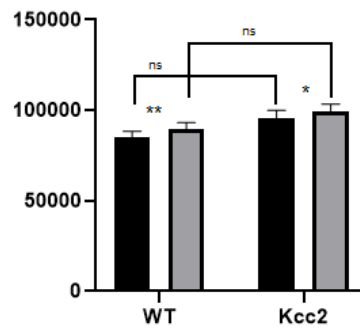
C.

SMALL isoguvacine-baseline



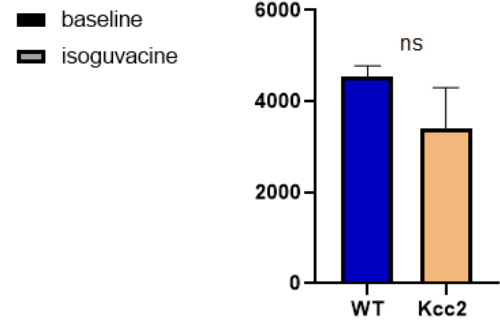
D.

BIG



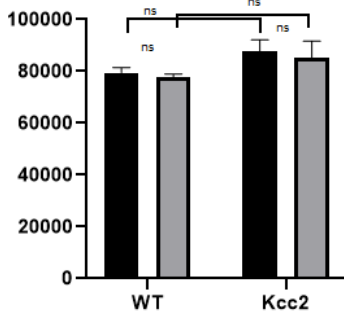
E.

BIG isoguvacine-baseline



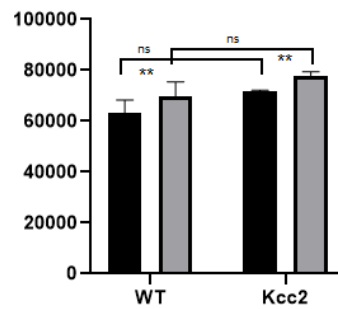
F.

BIG P2



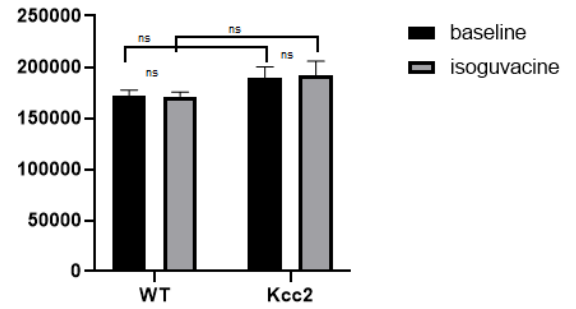
G.

BIG P3



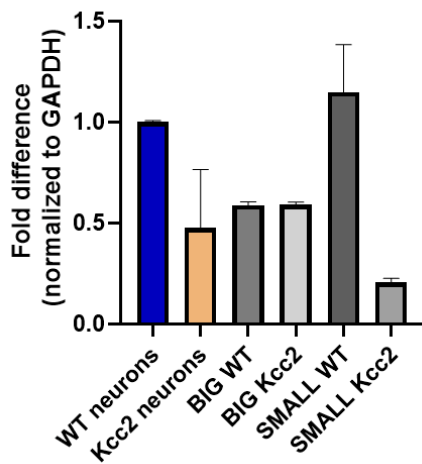
H.

BIG P4



I.

Neurod-1



J.

Meis2

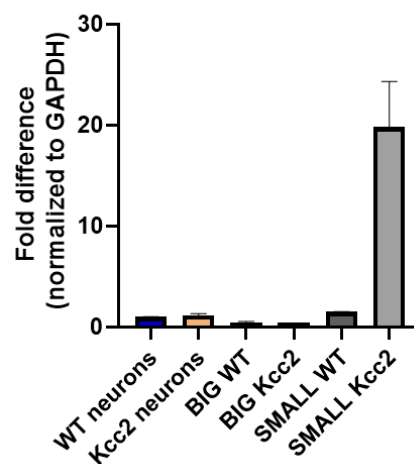


Figure 16. A. FACS gating strategy. BIG and SMALL cells were separated according to their size/structure. Due to their heterogeneity BIG cells were divided into P2, P3 and P4 sub-populations. B. Fluorescence level in baseline conditions and in response to GABA agonist - isoguvacine. Two-way ANOVA test revealed differences between baseline and GABA agonist response *** adjusted P Value = 0.0006, ** adjusted P value = 0.0045, and did not show any differences between WT and Kcc2-mut phenotype ns (baseline) adjusted P Value = 0.1214, ns (isoguvacine) adjusted P Value = 0.8778. C. Difference between WT and Kcc2-mut presented as a difference between GABA agonist response and baseline. Unpaired t test revealed differences between WT and Kcc2-mut * P = 0.0241. D. Fluorescence level in baseline conditions and in response to GABA agonist. Two-way ANOVA test revealed differences between baseline and GABA agonist response ** adjusted P value = 0.0045, * adjusted P value = 0.0126, ns (baseline) adjusted P value = 0.1546, ns (isoguvacine) adjusted P value = 0.2106. E. Difference between WT and Kcc2-mut presented as a difference between GABA agonist response and baseline. Unpaired t test did not reveal differences between WT and Kcc2-mut ns P value = 0.2922. F. Fluorescence level in baseline conditions and in response to GABA agonist. The two-way ANOVA test did not reveal any differences between baseline and GABA agonist response ns adjusted P value = 0.8667, ns adjusted P value = 0.7484, and between phenotypes ns (baseline) adjusted P value = 0.3472, ns (isoguvacine) adjusted P value = 0.4048. G. Fluorescence level in baseline conditions and in response to GABA agonist. The two-way ANOVA test revealed differences between baseline and GABA agonist response ** adjusted P value = 0,0035, ** adjusted P value = 0,0047, and did not reveal differences between phenotypes ns adjusted P value = 0.2990, ns adjusted P value = 0.3358. H. Fluorescence level in baseline conditions and in response to GABA agonist. Two-way ANOVA test did not reveal any differences between baseline and GABA agonist response ns adjusted P value = 0.8576, ns adjusted P value = 0.3475, and between phenotypes ns (baseline) adjusted P value = 0.3085, ns (isoguvacine) adjusted P value = 0.3940. I. RT-qPCR analysis of neuronal population subjected to aiFACS. The level of mRNA of excitatory neurons marker *Neurod-1* was evaluated. Neurons extracted from Kcc2-mut mice showed 0.5-fold decrease in abundance of this marker. J. RT-qPCR analysis of neuronal population subjected to aiFACS. The level of mRNA of inhibitory interneurons marker *Meis2* was evaluated. SMALL Kcc2-mut population seems to be significantly enriched in inhibitory interneurons.

Project 3. Studying the impact of reduction of *Fmr1* mRNA levels on the morphology and proteomics of cortical neurons derived from a FXTAS mouse model

Fragile X-associated tremor ataxia syndrome (FXTAS) is a rare, progressive neurodegenerative disorder that is mainly characterized by ataxia, cognitive decline and parkinsonism. It is linked to the presence of Fragile X premutation – a repeat of CGG sequence that varies between 55-200 CGG repeats in the 5' UTR of the *FMR1* gene. One of the molecular hallmarks of this disorder involves increased levels of *FMR1* mRNA in FXTAS patients and in the CGG-knockin (KI) mouse model of FXTAS. Interestingly, this elevated level of mRNA is not associated to higher FMRP protein level, which remains normal or slightly reduced. During this study, we were investigating the impact of reduction of *Fmr1* mRNA levels on the morphology and proteomics of cortical neurons derived from a FXTAS mouse model (CGG-KI). By studying the dendritic arborization of wild-type (WT) and CGG-KI cortical neurons, we could observed that KI-CGG neurons display a reduced arborization compared to WT neurons. This pathological phenotype was rescued by transduction with shRNAs that reduced the level of *Fmr1* mRNA. Transduction did not affect the protein level of FMRP. We could also confirm that axon length in CGG-KI mouse is decreased and we were able to show its rescue by transduction with shRNA. Subsequently, we concentrated on dendritic spine abnormalities that have never been studied in cultured cortical neurons at 20 days *in vitro* (DIV). Neurons from CGG-KI mouse were larger, longer and denser than WT controls. This abnormal phenotype was successfully rescued via shRNA transduction. These results suggest that an elevated level of *Fmr1* mRNA can contribute to morphological aberrations in CGG-KI neurons. At the end, we were analyzing the potential dysregulations at the protein level by performing proteomic analysis of neurons

transduced with shRNA and with control shRNA. We were able to identify many up-regulated and down-regulated proteins. Interestingly, some proteins return to normal levels when transduced with shRNA. Gene ontology analysis revealed enrichment in various biochemical pathways, such as RNA and GTP binding proteins. These results suggest that *Fmr1* mRNA metabolism can be regulated by a large number of RNA binding proteins. To conclude, we were able to demonstrate that reduction of *Fmr1* mRNA levels rescues the pathological features in cortical neurons derived from mouse model of FXTAS. Since FXTAS is mainly a neurodegenerative disorder, the identification of neurodevelopmental phenotypes at the cellular and at the molecular levels will open new possibilities to better understand the molecular bases of this disorder and to find treatments for those same phenotypes of patients affecting memory and behaviour during their infancy and youth.

My implication in the study: identification of molecular pathways underpinning some neurodevelopmental hallmarks of cultured cortical neurons obtained from a murine model of FXTAS

For the full paper, refer to the Annexes section – Publication 3 (Drozd *et al.*, 2019).

Discussion

Discussion Project 1. Functional characterization of new genes implicated in Early Onset Schizophrenia (EOS), Autism Spectrum Disorder (ASD) and Intellectual Disability (ID)

It is known that different neurodevelopmental disorders have a predisposition to co-occur. For instance, Attention Deficit Hyperactivity Disorder (ADHD) is frequently accompanied by reading and mathematics disorders. Moreover, epilepsy commonly goes together with ID, depression, ASD and schizophrenia (SCZ). The co-occurrence of various neurodevelopmental disorders is too common to consider this as a random event. Apart from their co-occurrence, many neurodevelopmental disorders are thought to have a common genetic background. There is a growing amount of evidence that support this concept. Due to such a huge genetic overlap and co-occurrence, some scientists postulated that phenotypes describing neurodevelopmental disorders should start being considered as common features that are at the root of a neurodevelopmental continuum. For this reason, they suggest to use more broad terms of developmental brain disfunctions to refer to both neurodevelopmental and neuropsychiatric disorders (Moreno-De-Luca *et al.*, 2013).

In the context of this work, the notion of developmental brain disorders is crucial due to the fact that diagnosis criteria for neurodevelopmental and neuropsychiatric disorder evolve all the time. There exist many cohorts of patients that initially were misdiagnosed or admitted to different studies according to different criteria (*e.g.*, Driver *et al.*, 2013). The Diagnostic and Statistical Manual of Mental Disorders (DSM) reference criteria change every

several years and are upgraded. For example, as mentioned in the Introduction, diagnosis of COS had not been established before the introduction of DSM-III (Asarnow and Forsyth, 2013). For these reasons, considering neurodevelopmental and neuropsychiatric disorders as a whole gives an opportunity to look for shared genetic causes of these disorders.

When considering the analysis of families subjected to our study, I cannot neglect the fact of slight heterogeneity existing between the patients in our cohort. Although all patients have been diagnosed with EOS, in some cases this phenotype was associated with other phenotypes, such as ID or ASD. Due to this fact, during our WES analysis, I was interested in the variants that could be associated with all of these disorders, not only in specific for EOS. Moreover, I was trying to obtain a broader view on this disorder by looking for common genetic pathways that can play an important role in the pathogenesis of DBDs.

With reference to family 5 analysis, the proband was diagnosed with COS and mild ID. Our candidate variant *STK33* had the strongest potential to influence the phenotype according to *in silico* analysis. It could be argued that due to its inherited character it was not the best candidate. However, the fact that the mother suffers from hallucinations, which are one of the features of COS, and the fact that the proband was separated from the mother during his early childhood support the hypothesis of an implication of this gene in disease development. Separation of the proband in his early childhood can be viewed as a traumatic event triggering the appearance of COS. Interestingly, the patient's mother was diagnosed to be at mild suicidal risk and it is known that SCZ and ASD patients are at higher risk of suicide attempt (Chen *et al.*, 2017; Fuller-Thomson and Hollister, 2016; Kato *et al.*, 2013; Mayes *et al.*, 2013). Moreover, a recent study suggested a link between *STK33* variants and suicidal behavior

(González-Castro *et al.*, 2019). Finally, re-occurrence of *STK33* in the context of studies associated with neurodevelopmental disorders, such as SCZ, support the suspicion of its potential involvement in these disorders (Homann *et al.*, 2016; Leonenko *et al.*, 2017; Utine *et al.*, 2017; Warnica *et al.*, 2015).

The analysis of *STK33* interactors with the STRING tool revealed its direct relation with *HSP90AA1*. During the analysis of patients from family 5, I detected a small *de novo* deletion (c.1093_1098delGAAGAA) in this gene. However, this change was predicted to be a polymorphism. Nevertheless, the presence of this variant can also support the potential impact of *STK33* on the patient's phenotype. I can hypothesize that variant in *HSP90AA1* can contribute to more deleterious influences on the *STK33* variant I characterized. Indeed, the postulate that different constellations of genetic variants can influence the pathogenesis of SCZ is well known (Stefanis *et al.*, 2013). HSP90 participates in the response to stress and in hormone signalling. This protein is important in restoration of proper hypothalamic-pituitary-adrenal (HPA) axis functioning after exposure to stressful events (Criado-Marrero *et al.*, 2018). Involvement of HSP90 in response to traumatic events can support the hypothesis that the SCZ phenotype in the patient was triggered by separation from the mother in his early childhood. I suspect that the presence of a variant in this gene can impact the response to stressful events. Interaction between HSP90 and *STK33* has been previously described. As mentioned in the introduction, inhibition of HSP90 can lead to degradation of *STK33* by the proteasome (Azoitei *et al.*, 2012). HSP90 was reported to be involved in some pathways that are associated in SCZ pathogenesis, such as Akt signalling, Wnt/ β -catenin pathway and ERK signalling (Dou *et al.*, 2005; Halder *et al.*, 2012). *STK33* involvement in ERK signalling has been

previously described. It is known that inhibition of ERK1/ERK2 leads to a decrease in *STK33* expression (Huang *et al.*, 2015). Interestingly, polymorphisms in other heat shock proteins have been associated with SCZ (Pae *et al.*, 2009). For instance, another member of the heat shock protein family – HSPA12A – was reported to be decreased in SCZ patients (Pongrac *et al.*, 2004). Immune reactivity related to antibodies against HSP90 and HSP70 was aberrant in a group of SCZ patients (Kim *et al.*, 2001). All the above evidences support the presumption of the plausible contribution of *HSP90AA1* variant to the worsening of the patient phenotype through its potential impact on the *STK33* variant.

STK33 as well as *HSP90* have been shown to be involved in the ERK signalling pathway. During this study, I decided to evaluate the phosphorylation levels of ERK1/ERK2. The results revealed reduction in their phosphorylation levels, both in homozygous and heterozygous cell lines. These results indicate the putative impact of *STK33* on ERK signalling. Our mutated cell lines do not possess variants in *HSP90* that were detected in the patient. Due to this fact, I cannot evaluate its potential implication in this molecular aberration. During a previous study the authors did not evaluate the impact of mutated *STK33* on ERK phosphorylation (Huang *et al.*, 2015). I can speculate that *STK33* can participate in ERK phosphorylation, but I cannot elucidate the mechanism through which this interaction can occur. It may be a direct interaction, or *STK33* could impact ERK signalling via other proteins, such as HSP90. In the future, it would be interesting to evaluate the potential impact of the presence of combined *STK33* and *HSP90* variants in SH-SY5Y cells. It could provide us with a deeper insight into the impact of this variant combination on ERK signaling.

I analyzed STK33 interactors involved in calcium signalling in order to get a deeper insight into the potential impact of the *STK33* variant on calcium homeostasis. As noted previously in the Introduction, anomalies in calcium homeostasis are thought to be involved in the pathogenesis of SCZ (Lidow, 2003). My results showed decreased mRNA levels of two calcium-related genes, *CABP7* and *HPCA*, in the homozygous cell line. Calcium-binding protein 7 (CaBP7) belongs to the calmodulin (CaM) superfamily. This protein participates in vesicle transport in neurons, being specialized in Golgi-to-plasma-membrane trafficking (McCue *et al.*, 2012; Mundhenk *et al.*, 2019). Interestingly, another member of the CaM family - CABP8 - is a candidate SCZ gene (Mundhenk *et al.*, 2019). The *HPCA* (hippocalcin) gene codes a neuronal calcium sensor that probably participates in voltage-dependent calcium channel regulation and calcium-dependent local activation of specific molecular targets. It is thought that hippocalcin may participate in pre- and post-synaptic signaling. *HPCA* is mainly expressed in brain and homozygous mutations have been associated with dystonia (Charlesworth *et al.*, 2015; Markova *et al.*, 2008). Interestingly, it was shown that *HPCA* is involved in neuronal differentiation in SH-SY5Y cells. It was demonstrated that *HPCA* levels increase during the differentiation process. Its overexpression stimulated neuronal differentiation, whereas its reduction led to inhibition of synaptophysin (SYP) expression and neurite outgrowth (Kang *et al.*, 2019). The hippocalcin promoting role on neuronal differentiation process was also demonstrated in neural stem cells (Park *et al.*, 2017). Variations in the differentiation process between cell lines might be associated with *STK33* variant impact on the mRNA level of *HPCA*. In the heterozygous cell line, I did not observe any significant changes in mRNA levels of the investigated *STK33* calcium-associated interactors. Maybe, the lack of one active copy of *STK33* is insufficient to impact significantly the level of its interactors. The other possibility is

the existence of mechanisms of compensation to balance the lack of one copy of *STK33*. On the other hand, I can not exclude the possibility that even slight changes, that by themselves are not significant, can impact the calcium homeostasis when their effects are combined. As previously mentioned, there exists a notion that the phenotype of developmental brain disorders can result from the accumulation of small genetic or molecular changes in many pathways. Decreased expression levels in some *STK33* calcium-associated interactors in the homozygous cell line suggest that this mutation can have an impact on calcium homeostasis.

STK33 belongs to the Ca^{2+} /calmodulin-dependent protein kinase family whose members participate in the regulation of calcium homeostasis that was shown to be altered in SCZ patients. For instance, it was shown that the level of calcium-calmodulin-dependent protein kinase II β mRNA in SCZ patients was elevated (Novak *et al.*, 2000). Another study demonstrated that mRNA levels of *CaMKII α* and *CaMKII β* were elevated in postmortem frontal cerebral cortex derived from SCZ patients (Novak *et al.*, 2006). Moreover, heterozygous *CaMKII α* knock-out mice model showed many traits that are characteristic of SCZ patients (Novak and Seeman, 2010). It was also suggested that *STK33* can influence calcium homeostasis in Fadu cells (Huang *et al.*, 2015). Due to these facts, I decided to investigate the potential impact of *STK33* silencing on calcium homeostasis. I performed preliminary experiments in order to verify if SH-SY5Y cells are able to respond to different stimuli and if transfection with siRNA can result in some differences. I evaluated the response to carbachol of WT cells and of cells transfected with a *STK33*-specific siRNA, that is a muscarinic receptor agonist and to the ionomycin ionophore, that triggers immediate calcium entry into the intracellular compartments. I could observe a lower response both to carbachol

and ionomycin in cells transfected with the *STK33* siRNA. The lower response to 0 Ca^{2+} plus ionomycin and 10 Ca^{2+} plus ionomycin in silenced cells suggests slower and lower entry of calcium ions compared to control. When the cells were stimulated with 0 Ca^{2+} , we could see a higher decrease in calcium levels in control cells. This can indicate faster extrusion of calcium ions in comparison to cells with a decreased level of *STK33*. Undeniably, these are results of my preliminary trial, so they should be treated with caution. Nevertheless, waiting for experiments on clone cells lines with a proper siRNA control, they gave me some indications for a possible role of *STK33*. For instance, they suggest that a decrease in *STK33* levels may have an impact on calcium ion flux through the membrane. Interestingly, it was shown that in Fadu cells, silencing of *STK33* inhibits the raise of *CAPN1* mRNA level upon ionomycin treatment (Huang *et al.*, 2015). This gave me a basis to hypothesize potential mechanisms by which *STK33* can modulate the calcium response. I can speculate that by regulating different calcium-associated genes or proteins, such as *CAPN1*, *STK33* can influence calcium homeostasis. Moreover, my results related to *STK33* calcium-associated interactors in mutated cell lines also support this hypothesis. These results gave us deeper insight into possible targets of *STK33* in the patient.

Carbachol triggers a response of the $\text{IP}_3/\text{Ca}^{2+}$ pathway via the M1 and M3 muscarinic receptors. A lower calcium response of cells transfected with the *STK33*-specific siRNA suggests a potential impact of *STK33* on the $\text{IP}_3/\text{Ca}^{2+}$ pathway. Interestingly, it was shown that activation of this pathway can lead to phosphorylation of vimentin via CaMK II activation (Ogawara *et al.*, 1995). As previously mentioned, vimentin is a direct interactor of *STK33*. Moreover, it was demonstrated that concomitant activation of IP_3 and PI_3K is indispensable

for neurotrophin-3 (NT3)-induced potentiation of synaptic transmission at the neuromuscular synapses (Yang *et al.*, 2001). These results might imply a similar role in the CNS. As noted in the Introduction, carbachol can activate ERK1/2 via MAPK. During my analysis, I was able to show that ERK1/2 phosphorylation is decreased in mutated cell lines. Both ERK1/2 and PI3K/Akt pathways were previously reported to be affected by anomalies in muscarinic receptors pathway (Vakalopoulos, 2014). These results indicate a probable role of *STK33* in calcium homeostasis and possible pathways that can be affected by its reduction.

With reference to the measurements of baseline calcium levels, I did not observe any difference between control and *STK33*-specific siRNA-transfected cells. Probably, basic calcium levels are not affected by *STK33* silencing. These data suggest that *STK33* does not influence the basic levels of calcium but can modulate the response to different stimuli.

Identification of unique proteins (proteins that were only detected in one of three cell types) from each cell type (WT, HOM and HET) revealed the presence of numerous proteins linked to psychological or neurological disorders. Pathway analysis of these proteins indicated involvement in CCKR, Wnt and Cadherin Signaling pathways, whose potential involvement in neurodevelopmental disorders was described in the Introduction. Moreover, I detected proteins associated with the muscarinic receptors pathway, Huntington disease, PI3K pathway, GABA-B receptor II signaling, axon guidance, synaptic vesicle trafficking, and Ras pathway. Abnormalities in these pathways can have potential influence on the neurological phenotype. For example, as noted in the introduction, DISC1 is a protein that has been previously associated with SCZ and autism and can affect muscarinic receptor signaling (Mulligan and Cheyette, 2016; Vakalopoulos, 2014). Moreover, it is involved in astrogenesis

during embryonic development by interacting with RAS/MEK/ERK signaling (Wang *et al.*, 2016). Among the detected proteins, also CAMKV (CaM Kinase Like Vesicle Associated) turned out to be associated with MAPK/ERK signaling. Moreover, it is a member of Development ERBB-family signaling (<https://pathcards.genecards.org>). The *CAMKV* gene has been associated with post-traumatic stress disorder (Le-Niculescu *et al.*, 2020). Chromogranin A (CHGA), that plays a role in the neuroendocrine system, has been previously reported to be decreased in the prefrontal cortex of SCZ patients. Some SNP markers in the *CHGA* gene were associated with the SCZ phenotype in a Japanese population (Takahashi *et al.*, 2006). Another SNP in mir-198, whose expression is associated with *Follistatin Like 1 (FSTL1)*, has been linked to the SCZ phenotype (Yamada *et al.*, 2018). Also variants in *Cytochrome B (CYTB)* were suggested to be linked to SCZ and bipolar disorders (Schulmann *et al.*, 2019). Protein levels of *CYTB* were found to be decreased in iPSC-derived neurons from patients with the 22q11.2 deletion syndrome and SCZ (Li *et al.*, 2019). The other identified protein Glutamate Receptor-Interacting Protein 1 (GRIP1) is known to participate in Alpha-amino-3-hydroxyl-5-Methyl-4-isoxazolePropionic Acid (AMPA) receptor trafficking to the synapse. Aberrations in AMPA trafficking are thought to be involved in SCZ (Hammond *et al.*, 2010). Taken together, proteomic analysis indicates various changes in multiple proteins and pathways that were previously related to neurodevelopmental disorders. I can hypothesize that small changes in multiple pathways can have a synergic impact on the phenotype, especially when I take into account the fact that the majority of the players involved in these pathways can influence one another. In the majority of cases I could find a link between identified protein and pathways that were previously related to DBDs, *e.g.*, *DISC1* that was associated with muscarinic receptors pathway and ERK signalling.

To conclude, I was able to show the effect of the variant in *STK33* that leads to deletion of exon 4 and causes the disappearance of one of the *STK33* isoforms. The presence of this mutation can have an impact on the proteome and affects multiple pathways. I was able to provide the first evidence suggesting that the silencing of *STK33* can disturb calcium homeostasis. Moreover, by demonstrating an association of *STK33* with various SCZ associated pathways, such as ERK signalling, I showed the potential of this gene to be involved in neurodevelopmental disorders.

Discussion Project 2. Characterization of a novel spontaneous mutation in the *Kcc2* gene in mice suffering from spontaneous tonic-clonic seizures starting at the age of four months

Epilepsy is included in DBDs, which enclose an extremely heterogenous group of neurodevelopmental disorders. Moreover, it is frequently accompanied by other neurodevelopmental disorders, such as ASD, ID and SCZ (Canitano, 2007; Clancy *et al.*, 2014; McGrother *et al.*, 2006; Mendez *et al.*, 1993). These disorders are thought to share a common genetic and molecular background. For instance, aberrations in GABAergic signaling, which is associated with maintenance of inhibition/excitation balance, are thought to be implicated in the pathogenesis of previously mentioned disorders (Coghlan *et al.*, 2012; Duy *et al.*, 2019; Hyde *et al.*, 2011). In the course of this study, I characterized a novel spontaneous mutation in the *Kcc2* gene in mice suffering from spontaneous tonic-clonic seizures starting at the age of four months. As mentioned in the introduction section, variants in *KCC2* have been shown to underlie many neurodevelopmental disorders (reviewed in Fukuda and Watanabe, 2019).

This protein is responsible for chloride extrusion from the cells that is implicated in the maintenance of the excitation/inhibition balance (Saito *et al.*, 2017; Saitsu *et al.*, 2016; Stöberg *et al.*, 2015). Due to the fact, that anomalies in the excitation/inhibition balance can be involved in other neurodevelopmental disorders apart from epilepsy (Coghlan *et al.*, 2012; Duy *et al.*, 2019; Hyde *et al.*, 2011), characterisation of this model can provide us with a deeper insight on the impact of KCC2 on their pathogenesis.

In order to characterize *Kcc2-mut* mouse model, I intended to correlate neuronal activity, seizure appearance and social and cognitive behavior with gene expression at different times of mouse development. As previously stated, so far, these functional studies were not performed due to the fact *Kcc2*-KO mice die a few days after birth and heterozygotes were never investigated in detail. The mutation I found is located at the same amino acid residue as the one found mutated in patient suffering from EIMFS (R857L corresponding to R880L in humans; described by Saito *et al.*, 2017), offering a unique opportunity to study *in vivo* the aetiology of this disorder and to correlate the behavioral phenotype with seizure progression.

Through the analysis of the pedigree of a large cohort of mice harbouring the mutation or not, I was able to confirm that the *Kcc2* R857G variant is responsible for the mouse phenotype and its autosomic dominant character. Visible epileptic phenotype was always accompanied by the presence of the *Kcc2* R857G variant which provides the proof of association between its presence and the observed phenotype. Bioinformatic evaluation of the identified variant with Provean and SIFT softwares indicated its potential deleterious and damaging impact on protein functions. Moreover, the R857G variant turned out to be

localized in a highly conserved region among multiple species, thus having high potential to be disease-causing (Li *et al.*, 2017). This variant is localized in the C-terminal domain of KCC2 where the majority of other variants linked to neurodevelopmental disorders were found (Fukuda and Watanabe, 2019). Aiming at performing more profound analysis and due to the lack of a KCC2 structure in PDB archive, I created a model of the KCC2 protein using I-TASSER tool, which enabled me to perform prediction of protein stability based on its potential structure. The analysis obtained with MUpro software showed that the R857G variant can decrease the stability of protein structure which can have potentially deleterious consequences in the terms of protein functions. Another prediction tool Dyna-mut indicated that this variant can affect flexibility and fluctuations of KCC2 structure. Taken together, all executed predictions were consistent and suggest a damaging impact of the identified variant on KCC2 protein functioning, which gave me a basis to consider this change as potentially pathogenic.

Due to the fact that aberrations in N-glycosylation have been linked to diseases affecting the nervous system (Patterson, 2005) and the absence of information about N-glycosylation sites in the mouse KCC2 sequence, I decided to look for these sites in the mouse KCC2 amino acid sequence. Bioinformatic analysis with the NetNGlyc tool revealed the presence of eight potential N-glycosylation sites in mouse KCC2. These results indicated that probably there is less N-glycosylation sites in the mouse sequence in comparison to the rat one previously suggested by Payne *et al.* (1996). None of these potential sites lie in the proximity of the R857G variant, which indicates that this mutation probably does not affect this process.

One of the main features of EIMFS are multifocal seizures that start before the age of six months in human patients (Caraballo *et al.*, 2015). Seizures are associated with abnormal neural discharge within the CNS that can have variable clinical manifestation involving involuntary movements or their cessation, alteration of consciousness and psychic disturbance. EEG recordings serve in describing these discharges and their classification (Fisher *et al.*, 2014). To get deeper insight into this part of the phenotype, in our mouse model, a preliminary electrophysiological analysis through intracranial ElectroEncephaloGraphy (iEEG) was performed in collaboration with the team of Dr. Mantegazza at the IPMC. iEEG recordings of *Kcc2-mut* mice at the age of two months showed the presence of interictal spikes typical for epileptic mice. During the recording, *Kcc2-mut* mice did not present the observed epileptic phenotype, which is in accordance to previous observation indicating the occurrence of seizures at the age of four months. Nevertheless, the presence of such epileptiform iEEG patterns can suggest a relatively high risk for having seizures. In the future, this experiment should be also performed at the age of four months in order to evaluate the presence of iEEG patterns during a visible seizures.

Aberrations in KCC2 function have been associated to anomalies in GABAergic signaling and with occurrence of neurodevelopmental disorders, such as ASD, ID, epilepsy and FXS. Aberration in post-translational modification of KCC2 can lead to communication deficits and social behavior alterations (Pisella *et al.*, 2019). Moreover, the human patient bearing a mutation localized in the same position exhibited deficits in intellectual development (Saito *et al.*, 2017). Taking into account all these premises, I decided to evaluate the socio-cognitive capabilities of *Kcc2-mut* mice in order to shed new light on its impact on the behavioral

phenotype. I performed USV and homing tests to search for potential aberrations in social communication and discrimination. These tests were performed before the peak of *Kcc2* expression in mouse brain, namely at PND12 and PND13, respectively. The results of these tests did not reveal any statistically significant differences between WT and *Kcc2-mut* mice suggesting that the R857G variant does not impact this type of behavioral features at this age when GABA switch is not yet concluded (Ben-Ari, 2014). In order to evaluate plausible autism-related behavioral defects and correlate them with seizure occurrence, I carried out social interaction tests at different time points: one month, four months and six months of age. I did not observe any differences between WT and *Kcc2-mut* mice, which may indicate that the presence of the identified variant does not have influence on autistic traits. I saw the decline in social interaction in both WT and *Kcc2-mut* mice at the age of four and six months in comparison to the mice at the age of two months. The presence of this decline in both mouse phenotypes suggests that it can be connected with the aging process. The NOR test aimed at estimating the learning and memory capabilities of the *Kcc2-mut* mice at the age of two months. Statistical analysis revealed significant changes between WT and *Kcc2-mut* male mice, while I did not observe any differences in the female group at the same age. These results are intriguing since they suggest differences between different sexes. Overall I can conclude that the spikes observed in mice at 2-3 months of age influence the memory and the cognition in these mice but not the social interaction. This behaviour will be likely influenced by the seizures. However, I cannot, at this stage of the study, explain the differences of behaviour between males and females. I would like to remind that the majority of behavioral studies of different *Kcc2* models have been carried out only in male animals. Nevertheless, there exist a lot of evidence suggesting sex-associated differences in GABAergic system,

including KCC2 expression that can help to elucidate observed results (Galanopoulou, 2010). Some sex differences in seizure types and symptoms in epileptic human patients have been already reported (Carlson *et al.*, 2014). However, changes between sexes in learning and memory capabilities have never been evaluated in the context of epilepsy.

A decline in psychomotor development is one of Epilepsy of Infancy with Migrating Focal Seizures (EIMFS) patient's characteristic (McTague *et al.*, 2013). Moreover, it is known that seizures can influence locomotor activity (Matos *et al.*, 2018). For these reasons, I decided to investigate the locomotor activity performance of *Kcc2-mut* mice at the age of two and four months. The 72 h recordings did not reveal any statistically significant differences between WT and *Kcc2-mut* mice neither at the age of two months nor at the age of four months. These results can indicate that the R857G variant does not impact the locomotor activity of *Kcc2-mut* mice at least in young adult mice.

In order to explore potential alterations of KCC2 at the protein level, I performed Western blot analysis on protein extracts derived from cortex, hippocampus and striatum of *Kcc2-mut* and WT mice. The results showed that the level of KCC2 at the age of two and four months is highly decreased in *Kcc2-mut* mice in comparison to WT in all three parts of the brain. These results indicate aberrations in the KCC2 protein level in mutated mice that can result from the presence of the identified *Kcc2* variant.

As mentioned in the Introduction, some variants in *KCC2* were suggested to cause changes in its localization at the membrane level. To further study such possibility in the context of the R857G variant, I investigated the possible shift in KCC2 localization with the cross-linking assay and immunohistochemical techniques. The cross-linking assay was

performed in cooperation with Dr. Alessandra Folci (CNR-Milan) and revealed a decrease in KCC2 level at the membrane in *Kcc2-mut* mice at the age of two months. Immunohistochemical analysis was performed at the age of twelve months and also indicated the decrease of KCC2 level at the membrane. These results suggest a possible shift in KCC2 localization at the membrane level probably due to the presence of R857G variant, which can have an impact on anchoring or transport of KCC2 to the membrane.

In the course of the biochemical studies concerning *Kcc2-mut* mice, I also analyzed the mRNA level of *Kcc2* and its main partner in maintaining the chloride balance *Nkcc1* in WT and *Kcc2-mut* mice by RT-qPCR technique. The results of these experiments did not reveal any statistically significant differences in cortex and striatum between WT and *Kcc2-mut* mice at the age of two months, nor in hippocampus and striatum between WT and *Kcc2-mut* mice at the age of four months. These results can suggest that the investigated *Kcc2* variant does not impact *Kcc2* and *Nkcc1* at the transcriptomic level. Together with previous results concerning KCC2 protein level and its localisation, I can suppose that the R857G variant can affect KCC2 at the protein level and that it does not have negative impact on the transcription process.

To further evaluate what is happening at the protein level, I searched for interactors of WT and *Kcc2-mut* protein in hippocampus and in cortex obtained by mice of both genotypes. The results revealed interesting differences between these two phenotypes. First, only two proteins - RAB3A and TUBA1B - are detectable in all WT samples, whereas they were not present in all samples derived from *Kcc2-mut* mice. In addition this analysis confirmed the reduced expression KCC2, in *Kcc2-mice* in comparison to WT mice. We can suspect the same scenario for the two other proteins.

RAB3A and TUBA1B have been associated with Synaptic vesicle trafficking and Gonadotropin-releasing hormone receptor pathways, respectively (according to the PANTHER database). Interestingly, both of these pathways have been previously described in the context of epilepsy (Bilo *et al.*, 1991; Elfving *et al.*, 2008; Fawley *et al.*, 2006; Li *et al.*, 2018).

As previously mentioned, the roles of TUBA1B involve cytoskeleton-dependent intracellular transport and translocation of SLC2A4 (GLUT4) to the plasma membrane (neXtProt, Zahn-Zabal *et al.*, 2020). Some mutations in genes encoding different isoforms of tubulin are known to underlie the tubulopathies, which involve various brain malformations. Interestingly, clinical features of tubulopathies include ID, motor deficits and epilepsy (Bahi-Buisson and Cavallin, 1993).

RAB3A is associated with intracellular protein transport, synaptic vesicle transport, GABA synthesis, release, reuptake and degradation, serotonin neurotransmitter release cycle, and protein localization to the plasma membrane (neXtProt, Zahn-Zabal *et al.*, 2020). Some mutations in Rab3 interacting molecule 3 (*RIM3*) have been reported in ASD patients. RIM3 interacts with VDCCs and can impact neurotransmitter release. Alterations in RIM3 were suggested to underlie synaptic dysfunctions which can contribute to autistic phenotype (Takada *et al.*, 2015). RAB3A was shown to participate in neurotransmitter release together with Synapsin II (*SYNII*). Mice with deleted *SynII* gene display an epileptic phenotype that is accompanied by excitatory/inhibitory imbalance. RAB3A deletion in these mice led to the rescue of the epileptic phenotype through restoration of excitatory/inhibitory homeostasis (Coleman and Bykhovskaia, 2010; Feliciano *et al.*, 2013).

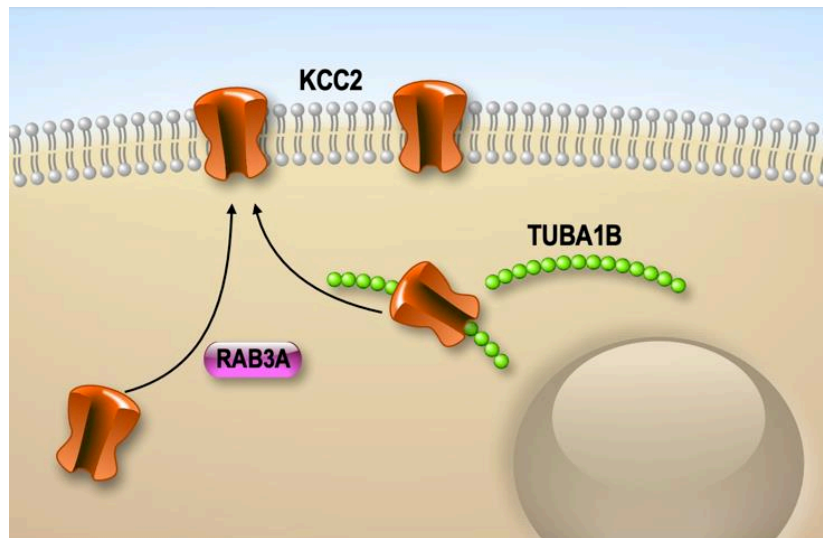


Figure 1. Schematic representation of TUBA1B and RAB3A potential involvement in KCC2 localisation at the plasma membrane.

Taken together, these observations suggest that these two proteins might be potentially implicated in KCC2 localization at the plasma membrane (Fig. 1). Moreover, their connection with epilepsy can suggest their potential role in observed epileptic phenotype in *Kcc2-mut* mice.

Axon guidance and dopaminergic synapse pathways were enriched in both hippocampus and cortex samples from *Kcc2-mut* mice. Moreover, *Kcc2-mut* cortex samples were enriched in long-term depression, axon guidance and dopaminergic synapse pathway. Dysregulations in all above mentioned pathways have been previously linked to many neurodevelopmental disorders (Kullmann *et al.*, 2000; Starr, 1996; Van Battum *et al.*, 2015). In *Kcc2-mut* hippocampus samples, I detected the proteins belonging to NMD pathways that are involved in degradation of RNA. It was suggested that NMD pathways can be associated with the process of epileptogenesis (Mooney *et al.*, 2017). The sphingolipid signaling pathway was found to be enriched in *Kcc2-mut* cortex samples. Sphingolipids are known to be involved

in brain development and its proper functioning. Aberrations in sphingolipid homeostasis can lead to alter plasma membrane organization and can underlie many neurological disorders, such as Huntington's disease, some types of epilepsy and Alzheimer's disease (Olsen and Færgeman, 2017). I can hypothesize that alterations in the sphingolipid pathway can contribute to the observed changes in KCC2 localization at the membrane level. Taken together, enrichment in these pathways in *Kcc2-mut* mice and their connection in neurodevelopmental disorders may suggest their potential implication in the observed *Kcc2-mut* mice phenotype.

KCC2 is involved in the GABAergic switch and its dysregulation is connected with excitation/inhibition imbalance. In the course of this study, it will be important to evaluate the impact of the R857G variant on the GABAergic switch. Due to the recent development of the aiFACS technique in our laboratory, I decided to evaluate if it will be possible to investigate changes in excitation/inhibition balance with this technique. In the first preliminary trials, the neurons derived from WT and *Kcc2-mut* mice (PND17) were subjected to stimulation with GABA agonist isoguvacine and sorted according to their response to this stimulant and to their morphological features (size/structure). The results showed that it is possible to detect a response of the cells to the GABA agonist both in neurons and interneurons (small and big cells respectively in my experiments of aiFACS). I was able to see that BIG cells sub-populations respond differently. Sub-populations P2 and P4 did not show any significant response to the GABA agonist. Also, the baseline fluorescence level was different between sub-populations. Previous data obtained in our laboratory suggested that BIG cells are enriched in excitatory neurons and SMALL cells are enriched in inhibitory interneurons. Our results showed that

SMALL WT cells are enriched in *Neurod1* that is marker for excitatory neurons, whereas the population of SMALL *Kcc2-mut* cells is enriched in inhibitory interneurons. Marker analysis can explain why I observed a smaller response to isoguvacine in SMALL and BIG *Kcc2-mut* cells when I analyzed the difference between isoguvacine and baseline. In theory, I would have expected a higher response in *Kcc2-mut* cells populations due to the lower level of the KCC2 protein. For further analysis, it will be crucial to determine proper separation of cell populations. For BIG cells, it will be essential to choose the sub-population that is enriched in excitatory cells. Moreover, if I manage to adapt the proper protocol for performing this kind of experiment, it will be important to confirm it with another well-established technique, such as the patch clamp method. Nevertheless, these results represent a promise for further evaluation of this technique in the context of the GABA switch.

Taken together, this result suggests that the R857G variant in the *KCC2* gene is likely implicated in the epileptic phenotype observed in *Kcc2-mut* mice. This mutation can be responsible for KCC2 decrease and changes in its localization at the membrane level. The proteomics results indicate some putative pathways that can be altered in *Kcc2-mut* mice and suggest involvement of the RAB3A and TUBA1B proteins. Further insight into these proteins and pathways can be useful in the development of novel therapies aiming at fighting epilepsy and other neurodevelopmental disorders.

Conclusions

In the course of my first project connected with the functional characterization of new genes implicated in Early Onset Schizophrenia, Autism Spectrum Disorder and Intellectual Disability:

- I detected common pathways in probands from families 1-9 that can be implicated in the observed phenotype of the patients;
- I identified a potential pathogenic variant in Family 5 in the *STK33* gene;
- I was able to show the effect of the variant in *STK33* that leads to the deletion of exon 4 and causes the disappearance of one of the *STK33* isoforms;
- I was able to show that SH-SY5Y cells are able to respond to different stimuli, such as carbachol and I provided the first evidence suggesting that the silencing of *STK33* can disturb calcium homeostasis;
- I created clone lines harbouring the mutation identified in the affected patient;
- Proteomics analysis suggested that the presence of the identified mutation can have an impact on the proteome and affects multiple pathways, such as ERK signalling.

During my second project associated with the characterization of a novel spontaneous mutation in the *Kcc2* gene in mouse suffering from spontaneous tonic-clonic seizures starting at the age of four months:

- I was able to identify a novel *Kcc2* variant (R857G) in mice suffering from spontaneous tonic-clonic seizures starting at the age of 4 months;
- Bioinformatic and pedigree analysis suggested that this variant can be possibly deleterious and responsible for the observed phenotype;
- The KCC2 protein level analysis showed decreased levels of this protein in *Kcc2-mut* mice. Immunohistochemical and cross-linking experiments revealed a decrease of KCC2 at the membrane level in *Kcc2-mut* mice;
- Proteomic analysis indicated some particular proteins and pathways which aberrations could be involved in the observed phenotype of *Kcc2-mut* mice;
- Transcriptomic analysis did not show alterations in *Kcc2* and *Nkcc1* levels, which can suggest that this mutation does not impair the transcription process;
- Behavioral studies did not reveal deficits in social communication, social discrimination, social interactions and locomotor activity in *Kcc2-mut* mice, which can indicate that the presence of the R857G variant does not impact these features. On the other hand, the NOR test demonstrated that *Kcc2-mut* mice exhibit learning and memory deficits, which can suggest the involvement of this variant in learning and memory processes;
- I showed the potential of the aiFACS technique in the context of excitation/inhibition balance studies.

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Supplementary Table 1. Unique proteins identified for WT hippocampus, *Kcc2-mut* hippocampus, WT cortex and *Kcc2-mut* cortex fractions that were used for enrichment analysis.

WT unique hippocampus	<i>Kcc2-mut</i> unique hippocampus	WT unique cortex	<i>Kcc2-mut</i> unique cortex
ADGRL2	A2ML1	ALDH1A2	AAK1
AP2B1	ABI1	ALDH1A3	AARS1
APCDD1	ABI2	ALDH1B1	ABAT
APOA1	ACLY	ALDH2	ACOT7
ARPC4	ACTR1A	ARID1A	ACTR1B
CAMK2B	ACTR1B	ATL1	ACTR2
CKMT1A	ADD1	ATP4A	ACTR3
CKMT1B	ADD2	BAZ2B	ADAP1
CPNE6	AHNAK	BLK	ADD1
DNM2	ALB	BRCA2	ADGRL2
FGA	ANK2	C3	AGAP3
FGG	AP2A2	CAMK2A	AHCY
FMNL1	AP2S1	CAMK2G	AHSG
FN1	ASAP2	CASP14	AKR1E2
GLDC	ATP2B2	CCT6A	ALB
GLS	ATP5F1A	CDK20	ALCAM
GLUD1	ATP6V1C1	CILK1	ALDH5A1
GLUD2	CAMK2B	CKMT1A	ALDH6A1
GNB3	CAMK2D	CKMT1B	ANXA1
GNB4	CAMKV	DGKI	ANXA6
GOT1	CATSPER4	DST	ARF5
GSTM2	CCT3	EPB41L2	ARFGAP1
GSTM5	CCT6A	EWSR1	ARHGAP1
HNRNPR	CCT7	FGFR1	ARHGDIA
HNRNPU	CCT8	FGFR2	ARPC1A
HSPA6	CDK5	FGFR3	ARPC4
HSPA7	CFL1	FGFR4	AUH
IDH3B	CLIC4	FGR	BIN1
IDH3G	CPA4	FYN	BPNT1
IGHM	CRNN	GNB3	CADM2
IGKV3D-7	CS	GNB4	CAMK4
KATNAL2	CSTB	GOT1	CAP1
KRT18	CYFIP2	GOT2	CAP2
KRT78	DARS1	GPR37L1	CAPZA1
KRT81	DCLK1	H2AC1	CAPZA2
Krt83	DCTN1	H2AC21	CATSPER4
MAP2K1	DDX17	H2AJ	CCT5
MDH1	DDX5	H2AX	CDC42
MYH7B	DGKI	H2AZ1	COPS6
NCAM1	DHX29	H2AZ2	CSRP1
NDRG2	DMXL2	H3-3A	CTBP1
PDHB	DNAH8	H3-3B	DCLK2
PFKP	DNM1L	H3-4	DDAH1
PGAM2	DSC3	H3-5	DNAJA2
POF1B	DSP	H3C1	DNAJC6
POT1	DYNC1H1	H3C10	DPYSL5
PPIA	EEF1A1	H3C11	DSTN
RPL36A	EEF1G	H3C12	EIF4A2
RTN1	EPB41L1	H3C13	ENO1

SH3GL1	EPB41L2	H3C14	FSCN1
SH3GL2	EPB41L3	H3C15	GAS7
SIGLEC16	EPS15L1	H3C2	GATD3A
SLC25A12	GAD1	H3C3	GATD3B
SPTAN1	GIT1	H3C4	GDA
SPTBN1	GNAI1	H3C6	GNA12
TGM1	GNAI2	H3C7	GNA13
TKT	GNAI3	H3C8	GNAI3
TTC39A	GNAL	HCK	GNAL
UBA1	GNAS	IGHV1-45	GNAS
UTS2	GNAT1	IGKV3-15	GNAT1
VDAC2	GNAT2	IGKV3D-7	GNAT2
WDR1	GNAT3	IGLC1	GNAT3
	GPBP1	IGLC2	GNAZ
	GPHN	IGLC3	GPBP1
	GSTP1	IGLL5	GPD1
	HBA	KPNB1	GPD2
	HNRNPH1	KRT33B	GPI
	HNRNPH2	KRT5	GPM6B
	HNRNPK	KRT6C	GPS1
	HSP90AA2P	KRT75	GSK3B
	HSP90AB2P	KRT77	GSTM3
	HSPA12A	Krt5	GSTM5
	HSPB1	LCK	HADHB
	IDH3A	LGALS7	HNRNPA2B1
	IGHA2	LGALS7B	HNRNPD
	IGHV3-72	LOC525947	HPRT1
	IMMT	LPL	HRAS
	INA	LRRC10B	HSP90B1
	KIF5A	LYN	HSPA6
	KIF5B	MAK	HSPA8
	KIF5C	MAPK15	IDH2
	KLC1	NDUFA10	IDH3G
	KLC2	NDUFS3	IGHV3-11
	KRT37	PCBP2	IGHV3-13
	KRT72	PCBP3	IGHV3-15
	MAP1A	PFKP	IGHV3-20
	MAP1B	PKLR	IGHV3-21
	MAP4	PKM	IGHV3-23
	MAP6	RPL13A	IGHV3-30-3
	MAPK1	RPL35	IGHV3-30-5
	MAPK15	RPL36A	IGHV3-33
	MAPT	RPL36AL	IGHV3-43
	MB21D2	RPL7	IGHV3-43D
	NCKAP1	RPS23	IGHV3-48
	NDUFS1	RTN1	IGHV3-49
	NEFL	SEPTIN4	IGHV3-53
	NEFM	SH3GL1	IGHV3-64
	NLRP12	SKOR2	IGHV3-66
	NPEPPS	SLC25A6	IGHV3-7
	P2RX4	SPTBN1	IGHV3-73
	PABPC1	SRR	IGHV3-74
	PCBP2	SYN2	IGHV3-9
	PCBP3	TFAP4	Impa1

	PFKM PHACTR1 PHYHIP PHYHIPL PLP1 POMGNT2 PPFIA1 PPFIA2 PPFIA3 PPM1E PPP2R2A PPP3CA PPP3CB PRKAR2B PRKCB PRPS1 PRPS1L1 PRPS2 PSMD2 RAB15 RAC3 RPL11 RPL12 RPL13 RPL18 RPL19 RPL24 RPL4 RPL7 RPL8 RPS14 RPS2 RPS3 RPS4X RPS6 RPS8 RPS9 RTN3 S100A11 S100A16 SEPTIN10 SEPTIN11 SEPTIN5 SEPTIN8 SEPTIN9 SERPINA12 SFN SGIP1 SKOR1 SLC25A18 SLC25A22 SLC25A5 SNAP91 SPIN4	TGM1 TRAP1 TRPM8 TXN WDR37 XDH YES1 ZKSCAN2	KATNAL2 KIF1A KIF1B KIF1C KIF5C KRAS KRT4 Krt2 Krt73 LGI1 MAP4 MOG MPP2 MTCH2 NCAM2 NDUFS1 NDUFS2 NDUFV1 NFASC NME1 NPTN NRAS OLA1 OTUB1 OXCT1 OXR1 PAFAH1B1 PAK1 PARK7 PDE1B PDIA3 PDXP PEBP1 PFKL PFKM PFN2 PGAM1 PGK1 PGM1 PHF24 PIP4K2A PIP4K2B PKP1 PLCB1 PPP1CB PPP1R7 PPP2CA PPP2CB PPP2R5B PPP2R5C PPP2R5D PPP2R5E PPP3CA PRKAR2A
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	SPRR1A SPRR1B SPRR2A SPRR2B SPRR2E SPRR2G SPRR3 SRCIN1 SUCLG1 SUCLG2 SYNGAP1 SYNJ1 TCP1 TFG TNFRSF10D TOMM70 TTC7A TUFM USP5 VPS35 WASF1 WDR87 WDR91 WRN XPNPEP3 YWHAB YWHAQ ZNF423		PRKAR2B PRPSAP1 PRPSAP2 PSAT1 PSMA6 PSMA7 PSMA8 PSMB1 PSMB6 PSMC5 PSMD2 PSMD3 PURA PYGM RAB11B RAB1A RAB1B RAB1C RAB2A RACK1 RAN RAP1A RAP1B RAP1GDS1 RHOB RIPK2 RPL12 RPL15 RPL17 RPL18 RPL3 RPL7A RPS15A RPS3A RPSA SCRN1 SEPTIN2 SERPINA1 SFPQ SFXN1 SGIP1 SH3GLB2 SIRT2 SLC6A11 SNAP25 SPATA5 STX1A SV2B TKT TOM1L2 TPI1 TPP2 TRIM2 TRIM6
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			TUBB1 TWF2 UCHL1 UGP2 USP5 VAMP2 VAMP3 VAT1L VDAC3 VPS26B WDR1 WDR7 WDR87 XPO1
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Annexes

Manuscript

Exploration and characterisation of the genetic profiles of patients with Early Onset Schizophrenia associated with Autism Spectrum Disorder through Whole Exome Sequencing

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Abstract

Diagnosis of COS is challenging not only due to its cooccurrence with other psychiatric disorders but also because of its genetic heterogeneity. Genetics studies of COS patients suggest that rare copy number variations (CNVs) are more common in COS than in Adult Onset Schizophrenia (AOS). Some of these rare CNVs were also associated with ASD and intellectual disability (ID) suggesting common causes of these diseases. With the purpose to better understand genetic and molecular bases of COS, we performed here the WES of 9 patients affected by COS, ASD and mild ID. We carried out gene prioritization by combining multiple bioinformatic tools such as RVIS, GDI, InegraGen tools, VarElect, DECIPHER, Panther and STRING allowing us to identify main pathways that could underpin the neurodevelopmental phenotype of these patients.

Introduction

Childhood Onset Schizophrenia (COS) is an extremely rare mental disorder that is a form of schizophrenia (SCZ) that manifests in children before the age of 13. In the general population, COS occurs in less than 1 out of 10,000 individuals (Burd and Kerbeshian, 1987; Gochman *et al.*, 2011). The diagnosis of COS is based on the same criteria as for adolescents and adults diagnosed with SCZ. SCZ with onset between 13 and 18 years old is referred as Early Onset Schizophrenia (EOS) (Grover and Avasthi, 2019). According to DSM-5, SCZ patients have to exhibit at least one of the following features: hallucinations (perception without external stimulus that seems to be real), delusions (firm convictions based on fake information that cannot be changed when confronting the reality) or disorganized speech (*e.g.* changing from one subject to another). Negative symptoms include lack of interest in social activities and interactions, reduction in showing feeling, lower self-motivation, poverty of the speech content, and anhedonia (Association, 2013). The diagnosis of COS is very challenging due to the fact that around 30 up to 50% of individuals suffering from affective or other atypical psychotic symptoms are misdiagnosed as COS (Driver *et al.*, 2020). Before introduction of DSM-III, the diagnostic criteria for COS haven't been established (Forsyth and Asarnow, 2020). Moreover, in the National Institutes of Mental Health (NIMH) studies of COS cohorts, 90% of patients initially diagnosed with COS received alternative diagnosis in the follow up studies (Driver *et al.*, 2020). Additionally, only in one third of articles related to COS the full phenotype of patients and its association with mutations is described (Fernandez *et al.*, 2019). The occurrence of COS is usually preceded by a period of normal development. Sometimes, atypical interests and beliefs can be confused with social deficits typical for Autism Spectrum Disorder (ASD) patients (Association, 2013). Around 27% of COS patients before manifestation

of the first psychotic symptoms meet criteria for ASD (Driver *et al.*, 2020). Hallucinations and delusions are traits that can help distinguish COS patients from ASD ones (Association, 2013). Diagnosis of COS is challenging not only due to its cooccurrence with other psychiatric disorders but also because of its genetic heterogeneity (Fernandez *et al.*, 2019). Genetics studies of COS patients suggest that rare copy number variations (CNVs) are more common in COS than in Adult Onset Schizophrenia (AOS). Some of these rare CNVs were also associated with ASD and Intellectual Disability (ID) (Addington and Rapoport, 2009). COS is thought to have higher familial transmission and higher prevalence of rare allelic variants than AOS. Risk for Schizophrenia Spectrum Disorder in parents of COS patients is higher than in parents of AOS. Schizophrenia Spectrum Disorder encompasses Schizoaffective Disorder, Schizophreniform and Atypical Psychosis, Schizotypal Personality Disorder, and Paranoid Personality Disorder. COS was associated with common polymorphisms in *Glutamate decarboxylase 1 (GAD1)*, *dysbindin-1 (DTNBP1)*, *Neuregulin 1 (NRG1)*, *G72/G30* and *Brain-derived neurotrophic factor (BDNF)*. Genome Wide Association Studies (GWAS) of AOS involving more than 16,161 individuals did not indicate significant changes. For this reason and due to a low prevalence of COS, GWAS on COS have not been performed yet (reviewed in Forsyth and Asarnow, 2020).

Gene prioritization is a challenging task considering the amount of data to process and the heterogeneity of the samples. There exist multiple bioinformatics tools and guidelines that aim to facilitate this laborious task (Richards *et al.*, 2015). Unfortunately, most of them are based on different approaches and referred to different reference data (*e.g.* different reference sequences, genome assemblies and different cohorts). Many researchers are trying to combine different approaches in the pursuit of elucidation of the best method of gene

prioritization (Richards *et al.*, 2015; Tranchevent *et al.*, 2011). In our study, the main objective is to identify disease causing mutations primarily in genes involved in neurodevelopmental pathways in a cohort of patients affected by COS. We carried out gene prioritization by combining multiple bioinformatic tools such as RVIS, GDI, InegraGen tools, VarElect, DECIPHER, Panther and STRING allowing us to identify main pathways that could underpin the neurodevelopmental phenotype of these patients.

Materials and Methods

TRIOs recruitment and clinical evaluation

Patients with SCZ (EOS and COS) were initially recruited through the 2011-2013 Interregional Hospital Clinical Research Program (NCT01512641) whose main goal was to estimate the prevalence of early and childhood onset schizophrenia in a population of children in child psychiatry care or medico-education structures (Dor-Nedonsel *et al.*, 2020). In addition, an enrolment in the GenAuDiss protocol study is offered to in- and out-patients corresponding to the inclusion criteria of the investigator, Child and Adolescent Psychiatry (CAP) centers, as well as their first-degree relatives (Fernandez *et al.*, 2018). Participants are included either directly at the study sites or after referral by child and adolescent psychiatrists of the Provence-Alpes-Côte d'Azur (PACA) region.

The investigator centers of the GenAuDiss protocol are the Children's Hospitals of Nice CHU-Lenval, the Marseille University Hospital, the Hospital La Fontonne (Antibes) and the Hospital Bonnet (Frejus). At inclusion (V1), clinical assessments (both psychiatric and neurocognitive)

are performed in patients as well as first-degree relatives (parents and siblings). Quantitative and qualitative measures are assessed during the study:

- Clinical parameters from medical history (including pregnancy and birth) and biographic parameters, types and dates of significant life events including trauma and environmental exposures (*e. g.*, drugs and substances);
- Clinical parameters from physical examination (*e. g.*, body weight, BMI, arterial pressure);
- Semistructured interviews to assess main diagnosis at inclusion (K-SADS-PL, ADI-R, MINI);
- Clinical heteroassessments with specific neurocognitive rating scales (WISC V, TMTA, TMTB, Verbal fluency) and specific psychiatric rating scales (PANSS, SANS);
- Self-report questionnaires (Cloninger's TCI 226 to assess personality trouble and Baron Cohen's AQ to assess autistic traits);

Sample collection

Blood samples were collected by personnel of Children's Hospitals of Nice CHU-Lenval (Nice, France) in a second visit (V2) within 6 months. DNA from blood was isolated with GenElute Mammalian Genomic DNA Miniprep Kit (SIGMA Cat. No. G1N70). The biobank of IPMC (CNRS laboratory) manage the anonymized biological samples of patients. First, we performed current testing (including DNA testing for fragile-X syndrome, high resolution karyotype and CGH array). Then, in case of negative result from this previous step, we performed an exome sequencing on trio (mother, father and child).

Whole Exome Sequencing

Isolated DNA samples were sent for WES analysis that was performed by IntegraGen SA (Evry, France). Targeted exome sequencing included library preparation, exome capture, sequencing, and final pair-end sequencing on Illumina HiSeq4000 as paired-end 75 bp reads. Base calling was performed according to IntegraGen software with default parameters. Sequence reads were mapped to the human genome build (hg19/GRCh37). Sequencing data were further subjected to bioinformatic analysis.

Bioinformatic analysis

Gene prioritization was performed by combining multiple bioinformatic tools such as RVIS, GDI, InegraGen tools, VarElect, DECIPHER, Panther and STRING. RVIS (Residual Variation Intolerance Score) is based on assumption that genes with common genetic functional variation are more “tolerant” and the variations occurring in those genes are more common (higher RVIS score) and are less probable to be disease-causing. On the other hand, genes with lower genetic variation (lower score) are considered to be “intolerant” and have higher chances to be disease-causing. RVIS percentile refers to a percentage of intolerant genes among which the gene of interest is found (Petrovski *et al.*, 2013). Gene Damage Index (GDI) predicts if a given gene is prone to harbour disease-causing variants. The index evolved from the observation that more than a half of rare variants found in protein coding sequences are located in 2% of the genes (Itan *et al.*, 2015). STRING is a protein-protein interactions database that shows data based on experiments (co-crystallization, co-purification and genetic interaction), databases (pathways and protein complexes), textmining (names of proteins that are often mentioned together), cooccurrence of gene patterns across genomes and

coexpression (Franceschini *et al.*, 2013). IntegraGen tools such as ERIS and SIRIUS enables WES analysis. They enable to follow family transmission of variants, predict the impact of variants, get access to external databases like 1000Genomes, ExAC and prediction programs like SIFT and PolyPhen. VarElect is a tool that enables gene prioritization according to the disease and/or phenotype association. It also includes access to tools such as RVIS and GDI (Stelzer *et al.*, 2016). DatabasE of genomiC variation and Phenotype in Humans using Ensembl Resources (DECIPHER) is a database that combines multiple tools to facilitate evaluation of the impact of a given variant (Firth *et al.*, 2009). The Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system facilitate protein and genes categorization according to family and subfamily classes, molecular function, biological process and pathways (Mi *et al.*, 2019).

Statistics

two-tails Mann Whitney test was performed for all the analysis. Data were presented in the form of violin plot. The statistical analysis was performed using Prism Software 7 and 8 version (GraphPad Software, Inc.).

Ethics

The study protocol was approved by the Local Ethics Committee 'Sud Méditerranée V' (number 14.002) and authorized by the French National Agency for Medicines and Health Products Safety (ANSM 2013-A01699-36). All patients, their parents and siblings signed informed consent upon enrolment in the study. The GenAuDiss study was funded by Monaa (Monaco Against Autism), the Conseil Général des Alpes-Maritimes (CG-06), FRM (*Fondation*

pour la Recherche Médicale, ING20140129004) and by ANR (*Agence Nationale de la Recherche*, ANR-11-LABX-0028-01). It is an ongoing study with the inclusion period starting in April 2014 and ending in November 2021. It has been registered on ClinicalTrials.gov (NCT02565524).

RESULTS

***De novo* variant analysis**

Previous data suggest that there is a difference between RVIS and GDI index of inherited and *de novo* mutations. *De novo* mutations are thought to occur more frequently in genes that are more prone to harbour disease-causing variants and have less common variation (Ambalavanan *et al.*, 2016). We compared RVIS and GDI of inherited and *de novo* mutations but statistical analysis with the two-tails Mann Whitney test did not reveal statistical differences between those two groups (Fig. 1A and B). Even when we considered only missense *de novo* mutations, we did not obtain the same pattern as in previous studies (Table 4; APPENDIX; Fig. 1). Actually, more missense variants occurred in genes that were predicted to have more common variation that is opposite to what was found by other groups (Ambalavanan *et al.*, 2016).

In order to find a link between TRIOs, we created a Venn Diagram with *de novo* variants identified in families 1-9 (Fig. 2). We focused on exon, exon plus splice intron, missense, nonsense plus splice exon, frameshift, inframe deletion/insertion, and splice acceptor variants. Due to splice variant identification, some changes were localized in the intronic part

of the genes. We focused on *de novo* variants in genes that were common for at least three families from our dataset (Table 2.).

In families 1-5 and 7 we identified many *de novo* changes in *Zinc Finger Protein 717 (ZNF717)* that is transcription regulator coding the KRuppel-Associated Box (KRAB) zinc-finger protein. According to DECIPHER database, the *ZNF717* gene was found within the CNV region in five patients suffering from ID and in two patients with ASD. All reference SNPs (rs2918517, rs3009004, rs78135954, rs80214016, rs74357986, rs77110669, rs79138891, rs73843014, rs75737034, rs150497643, rs149076283, rs77378861, and rs776210532) do not have ClinVar reference. Most altered alleles have low frequency or data are not available. GDI predicts that changes within the *ZNF717* gene have moderate chances to be disease-causing. In 2018, a *de novo* variant in *ZNF717* was reported as a gene potentially associated with growth retardation and ID (Diao *et al.*, 2018).

Variants in *Mucin 6 (MUC6)* were identified for families 1-5. Only two *de novo* variants in this gene had reference in SNPdb. SNP rs78848170 turned out to have high frequency [G=0.40791 (37335/91528, ExAC)], which indicates that it is a polymorphic change frequently present in the global population. Another SNP rs34649796 has unknown frequency. Both of previously reported SNPs do not have any references in the ClinVar database. RVIS predicts this gene to have more common functional variation which means that changes in this gene usually are not disease-causing. GDI predicts this gene to have little chances to harbour disease-causing mutations. *MUC6* is actually one of the most frequently mutated genes in the general population (Itan *et al.*, 2015).

FRG1 (FSHD Region Gene 1) is a gene that was previously associated with the neuromuscular disorder fascioscapulohumeral muscular dystrophy (FSHD). Variants in this gene were found

in families 1-3. This gene was linked to SCZ and BD by Pedrosa *et al.* who suggested that many candidate genes for SCZ and BD may belong to the GSK3 β / β -catenin signalling pathway. They performed microarrays (ChIP-chip) targeting genes that bind to β -catenin. Their results indicated the *FRG1* gene as a potential target of β -catenin. Moreover, *FRG1* is a gene that was deleted in patient with ASD (Pedrosa *et al.*, 2010). The *FRG1* gene was also found in the CNV region 4q35.1-q35.2 in SCZ patients (Bulayeva *et al.*, 2016).

Another gene shared by families 1-3 is *Poly(A) Binding Protein Cytoplasmic 1 (PABPC1)*. This gene was shown to be upregulated in male patients with SCZ (Qin *et al.*, 2016). According to GDI prediction, *PABPC1* has moderate chances to harbour disease-causing mutations. On the other hand, RVIS predicts that this gene has less common genetic variation. This gene has never been associated with SCZ, ASD and BD.

The variants in *Aggrecan (ACAN)* were found in families 2, 3 and 4. Variant found in family 4 (rs12899191) turned out to have a high frequency in the general population [G=0.27107 (3538/13052, GnomAD)] and was not reported in ClinVar database. DECIPHER analysis showed that this gene is extremely intolerant of loss-of-function variation. *ACAN* was shown to be downregulated in the brain cortex region of SCZ individuals (Pietersen *et al.*, 2014). Both RVIS and GDI predict this gene as highly mutated which means that there is little probability to be a disease-causing gene.

The same variant in *RNA-Binding Motif Protein, X-Linked-Like-3 (RBMXL3)*, was found in families 1, 3 and 4. DECIPHER database provides reports of two patients with abnormalities in the nervous system who possess variants in the *RBMXL3* gene. Mutation tester showed that deletion in this gene might be disease-causing. GDI places this gene among ones that have

moderate chances to harbour disease-causing mutations. The RVIS database does not contain information about this gene.

VarElect analysis of *de novo* variants

In order to group the genes harbouring *de novo* variants according to their association with certain diseases or phenotypes, we carried out analysis with VarElect tool. As a query, we used the following phenotypes: SCZ/ASD/ID/neurodevelopmental disorder. We concentrated on the first 10 best scored genes (Table 3.).

A missense mutation in *Potassium Voltage-Gated Channel Subfamily B Member 1 (KCNB1)* gene was found in a patient of family 6. This mutation was predicted by the IntegraGen tool to have a moderate impact. SIFT and PolyPhen predicted this change as deleterious (score 0) and probably damaging (score 0,999), respectively. Mutation tester indicates this change as disease-causing. *KCNB1* is mainly linked to Early Infantile Epileptic Encephalopathy (de Kovel *et al.*, 2017). The proband was, apart from EOS, diagnosed with idiopathic epilepsy, thus, this mutation identified in *KCNB1* can be associated with the epileptic phenotype of the patient.

The second gene predicted by VarElect is Major Histocompatibility Complex, Class II, DR Beta 1 (*HLA-DRB1*). A missense variant in this gene was identified in family 4. Mutation tester predicts this variant to be of polymorphic nature. Moreover, this variant (rs201929247) turned out to be very frequent [A=0.147043 (14841/100930, ExAC)] and was not reported in ClinVar. According to GDI and RVIS predictions, variations in this gene are very common. For those reasons, its impact on the phenotype is very dubious.

The variant for third predicted gene *Bromodomain PHD Finger Transcription Factor (BPTF)* was identified in family 8. *BPTF* is associated with neurodevelopmental disorder with dysmorphic

facies and distal limb anomalies. The characteristics of this disorder encompass ID features (Midro *et al.*, 2019). IntegraGen tool predicts the impact of this change (inframe insertion) as moderate. GDI analysis suggests that *BPTF* is among moderately-damage genes. RVIS predicts this gene as a gene of less common variation.

A missense variant identified in *HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2 (HERC2)* was found in family 1. Mutations in *HERC2* were associated with developmental delay with Angelman-like features (includes strong ID) (Harlalka *et al.*, 2013). This variant (rs146883683) is known to have high frequency [G=0.11394 (9547/83792, ExAC)] and is not reported in the ClinVar database. Moreover, Mutation Tester predicts this variant as polymorphism.

A missense variant in *Cystathionine Beta-Synthase (CBS)* was identified in family 3. According to ClinVar, the variant found in a patient (rs5742905) is associated with homocystinuria. Interestingly, this disorder is frequently accompanied by ID. Mutation Tester predicts this change as disease causing. GDI analysis suggests that *CBS* is among moderately damage genes and RVIS predicts this gene as a gene of less common variation.

An insertion in *Neurogenic Locus Notch Homolog Protein 4 (NOTCH4)* was identified in family 4. Mutation tester predicts this variant as polymorphism. Interestingly, polymorphic variants in this gene were associated with SCZ and schizoaffective disorder (Ujike *et al.*, 2001; Zhang *et al.*, 2015). Both, GDI and RVIS predictions indicate that variants in this gene are not prone to be disease-causing.

A missense variant in *Folate Hydrolase 1 (FOLH1)* was found in family 3. This variant (rs75111588) is frequent in the general population [A=0.187153 (20786/111064, ExAC)], thus, there are little chances that this change influences the patient's phenotype.

A missense variant in *Filaggrin (FLG)* was identified in family 5. It turned out to be frequent in the general population [G=0.08408 (1087/12928, GO-ESP; G=0.117 (70/600, NorthernSweden)], which suggests that this variant does not impact the phenotype.

An inframe deletion in *Achaete-Scute Family BHLH Transcription Factor 1 (ASCL1)* was identified in family 6. IntegraGen tool predicts this change to have a moderate impact. GDI places this gene among the genes with moderate chances to harbour disease-causing variants. *ASCL1* promotes neural differentiation (Castro *et al.*, 2011). *ASCL1* was suggested to increase the risk of Parkinson's disease (Ide *et al.*, 2005). According to genomAD browser this variant is quite frequent (delGCA=0.01150; 340/29556). Mutation tester predicts this change as potential polymorphism.

In family 6, we identified a frameshift variant in *Zinc Finger Protein 148 (ZNF148)*. IntegraGen predictions indicate high impact of this variant. Mutations in *ZNF148* were associated with Global Developmental delay, Absent or hypoplastic Corpus Callosum and dysmorphic Facies (GDACCF). Apart from this disorder, the phosphorylation level of *ZNF148* was shown to be decreased in SCZ patients, but its overall level was unchanged (Jaros *et al.*, 2012). GDI analysis suggests that CBS is among moderately-damage genes and RVIS predicts this gene as a gene of less common variation.

In general, VarElect analysis has provided a further insight into genes that are already implicated into the pathogenesis of neurological disorders. We could find interesting variants in genes such as *ZNF148* and *KCNB1* that could impact the phenotype of the proband from family 6. We were able to exclude some variants of polymorphic nature that turned out to be common in the general population and that were not associated with any neurological disorders. We came across polymorphisms that could increase the risk of SCZ (*NOTCH4*). The

VarElect analysis enables to extract variants that sometimes can be overlooked in general analysis (e.g. some polymorphisms that frequently are excluded from candidate genes).

Inherited variant analysis

We created a Venn Diagram for inherited variants to find some genes that are common for at least three families (Fig. 3). We found variants in the following genes: *ZNF717*, *FAM182B*, *LRP5L*, *HRNR*, *OR4C5*, *APOL3*, *BCLAF1*, *FLG*, *RP1L1*, *MUC6*, *HYDIN*, *PLEC*, *TTN*, *NRAP*, *ADAMTS8*, *SSPO*, *FAM104B*, *DAAM2*, *ZFH3*, and *AHNAK2*. According to GDI and RVIS predictions, most of these genes are less prone to harbour disease-causing mutations and are belonging to genes that are have more variability. We focused on genes that did not have RVIS reference.

We found 17 missense variants, two frameshift and one insertion in *ZNF717* in families 1 to 7. This gene was previously found during *de novo* mutations analysis. Considering the amount of variants found and the fact that the majority of them have references in SNPdb, it is possible that variants in this gene are very common and are not prone to be disease-causing.

In families 3 and 4, we found variants in *Family With Sequence Similarity 182 Member B* (*FAM182B*). In family 4, we found a variant in the 5'-UTR, in family 3, a variant in the 5'-UTR and one synonymous variant and in family 4 a missense variant. This gene is predicted by GDI to have medium chances to harbour disease-causing mutations.

In families 1, 2 and 9 we identified variants in *Hornerin* (*HRNR*). All of them have references in SNPdb (rs79513582, rs76694305, rs61814943, rs61814936) and have high frequencies in the general population.

We found some variants in *Olfactory Receptor Family 4 Subfamily C Member 5* (*OR4C5*): i) the same deletion in families 1 and 3 that was reported in SNPdb (rs66829866) and it is known to

have a high frequency; ii) an insertion in family 1 (rs114053360) that has low frequency and no report in ClinVar; iii) an insertion in family 4. *OR4C5* is a gene predicted by GDI to be highly damaged.

We identified three variants in *Plectin* (*PLEC*). Missense variant in family 2 is predicted by PolyPhen2 to be benign (score 2). In family 4, we found a synonymous variant described in SNPdb (rs113133985) with low frequency. A missense variant in family 5 also turned out to have low frequency and no record in ClinVar database. GDI was predicted to be high, whereas RVIS score was very low.

Overall, all inherited variants that we found seem to have benign consequences.

Interaction and pathway analysis

We carried out interaction analysis with the STRING database. The analysis of genes that were inherited after the mother revealed that “the network between them had significantly more interactions than expected”. This means that these proteins have more interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates that the proteins are at least partially biologically connected. Molecular functional enrichment in this network includes transferase activity, catalytic activity (acting on protein), adenylyl ribonucleotide binding, ATP-binding and ubiquitin protein ligase activity. Considering PFAM protein domains, enrichment was observed in protein kinase domains, protein tyrosine kinase, microtubule-binding and kinesin motor domains.

We performed pathway analysis with PANTHER for genes that were inherited from the mother. The results showed that all nine families share variants in genes belonging to the Wnt and Cadherin pathways (Fig. 4).

In addition, families 1 to 8 share additionally cholecystokinin receptor (CCKR) signalling pathway. Cholecystokinin activates the CCK(1) receptor which belongs to G-protein-coupled receptors.

Discussion

In our study we correlated the phenotype and the genotype of nine patients affected by complex neurodevelopmental disorders having in common EOS, ASD and mild ID.

Even if we cannot exclude the hypothesis that minor changes in these genes could contribute to the phenotype of patients if they are co-occurring, an interesting aspect of our analysis was the definition of pathways deregulated by the presence of variants in disorder-candidate genes: WNT, cadherin signaling and CCK(1) receptor signalling.

Aberrations in WNT signalling are related to the pathogenesis of diverse neurodevelopmental diseases such as SCZ, ASD and bipolar disorder. WNT pathway participates in many processes such as regulation of gene transcription, apoptosis, proliferation, cell migration and cytoskeletal dynamics. It plays an important role in CNS development, in particular, in differentiation of neural progenitors, synaptogenesis, neuronal migration and plasticity. Mutations in WNT signalling genes are associated with many psychiatric illness. Genes such as the *APC Regulator of WNT Signalling Pathway (APC)*, *chromodomain helicase DNA-binding protein 8 (CHD8)*, *Disrupted in schizophrenia 1 (DISC1)*, *Phosphatase And Tensin Homolog (PTEN)*, *WNT Family Member 1 (WNT1)*, *WNT2*, *WNT3*, *WNT7A*, *β -catenin (CTNNB1)*,

Transcription Factor 4 (TCF4), and *TCF7* were linked to ASD. Wnt/ β -catenin pathway loci were identified in the genomic analysis of SCZ patients, and some of them were suggested to be a risk factor for this disorder. Among them, *CHD8*, *CTNNB1*, *DISC1*, *TCF4*, (Dickkopf WNT Signaling Pathway Inhibitor 1) *DKK1* and *DKK4* (Mulligan and Cheyette, 2016). It was shown that the mRNA expression levels of WNT signalling members - *Frizzled Class Receptor 7 (FZD7)* and *Nuclear Factor Of Activated T Cells 3 (NFATC3)* - are increased in SCZ patients (Hoseth *et al.*, 2018). Moreover, the plasma level of dickkopf 1 and sclerostin were lower in SCZ patients (Hoseth *et al.*, 2018). It is interesting to underline that that some antipsychotic drugs used to treat patients affected by various forms of neurodevelopmental disorders (*e.g.* haloperidol, clozapine, fluoxetine and Ritalin) are Wnt pathway modulators (Bae and Hong, 2018) This information can also be useful to treat our patients' cohort.

The Cadherin signalling pathway – involving many cell adhesion molecules - plays a role in CNS development and is associated with WNT. Indeed, cadherins negatively regulate WNT signalling by sequestering β -catenin and play an important role in the regulation of β -catenin-dependent transcription (Howard *et al.*, 2011). Cadherins participate in neural circuit formation, synapse development, differentiation of grey matter, neuronal migration and spine morphology. Cadherins that are present in brain display spatio-temporal specific expression (Redies *et al.*, 2012). GWAS studies revealed many genes associated to different neuropsychiatric diseases such as ASD, SCZ, epilepsy, bipolar disorder and ID. *CDH15* and *PCDH19* were linked to ID; *CDH5*, *CDH8*, *CDH9*, *CDH10*, *CDH13*, *CDH15*, *PCDH10*, *PCDH19* and *PCDHb4* were associated with ASD; *CDH7*, *CDH12*, *CDH18*, *PCDH12* and *FAT* have been related to SCZ and bipolar disorder (Redies *et al.*, 2012).

The CCK(1) receptor is responsible for regulation of serotonin neuron activity and maintenance of proper dopamine level in raphe nuclei (cluster of cell group localized in the brainstem). It also influences hormones and neurotransmitters regulation in hypothalamus. Due to its functions, CCK was suggested to be a target in the treatment of SCZ, mood disorders and drug addiction (Ballaz, 2017). Especially, the relation between CCK and dopaminergic circuits was proposed to play a role in the development of SCZ. The concentration of CCK was showed to be lowered in some brain regions of SCZ untreated patients, leading to the conclusion that CCK may display antipsychotic effects in sub-population of SCZ patients. Last, antipsychotic drug treatment can boost CCK and CCKR levels (Wang *et al.*, 1984).

All these pathways are well known to be involved in ASD as well as in SCZ and EOS reinforcing the idea that a link exist between these two disorders that should be better explored in the future (Curley and Lewis, 2012; Hoseth *et al.*, 2018; Kwan *et al.*, 2016; Panaccione *et al.*, 2013; Zhang *et al.*, 2014)

FIGURES

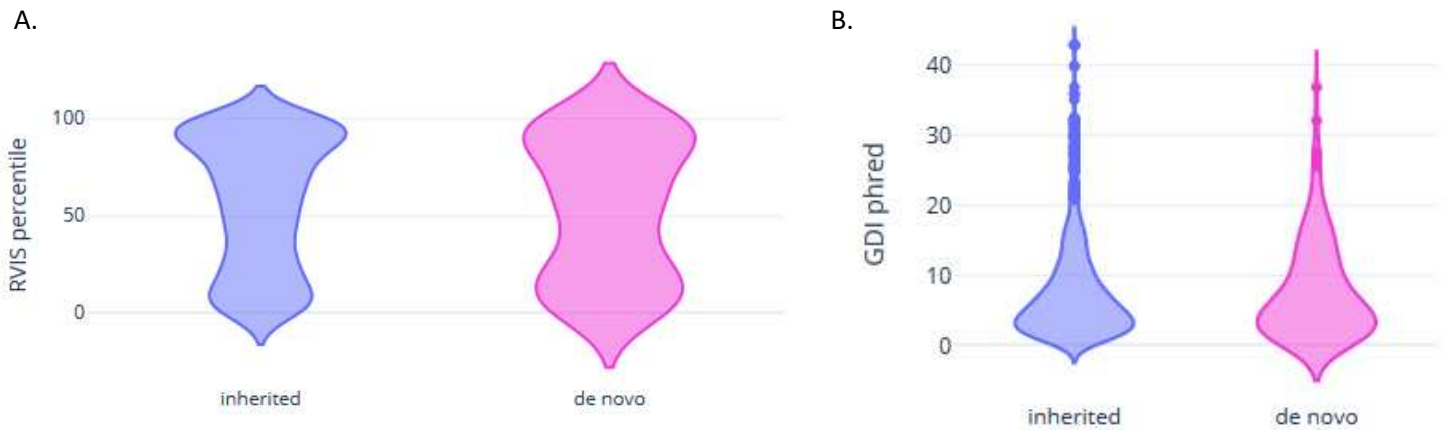


Figure 1. A. Violin plot showing the RVIS percentile. Lower percentile indicates more “intolerant” variants. Statistical analysis with two-tails Mann Whitney test did not reveal statistical differences between inherited and de novo mutations. B. Violin plot showing the GDI phred. Lower GDI values indicate genes more prone to harbour disease-causing mutations. Statistical analysis with two-tails Mann Whitney test did not reveal statistical differences between inherited and de novo mutations.

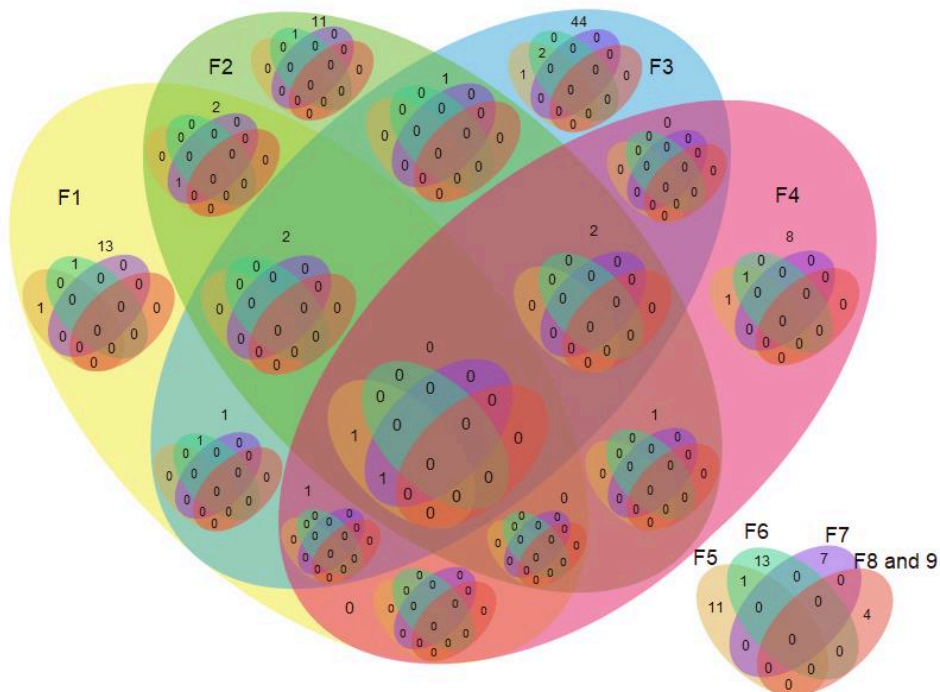


Figure 2. Venn diagram showing the genetic overlap between de novo mutations of the nine families. From technical reasons families 8 and 9 were combined together.

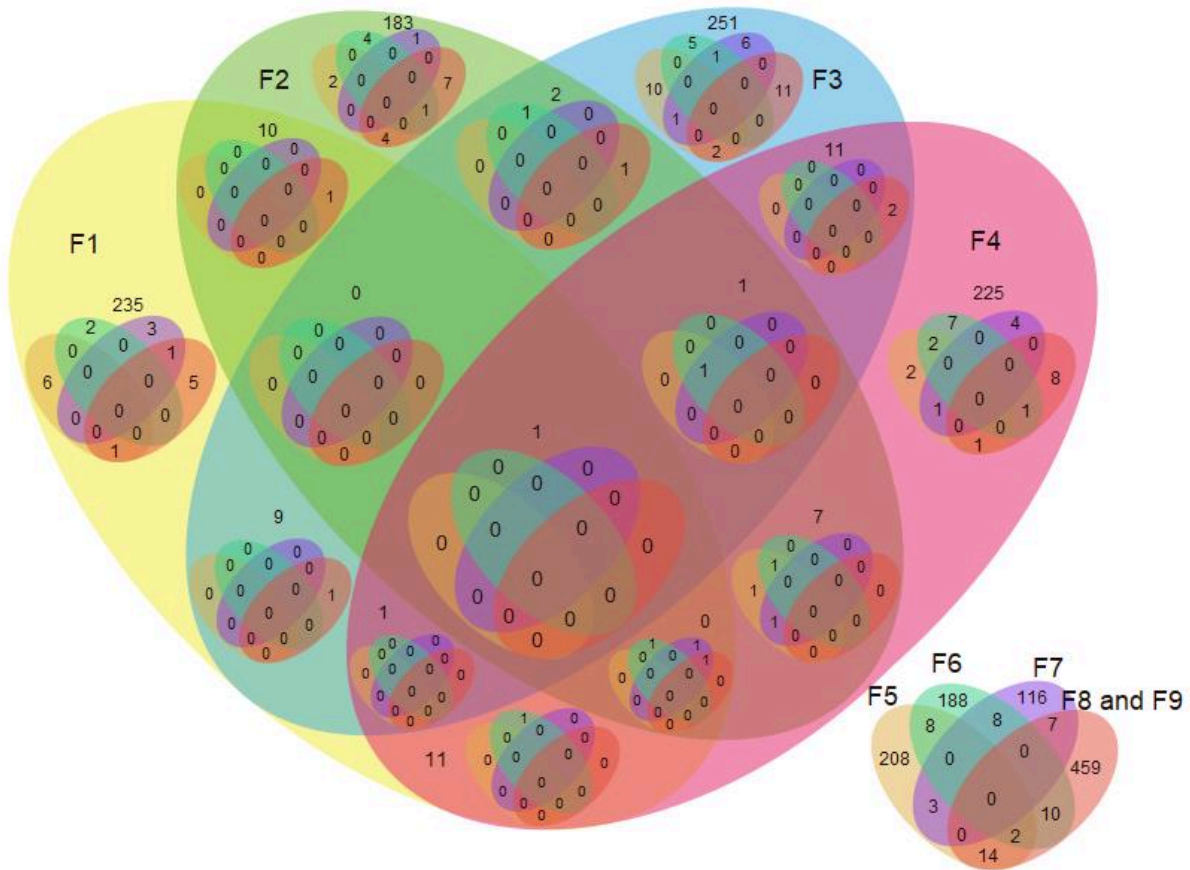


Figure 3. Venn diagram showing the genetic overlap between inherited mutations of the nine families. For technical reasons families 8 and 9 were combined together.

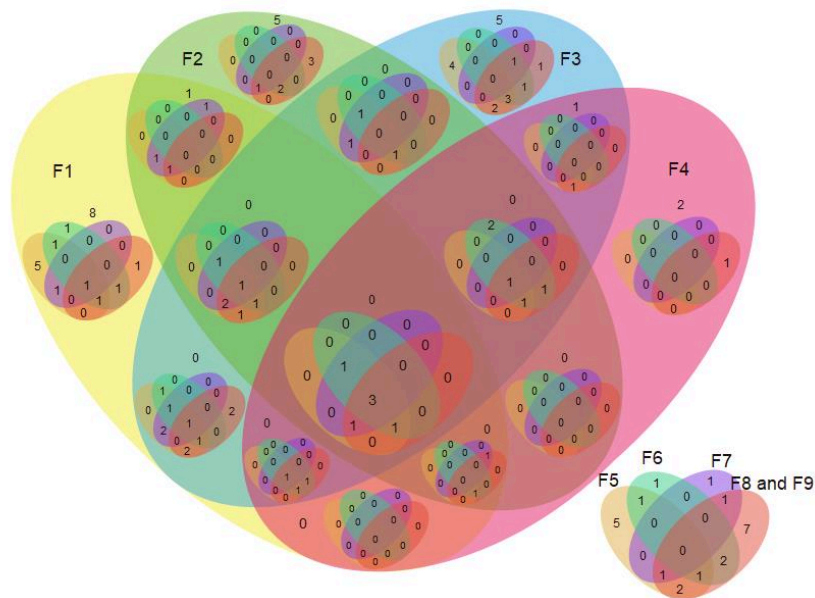


Figure 4. Venn diagram representing overlap between pathways of the nine families. For technical reasons, families 8 and 9 were combined together.

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Table 1 : Summarized Phenotypes of patients from families 1 to 9.

	Age	sex	Onset	IQ	ASD	ADHD
Family 1	20	F	17	96*	++++	
Family 2	13	F	8	62*		+
Family 3	13	F	5	53*	++++	
Family 4	19	F	16	n/a		
Family 5	20	M	12	47	++	+
Family 6	22	M	14	40		
Family 7	16	F	7	69*	++++	+
Family 8	14	M	7	71*	++++	+
Family 9	18	M	12	63*	+++	+

ADHD :Attention
Deficit
Hyperactivity
Disorder

ASD : Autism Spectrum Disorder

IQ : Intellectual Quotient (WISC)

onset : year of onset of schizophrenia

*heterogeneous IQ

n/a : not available

+, ++, +++, +++++ means 1, 2, 3 or 4 positive eras to ADI-R

Table 2. De novo variants in genes common for at least three families.

Chr	position	Gene	Ref seq	Protein variant	Variant type	Rs
Fam 1						
chr3	>75786942"	ZNF717	NM_001290209	R561I	missense	rs2918517
			NM_001128223	R611I		
			ENST00000422325	R611I		
			ENST00000478296	R561I		
			NM_001290208	R611I		
			ENST00000400845	R604I		
chr3	>75788158"	ZNF717	NM_001290209	L156V	missense	rs3009004
			ENST00000400845	L199V		
			ENST00000422325	L206V		
			NM_001128223	L206V		
			ENST00000478296	L156V		
			NM_001290208	L206V		
Fam2						
chr3	>75786175"	ZNF717	NM_001290208	E867*	nonsense	rs78135954
			ENST00000478296	E817*		
			ENST00000422325	E867*		
			ENST00000400845	E860*		
			NM_001290209	E817*		
			NM_001128223	E867*		
chr3	>75786687"	ZNF717	NM_001290208	L696H	missense	rs80214016
			ENST00000478296	L646H		
			NM_001290209	L646H		
			ENST00000400845	L689H		
			ENST00000422325	L696H		
			NM_001128223	L696H		
chr3	>75787199"	ZNF717	NM_001290209	H475Q	missense	
			NM_001128223	H525Q		
			NM_001290208	H525Q		
			ENST00000400845	H518Q		
			ENST00000478296	H475Q		
			ENST00000422325	H525Q		

Fam3

chr3	>75788068"	ZNF717	NM_001128223	F236V	missense	rs74357986
			ENST00000478296	F186V		
			ENST00000400845	F229V		
			NM_001290208	F236V		
			ENST00000422325	F236V		
			NM_001290209	F186V		

chr3	>75788199"	ZNF717	ENST00000422325	H192R	missense	rs77110669
			NM_001128223	H192R		
			NM_001290209	H142R		
			NM_001290208	H192R		
			ENST00000478296	H142R		
			ENST00000400845	H185R		

Fam4

chr3	>75786252"	ZNF717	NM_001290208	P841H	missense	rs79138891
			ENST00000422325	P841H		
			NM_001290209	P791H		
			NM_001128223	P841H		
			ENST00000400845	P834H		
			ENST00000478296	P791H		

chr3	>75787405"	ZNF717	NM_001290209	G407R	missense	rs73843014
			ENST00000400845	G450R		
			ENST00000478296	G407R		
			ENST00000422325	G457R		
			NM_001128223	G457R		
			NM_001290208	G457R		

chr3	>75787620"	ZNF717	ENST00000400845	H378R	missense	rs75737034
			NM_001290208	H385R		
			ENST00000422325	H385R		
			NM_001128223	H385R		
			ENST00000478296	H335R		
			NM_001290209	H335R		

Fam5

chr3	>75786041"	ZNF717	NM_001128223.1	S911X	frameshift	rs77747132&rs150497643
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Fam7

chr3	>75786035"	ZNF717	NM_001128223.1	F915FX	frameshift	rs149076283
chr3	>75787081"	ZNF717	NM_001128223.1	C565S	missense	rs77378861
chr3	>75787266"	ZNF717	NM_001128223.1	TG504TX	frameshift	rs776210532
chr3	>75788403"	ZNF717	NM_001128223.1	T124NX	frameshift	

Fam1

chr11	>1017444"	MUC6	NM_005961		Exon	
chr11	>1017498"	MUC6	NM_005961	S1768T	missense	
chr11	>1017746"	MUC6	NM_005961	L1685F	missense	rs78848170

Fam2

chr11	>1016989"	MUC6	NM_005961		Exon	
chr11	>1018419"	MUC6	NM_005961	T1461I	missense	

Fam3

chr11	>1017084"	MUC6	NM_005961	P1906L	missense	rs34649796
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Fam4

chr11	>1017498"	MUC6	NM_005961	S1768T	missense	
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Fam5

chr11	>1017443"	MUC6	NM_005961.2	TT1786T	Inframe deletion	
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RBMXL3

Fam1

chrX	>114425105"	RBMXL3	NM_001145346		exon deletion	
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Fam3

chrX	>114425105"	RBMXL3	NM_001145346		exon	
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deletion

Fam4

chrX >114425105" RBMXL3 NM_001145346 exon
deletion

ACAN

Fam2

chr15 >89399701" ACAN NM_001135 D1295E missense
NM_013227 D1295E

Fam3

chr15 >89399758" ACAN NM_013227 D1314E missense
NM_001135 D1314E

Fam4

chr15 >89400023" ACAN NM_013227 T1403A missense rs12899191
NM_001135 T1403A

PABPC1

Fam1

chr8 >101721839" PABPC1 NM_002568 V365L missense

Fam2

chr8 >101721933" PABPC1 NM_002568 exon
deletion

Fam3

chr8 >101719035" PABPC1 NM_002568 intron+splice
intron

FRG1

Fam1

chr4	>190878556"	FRG1	NM_004477	exon Deletion
Fm2				
chr4	>190864422"	FRG1	NM_004477	exon insertion
Fam3				
chr4	>190862999"	FRG1	NM_004477	intron deletion

Table 3. 10 first genes according to VarElect predictor.

Symbol	Description	Matched Phenotypes	Matched Phenotypes	-LOG10(P)	Score	Average Disease Causing Likelihood
KCNB1	Potassium Voltage-Gated Channel Subfamily B Member 1	schizophrenia, autism, intellectual, disability, neurodevelopmental	5	2,27	69,28	62,07%
HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1	schizophrenia, autism, intellectual, disability, neurodevelopmental	5	2,25	68,54	0,99%
BPTF	Bromodomain PHD Finger Transcription Factor	autism, intellectual, disability, neurodevelopmental	4	2,27	62,32	67,63%
HERC2	HECT And RLD Domain Containing E3 Ubiquitin Protein Ligase 2	schizophrenia, autism, intellectual, disability, neurodevelopmental	5	2,1	59,22	56,87%
CBS	Cystathionine Beta-Synthase	schizophrenia, autism, intellectual, disability, neurodevelopmental	5	1,52	34,34	54,73%
NOTCH4	Notch Receptor 4	schizophrenia, autism, intellectual, disability, neurodevelopmental	5	1,43	30,96	20,47%

FOLH1	Folate Hydrolase 1	schizophrenia, autism, intellectual, disability	4	1,19	19,64	30,23%
FLG	Filaggrin	autism, intellectual, disability, neurodevelopmental	4	1,11	16,91	0,09%
ASCL1	Achaete-Scute Family BHLH Transcription Factor 1	schizophrenia, intellectual, disability, neurodevelopmental	4	1,09	16,51	69,21%
ZNF148	Zinc Finger Protein 148	intellectual, disability, neurodevelopmental	3	1,16	16,04	92,24%

Table 4. Missense de novo mutations.

Chr	Position	Gene	Ref seq	Protein variation	Variation type	rs
chr1	>89449483"	RBMXL1	NM_019610 NM_001162536	K9N K9N	missense	rs74100106
chr1	>117146504"	IGSF3	NM_001007237 NM_001542	R456C R476C	missense	rs61786577
chr2	>96617102"	ENSG00000174501	ENST00000528268 ENST00000456556	E420K E420K	missense	
chr3	>75786942"	ZNF717	NM_001290209 NM_001128223 ENST00000422325 ENST00000478296 NM_001290208 ENST00000400845	R561I R611I R611I R561I R611I R604I	missense	rs2918517
chr3	>75788158"	ZNF717	NM_001290209 ENST00000400845 ENST00000422325 NM_001128223 ENST00000478296 NM_001290208	L156V L199V L206V L206V L156V L206V	missense	rs3009004

chr7	>100551035"	MUC3A	NM_005960	S539T	missense	rs62483695
chr8	>101721839"	PABPC1	NM_002568	V365L	missense	
chr11	>1017498"	MUC6	NM_005961	S1768T	missense	rs67507057
chr11	>1017746"	MUC6	NM_005961	L1685F	missense	rs78848170
chr15	>28447562"	HERC2	NM_004667	S2471P	missense	
chr16	>24788455"	TNRC6A	NM_014494	P122Q	missense	
chr17	>39346622"	KRTAP9-1	NM_001190460	C162S	missense	rs79470847
chr19	>56283297"	RFPL4AL1	NM_001277397 ENST00000341750	D43N D43N	missense	rs75204738
chr1	>117660667"	TRIM45	NM_025188	L404P	missense	
chr1	>145281656"	ENSG00000213240	ENST00000362074 ENST00000468030 NM_203458 ENST00000344859 ENST00000369340	T196S T196S T196S T196S T196S	missense	rs4649852
chr3	>75786687"	ZNF717	NM_001290208 ENST00000478296 NM_001290209 ENST00000400845 ENST00000422325 NM_001128223	L696H L646H L646H L689H L696H L696H	missense	rs80214016
chr3	>75787199"	ZNF717	NM_001290209 NM_001128223 NM_001290208 ENST00000400845 ENST00000478296 ENST00000422325	H475Q H525Q H525Q H518Q H475Q H525Q	missense	
chr6	>30996959"	MUC22	NM_001198815	E1251K	missense	rs115182197
chr6	>32714125"	HLA-DQA2	NM_020056 ENST00000374940	Q241R Q241R	missense	

chr7	>72413581"	POM121	NM_172020 NM_001257190	V752I V752I	missense	rs71554687
chr7	>72413593"	POM121	NM_001257190 NM_172020	Y756H Y756H	missense	rs71554688
chr7	>100550795"	MUC3A	NM_005960	T459I	missense	
chr9	>33385656"	AQP7	NM_001170	Q245L	missense	
chr11	>1018419"	MUC6	NM_005961	T1461I	missense	
chr11	>48387237"	ENSG00000176540	ENST00000319813	V261I	missense	rs72898877
chr15	>89399701"	ACAN	NM_001135 NM_013227	D1295E D1295E	missense	
chr2	>96604606"	ENSG00000174501	ENST00000456556	G535V	missense	rs80350011
chr3	>75786211"	ENSG00000227124	ENST00000422325 NM_001290209 NM_001290208 NM_001128223 ENST00000478296 ENST00000400845	S855P S805P S855P S855P S805P S848P	missense	rs77715040
chr3	>75786759"	ENSG00000227124	ENST00000422325 NM_001290208 NM_001290209 ENST00000478296 ENST00000400845 NM_001128223	K672R K672R K622R K622R K665R K672R	missense	rs75759940
chr3	>75788068"	ZNF717	NM_001128223 ENST00000478296 ENST00000400845 NM_001290208 ENST00000422325 NM_001290209	F236V F186V F229V F236V F236V F186V	missense	rs74357986
chr3	>75788199"	ENSG00000227124	ENST00000422325 NM_001128223 NM_001290209 NM_001290208	H192R H192R H142R H192R	missense	rs77110669

			ENST00000478296	H142R		
			ENST00000400845	H185R		
chr5	>156479571"	HAVCR1	NM_012206	M158I	missense	rs75340804
			NM_001173393	M158I		
			NM_001099414	M158I		
chr7	>72081774"	ENSG00000254184	ENST00000435769	C557R	missense	rs3015854
chr7	>72413581"	POM121	NM_172020	V752I	missense	rs71554687
			NM_001257190	V752I		
chr7	>72413593"	POM121	NM_001257190	Y756H	missense	rs71554688
			NM_172020	Y756H		
chr7	>100677279"	MUC17	NM_001040105	S861T	missense	rs76184171
chr7	>100677285"	MUC17	NM_001040105	G863E	missense	rs74852422
chr7	>100678481"	MUC17	NM_001040105	T1262S	missense	rs73168389
chr7	>100678560"	MUC17	NM_001040105	T1288K	missense	rs77199586
chr7	>100678568"	MUC17	NM_001040105	T1291S	missense	rs75492258
chr7	>100682624"	MUC17	NM_001040105	M2643L	missense	
chr11	>1017084"	MUC6	NM_005961	P1906L	missense	rs34649796
chr11	>49208267"	FOLH1	NM_001014986	R190W	missense	rs75111588
			NM_001193472	R175W		
			NM_001193471	R175W		
			NM_004476	R190W		
chr12	>11183722"	TAS2R31	NM_176885	F71L	missense	rs78562467
chr14	>60074085"	RTN1	NM_206852	A63T	missense	
			NM_021136	A631T		
chr15	>89399758"	ACAN	NM_013227	D1314E	missense	
			NM_001135	D1314E		
chr17	>39274364"	KRTAP4-11	NM_033059	R68S	missense	rs425784
chr21	>11049596"	BAGE2	NM_182482	C102Y	missense	rs28571918
			NM_182481	C102Y		

chr21	>44483184"	CBS	NM_001178008 NM_001178009 NM_000071	I278T I278T I278T	missense	rs5742905
chr1	>17263214"	CROCC	NM_014675	R347W	missense	
chr3	>75786252"	ZNF717	NM_001290208 ENST00000422325 NM_001290209 NM_001128223 ENST00000400845 ENST00000478296	P841H P841H P791H P841H P834H P791H	missense	rs79138891
chr3	>75787405"	ZNF717	NM_001290209 ENST00000400845 ENST00000478296 ENST00000422325 NM_001128223 NM_001290208	G407R G450R G407R G457R G457R G457R	missense	rs73843014
chr3	>75787620"	ENSG00000227124	ENST00000400845 NM_001290208 ENST00000422325 NM_001128223 ENST00000478296 NM_001290209	H378R H385R H385R H385R H335R H335R	missense	rs75737034
chr5	>40853031"	CARD6	NM_032587	R533C	missense	
chr6	>32549588"	HLA-DRB1	NM_002124 NM_001243965	S133L S124L	missense	rs117994013
chr6	>32714164"	ENSG00000237541	ENST00000374940 NM_020056	L254P L254P	missense	rs115121776
chr11	>1017498"	MUC6	NM_005961	S1768T	missense	rs67507057
chr15	>89400023"	ACAN	NM_013227 NM_001135	T1403A T1403A	missense	rs12899191
chr16	>70954691"	HYDIN	NM_001270974	K2530E	missense	rs1798528
chr19	>44891043"	ZNF285	NM_001291491 NM_001291488 NM_152354	P300Q P462Q P455Q	missense	rs77661661

			NM_001291489	P455Q		
			NM_001291490	P300Q		
chr1	>152278856"	FLG	NM_002016.1	S2836R	missense	rs11582087
chr10	>47087078"	NPY4R	NM_005972.5	A99S	missense	
			NM_001278794.1	A99S		
chr17	>39471763"	KRTAP17-1	NM_031964.1	G47D	missense	rs74252500
chr19	>55105722"	LILRA1	NM_001278318.1	V5L	missense	
			NM_006863.3	V5L		
			NM_001278319.1	V5L		
chr19	>55358686"	KIR2DS4	NM_012314.3	K247N	Missense	rs112697729
chrX	>55172659"	FAM104B	NM_138362.3	I69T	missense	rs5018687
			NM_001166702.1	I66T		
			NM_001166699.1	I70T		
			NM_001166700.1	I70T		
			NM_001166703.1	I68T		
			NM_001166701.1	I69T		
chr4	>191003044"	DUX4L4	NM_001177376.2	R258W	missense	rs370569001
chr10	>46967616"	SYT15	NM_031912.4	S154L	missense	rs74128855
			NM_181519.2	S154L		
chr16	>70891640"	HYDIN	NM_001270974.1	K4088R	missense	rs1774416
chr16	>70908771"	HYDIN	NM_001270974.1	P3537A	missense	
chr16	>70926334"	HYDIN	NM_001270974.1	T3116R	missense	rs1774423
chr16	>90095620"	C16orf3	NM_001214.3	V44A	missense	rs61740023
chr19	>17397483"	ANKLE1	NM_001278444.1	V639L	missense	rs367668712 &rs60338123
chr20	>47991056"	KCNB1	NM_004975.2	S347R	missense	
chr3	>75787081"	ZNF717	NM_001128223.1	C565S	missense	rs77378861
chr11	>1267825"	MUC5B	NM_002458.2	A3239P	missense	rs200304875
chr11	>1267852"	MUC5B	NM_002458.2	A3248T	missense	rs201342042

chr11	>1856582"	SYT8	NM_138567.3	C65G	missense
chr19	>9271435"	ZNF317	NM_020933.4 NM_001190791.1	R372C R340C	missense

APPENDIX

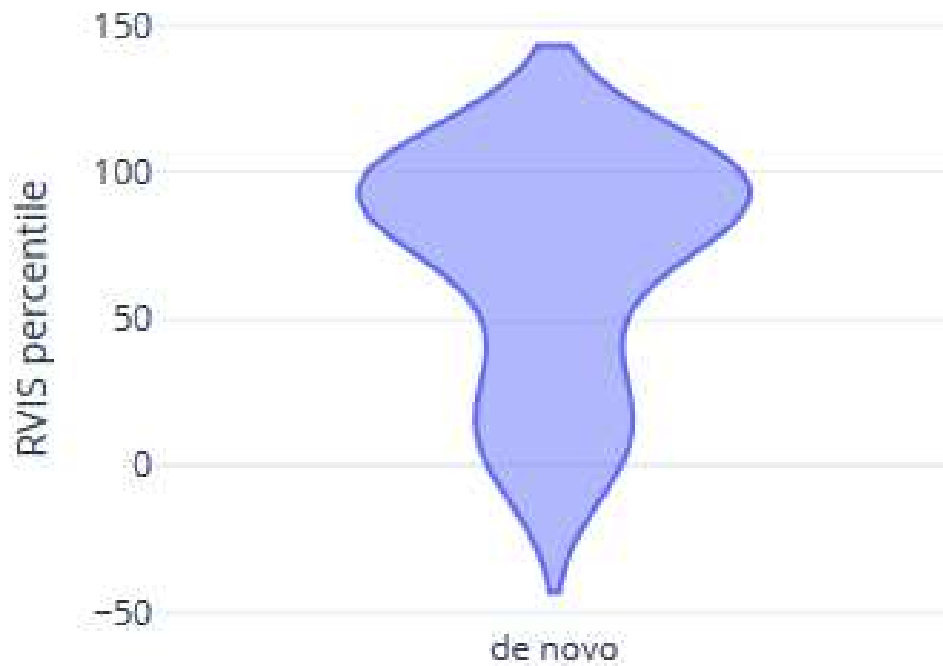
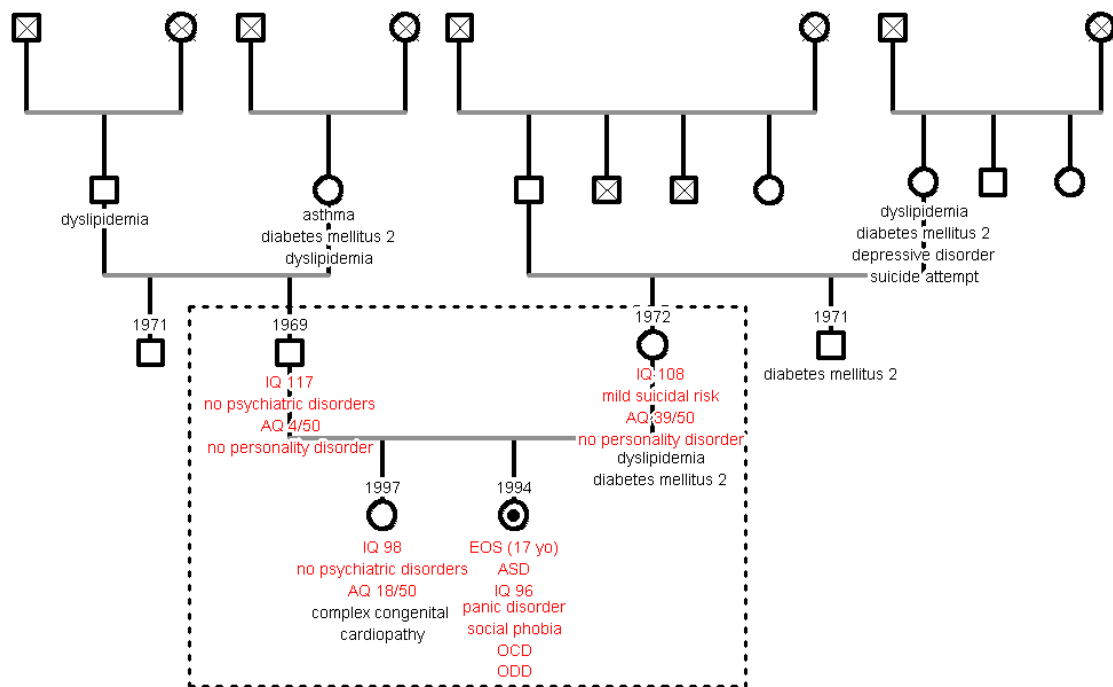


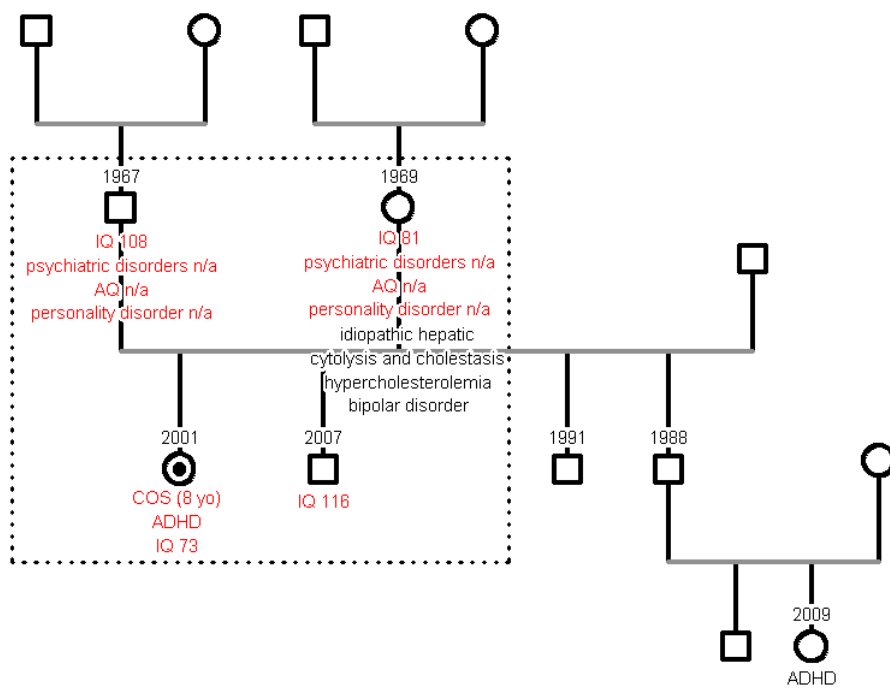
Figure 1. Violin plot representing RVIS percentile for missense mutations. Lower percentile indicates more “intolerant” variants. Our data showed opposite results to one previously published (Ambalavanan et al., 2016). The genes with missense mutations are predicted to belong to a group of more “tolerant” genes.

Figure 2. Trees of families 1 to 9 exhibiting principal results of standardized neuropsychological and psychiatric assessments.

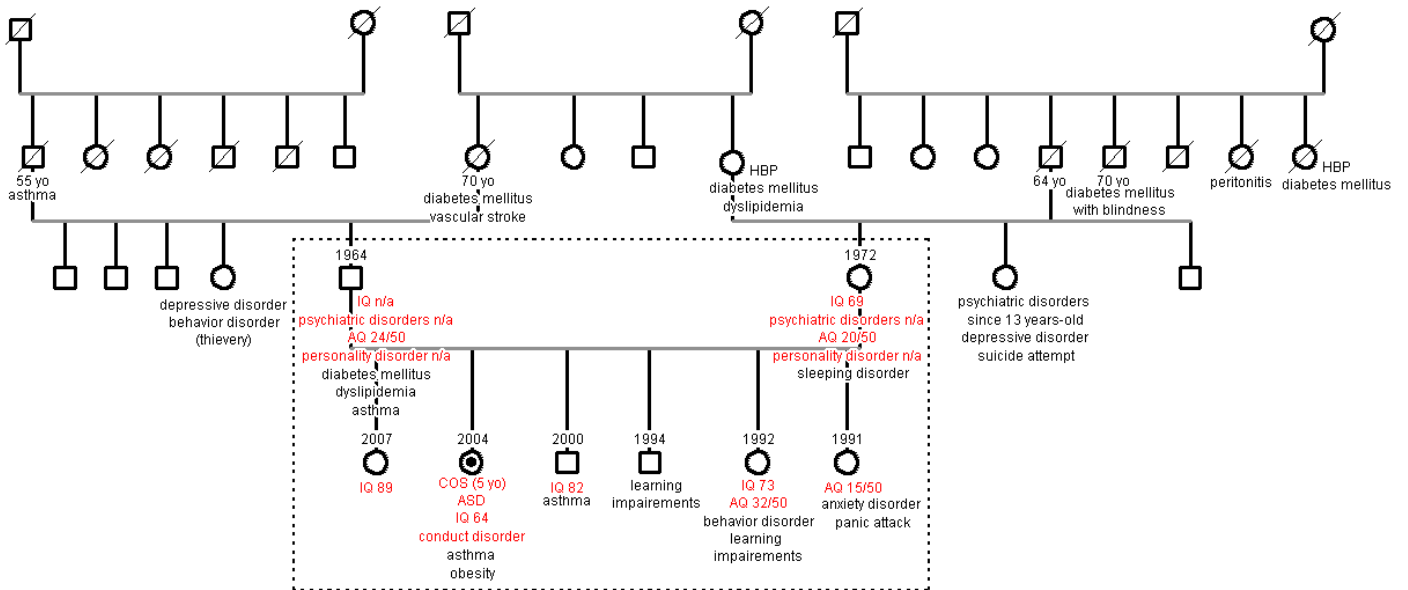
Family 1 :



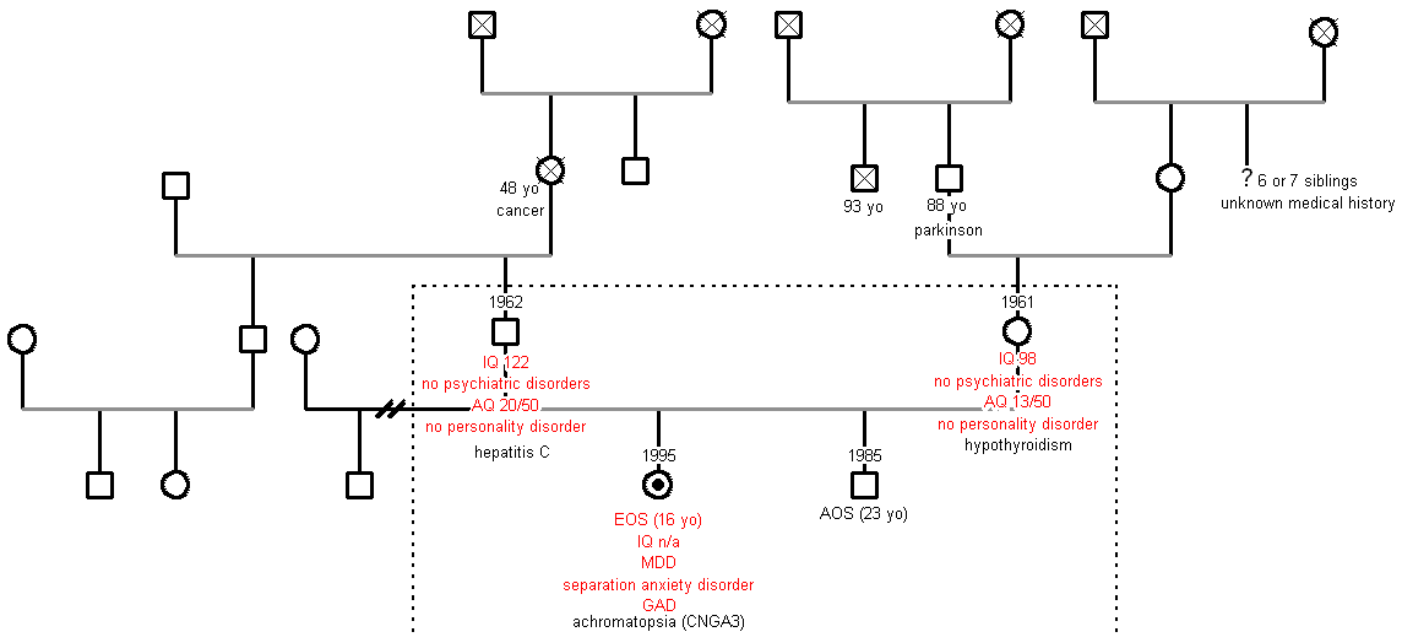
Family 2 :



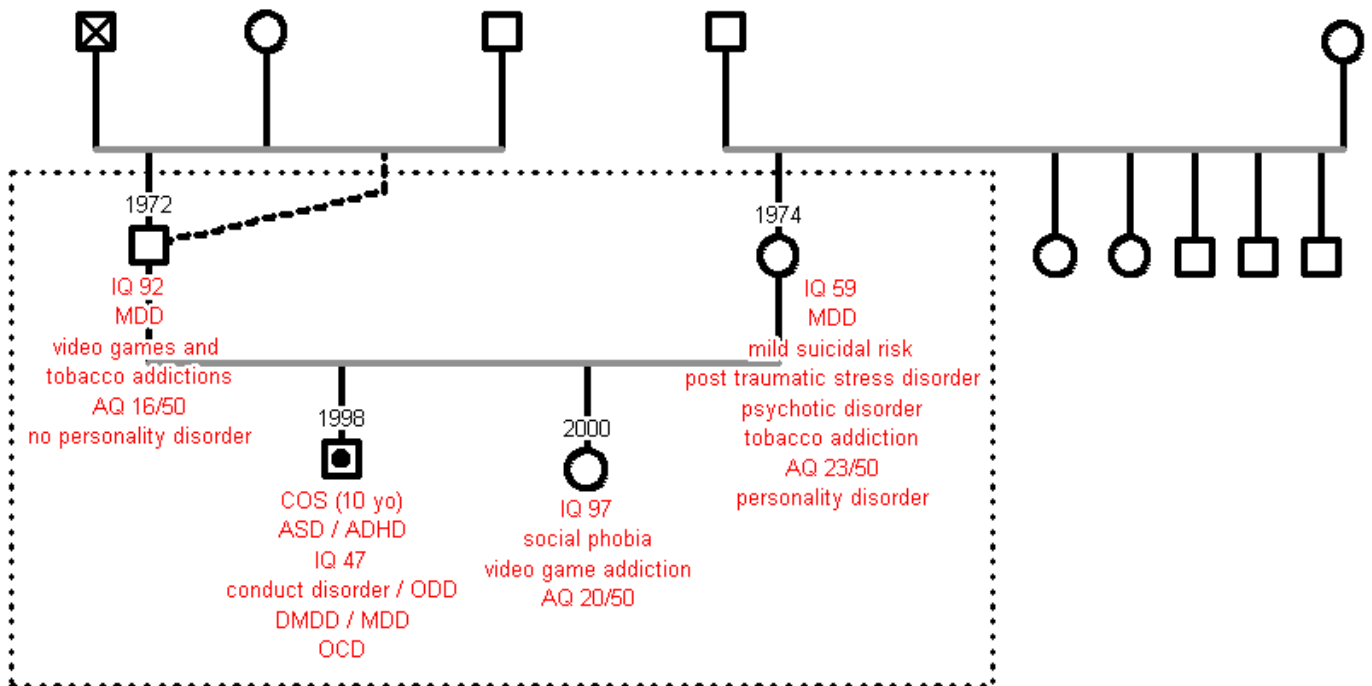
Family 3:



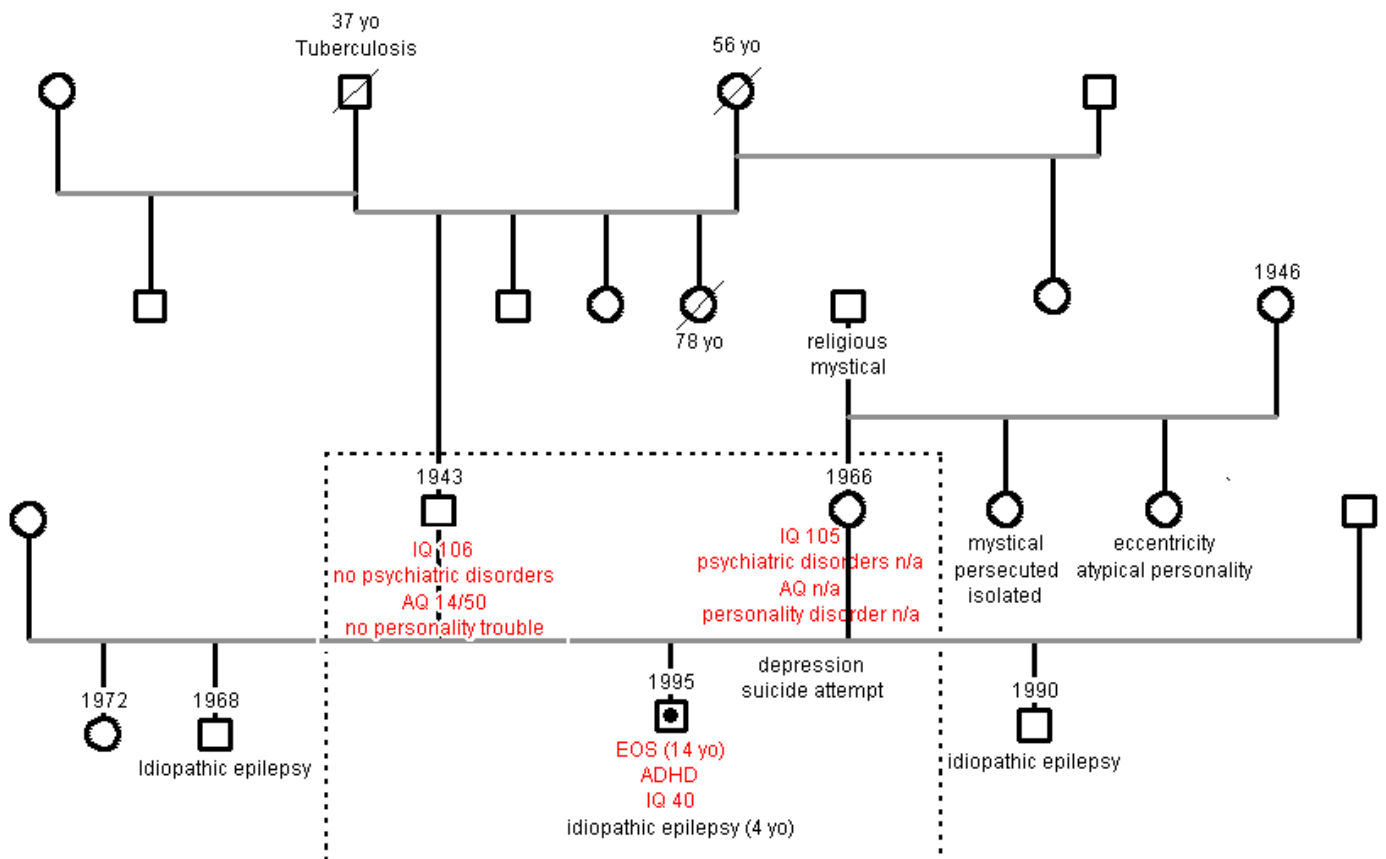
Family 4:



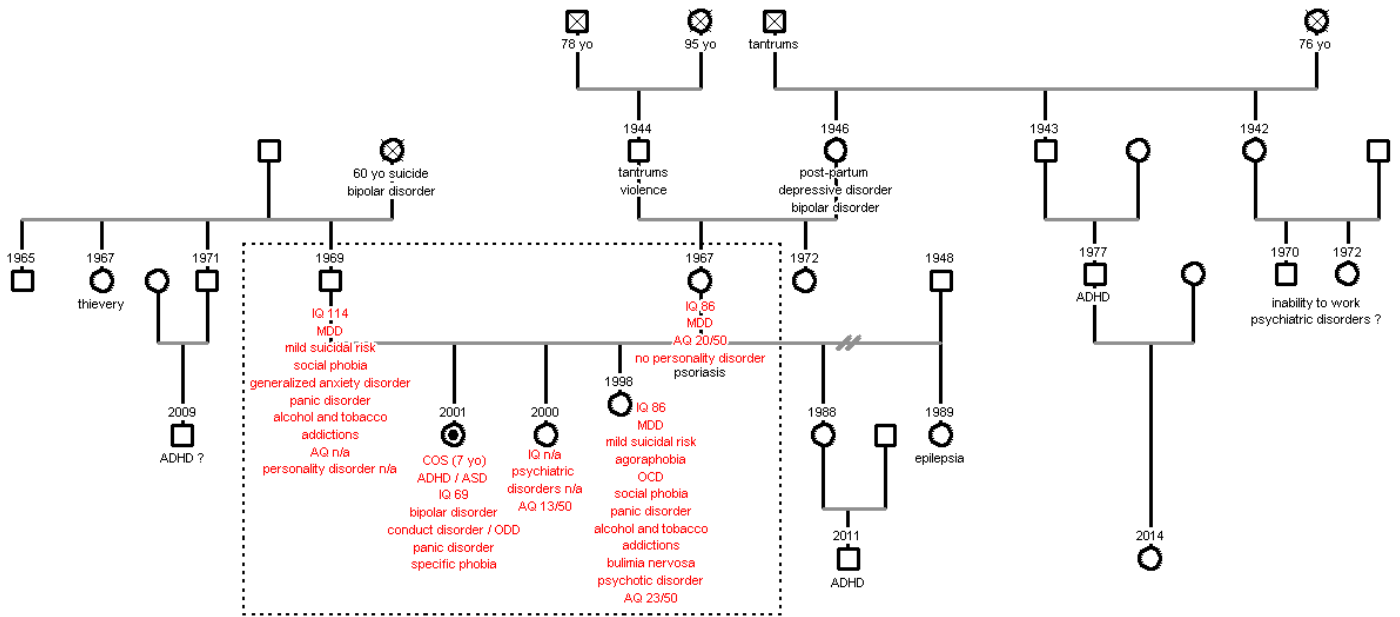
Family 5:



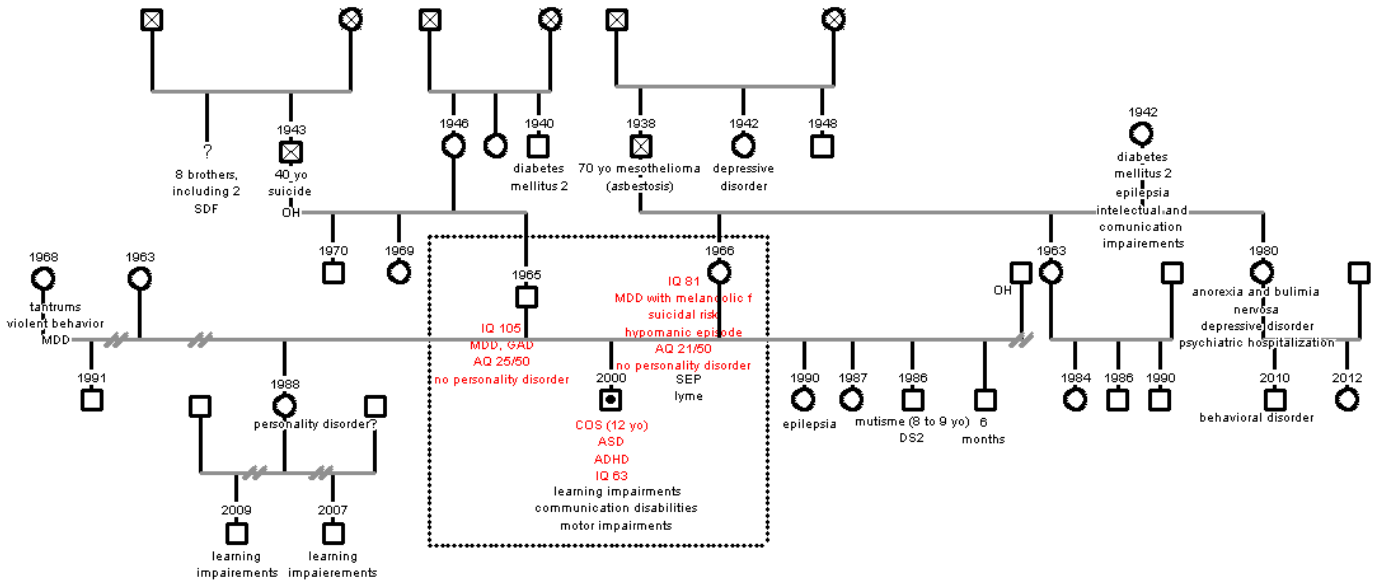
Family 6:



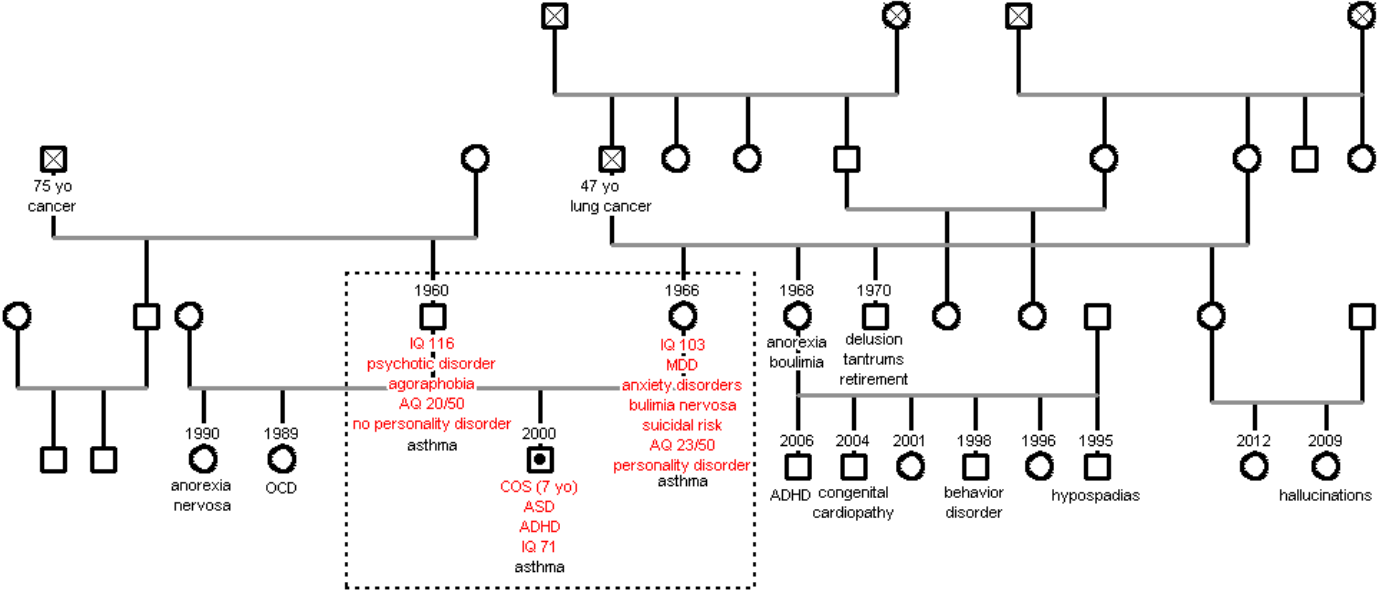
Family 7:



Family 8:



Family 9:



Publication 1

BMJ Open Exploration and characterisation of the phenotypic and genetic profiles of patients with early onset schizophrenia associated with autism spectrum disorder and their first-degree relatives: a French multicentre case series study protocol (GenAuDiss)

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ABSTRACT

Introduction Early-onset schizophrenia (EOS) is a rare and severe condition. A higher rate of neurodevelopmental abnormalities, such as intellectual or communication impairments as well as attention deficit hyperactivity disorder, is observed in EOS compared with adult-onset schizophrenia. Early signs of autism spectrum disorders (ASD) are present in about 30% of patients. Genetic abnormalities, including copy number variations, are frequent in neurodevelopmental disorders and have been associated to ASD pathophysiology. Implicated genes encode proteins involved in brain development, synapses morphology and plasticity and neurogenesis. In addition, an increasing number of genetic abnormalities are shared by EOS and ASD, underlying the neurodevelopmental hypothesis of EOS. The main objective of our study is to identify disease-causing genetic mutations in a cohort of patients affected by both EOS and ASD. Special attention will be paid to genes involved in neurodevelopmental pathways.

Methods and analysis We describe a multicentric study in a paediatric population. The study started in April 2014. Inclusion criteria are: age 7–22 years, diagnosis of EOS with comorbid ASD and IQ >50; Parents and siblings are also enrolled. We perform psychiatric assessments (Mini International Neuropsychiatric Interview, Kiddie Schedule for Affective Disorders and Schizophrenia -Present and Lifetime Version, Positive and Negative Syndrome Scale and Scale for the Assessment of Negative Symptoms) together with neurocognitive evaluations (IQ, Trail Making Test A/B and verbal fluency). Then, we study variants of the coding part of DNA (exome), using next-generation sequencing process on trio (mother, father and child). Bioinformatics tools (RVIS and PolyPhen-2) are used to prioritise disease-causing mutations in candidate genes. The inclusion period will end in November 2019.

Ethics and dissemination The study protocol was approved by the Local Ethic Committee and by the French

Strengths and limitations of this study

- University Child Psychiatry Department has recognised specialists in rare and severe disorders such as Early-Onset Schizophrenia (EOS).
- Innovative clinical research protocol in the field of child psychiatry associating psychiatric assessments, neuropsychological explorations and DNA next-generation sequencing of patients as well as their parents and siblings.
- Collaboration with research may provide new understanding of the molecular mechanisms of the condition and could provide biomarkers for diagnosis and treatment.
- Clinical research very challenging in EOS patients and families (severity of illness and impact on individual and family life), with limited sample size (rare condition and regional enrolment) and difficulties in involving both parents (separation, conflict, death and so on).
- Exome analysis may miss causative mutations in non-coding part of the genome.

National Agency for Medicines and Health Products Safety. All patients signed informed consent on enrolment in the study. Results of the present study should help to unravel the molecular pathology of EOS, paving the way for an early therapeutic intervention.

Trial registration number NCT0256552; Pre-results.

INTRODUCTION

Early-onset schizophrenia (EOS) is a rare (1/5000 to 1/20 000) and severe chronic psychiatric condition defined by an onset of schizophrenia-positive symptoms (delusions,

hallucinations and disorganised speech or behaviour) before the age of 18.^{1 2} Onset before 13 years of age is called childhood-onset schizophrenia (COS).^{3 4}

A higher rate of neurodevelopmental abnormalities is observed in EOS compared with adult-onset schizophrenia (AOS), and patients typically have frequent deficits in cognition, communication or neuromotor impairments associated with attention deficit, hyperactivity disorder (ADHD) or autism spectrum disorder (ASD).^{5–7} Furthermore, 28% of patients with COS in the US cohort of the National Institute of Mental Health Child Psychiatry Branch met criteria for comorbid ASD,⁶ and >80% of children with schizophrenia or schizoaffective disorder might present comorbid ADHD.⁷ In addition, EOS (including COS) is a complex disorder related to other neurodevelopmental disorders, and it represents a real challenge for clinical diagnosis with,⁴ to date, no objective test based on genetics. Therefore, it is critical to conduct a rigorous and homogeneous phenotypic characterisation by using international classifications such as International Classification of Diseases (ICD) or Diagnostic and Statistical Manual of Mental Disorders (DSM)^{8–10} and by using standardised and internationally validated psychiatric categorical assessments.

A few genetic studies of EOS are reported, due to the very low prevalence^{1 2} and to the nosographic difficulties which made it difficult to obtain a consensual clinical definition of this disorder and to carry out aetiological studies. Interestingly, DSM, Fifth Edition (DSM-5) classification provides clarification in this area with schizophrenia no longer excluding the diagnosis of ASD and formalising the overlap between these two disorders. On the other hand, DSM-5 does not recognise the specificities of EOS compared with adult onset schizophrenia.¹⁰

Submicroscopic cytogenetic abnormalities, such as disease-related copy number variations (CNVs), are more frequent in COS than in AOS (about 11%^{11 12} vs 2%–5%, $p < 0.0001$ ¹²). A high heritability rate of COS (>80%) has also been suggested in early adoption/twin studies and has been confirmed by familial aggregation studies.^{13 14} The fact that genome-wide association studies failed to yield a genome-wide significant result in patients with AOS appears to argue in favour of a polygenic basis of EOS with hundreds of genes with small individual effects being involved.¹⁵ In EOS pathogeny, recent studies also demonstrated the involvement of mutations in *NRXN1* (2p16.3) and *UPF3B* (Xq24), crucial for development and function of the brain.^{12 16}

Next-generation sequencing (NGS) is a process to determine the entire DNA sequence of a given individual with unprecedented throughput, scalability and speed. Whole-exome sequencing (WES), a NGS method, corresponds to an exhaustive analysis of the exome (the coding part of the genome) thus helping to understand complex neurodevelopmental disorders such as EOS.¹⁷ To our knowledge, until now, only two studies using WES have been published in the field of EOS. Smedemark-Margulies and colleagues described de novo heterozygous mutation

(c.385G>A) in the *ATPIA3* gene (19q13.2).¹⁸ Another cohort study using WES identified 20 de novo variants in 17 COS probands (rate: 1.17)¹⁹ in genes previously linked to neuronal function or to psychiatric disorders.¹⁹ These arguments (phenotypic overlap with other neurodevelopmental disorders, high heritability, disease-related CNVs and de novo SNPs rates) strongly support the neurodevelopmental basis of schizophrenia²⁰ particularly of the early onset subclass. In addition, our trio approach, both at the clinical and genetic level, will help to better understand transgenerational inheritance and to differentiate de novo mutations from inherited ones.

METHODS AND ANALYSIS

Objectives

The main objective of this study is to identify disease causing mutations primarily in genes involved in neurodevelopmental pathways in a cohort of patients affected by EOS.

The secondary objectives are the characterisation of the clinical and neurocognitive profiles of patients and their first-degree relatives.

Trial design

GenAuDiss is a translational multicentre study. Patients with EOS were initially recruited through the 2011–2013 Interregional Hospital Clinical Research Program (NCT01512641) whose main goal was to estimate the prevalence of dissociative disorders in a population of children in child psychiatry care or medico-education structures. A total of 6 patients and 14 first-degree relatives have been enrolled from this completed programme. In addition, an enrolment in the present study is offered to inpatients and outpatients corresponding to the inclusion criteria of the investigator, Child and Adolescent Psychiatry (CAP) centres, as well as their first-degree relatives. Participants are included either directly at the study sites or after referral by child and adolescent psychiatrists of the Provence-Alpes-Côte d'Azur (PACA) region.

The investigator centres of the GenAuDiss are the Children's Hospitals of Nice CHU-Lenval, the Marseille University Hospital, the Hospital La Fontonne (Antibes) and the Hospital Bonnet (Frejus). To date, 13 patients and 31 first-degree relatives (44 subjects in total) have been included in the GenAuDiss study protocol. At inclusion (V1), clinical assessments (both psychiatric and neurocognitive) are performed in patients as well as first-degree relatives (parents and siblings). Blood test are performed in a second visit (V2) within 6 months. Study procedure, visits and inclusion criteria are presented in [table 1](#).

Inclusion criteria

The study is proposed to children and adolescents presenting EOS with comorbid ASD and their first-degree relatives.

Major inclusion criteria for patients:

- ▶ Age of 7–22 years.

Table 1 GenAuDiss study procedure and evaluation criteria

Patient	V0: Selection	V1: Inclusion	V2
Inclusion criteria	+		
Informed consent	+		
Medical history+genealogical tree		+	
Psychiatric evaluation			
K-SADS-PL psychotic disorders	+		
ADI-R	+		
K-SADS-PL (full) (DSM 4/5)		+	
SANS		+	
PANSS		+	
Neuropsychological evaluation			
WISC-IV (abridged version)	+		
IQ (full)*†		+	
Verbal fluency		+	
TMT A/TMT B		+	
Genetic testing			
Blood tests			+
Siblings/Parents	V0: selection	V1: Inclusion	V2
Inclusion criteria	+		
Informed consent	+		
Medical history		+	
Psychiatric evaluation			
K-SADS-PL (full version) or MINI/MINI-S (DSM 4/5)*‡		+	
AQ		+	
TCI 226 (parents only)		+	
Neuropsychological evaluation			
IQ (abridged version) *†		+	
Genetic testing			
Blood tests			+

*Clinical tools are selected dependent on patient's age.

†Neurocognitive tools: < 7: WPPSI; 7-16: WISC; > 16: WAIS.

‡Psychiatric tools: < 17: K-SADS-PL; > 16: MINI.

ADI-R, Autism Diagnostic Interview-Revised; AQ, Autism Quotient; IQ, intelligence quotient; DSM, Diagnostic and Statistical Manual of Mental Disorders; K-SADS-PL, Kiddie Schedule for Affective Disorders and Schizophrenia-Present and Lifetime Version; MINI-S, Mini International Neuropsychiatric Interview-Simplified; PANSS: Positive and Negative Syndrome Scale; SANS, Scale for the Assessment of Negative Symptoms; TCI, Temperament and Character Inventory; TMT, Trail Making Test; WAIS, Wechsler Adult Intelligence Scale; WISC, Wechsler Intelligence Scale for Children; WPPSI, Wechsler Preschool and Primary Scale of Intelligence.

- ▶ Diagnosis of EOS (onset before age 18), diagnosed by the semistructured interview *Kiddie Schedule for Affective Disorders and Schizophrenia-Present and Lifetime Version* (K-SADS-PL) psychosis section.

- ▶ Diagnosis of ASD (*Autism Diagnostic Interview-Revised* (ADI-R).
- ▶ IQ ≥ 50 at *Wechsler Intelligence Scale for Children* (WISC-IV, abridged version).

Major inclusion criteria for parents and siblings:

- ▶ First-degree biological relationship (no adoption) with the index patient.

Exclusion criteria

- ▶ Refusal or withdrawal of consent.
- ▶ Children without verbal language.
- ▶ Adults protected by law (relatives).

Measures

Quantitative and qualitative measures are assessed during the study (summarised in table 1):

- ▶ Clinical parameters from medical history (including pregnancy, birth and so on) and biographic parameters, types and dates of significant life events including trauma and environmental exposures (eg, drugs and substances).
- ▶ Clinical parameters from physical examination (eg, body weight, body mass index, arterial pressure).
- ▶ Semistructured interviews to assess main diagnosis at inclusion (K-SADS-PL,²¹ ADI-R,²² Mini International Neuropsychiatric Interview (MINI)^{23 24}).
- ▶ Clinical heteroassessments with specific rating scales (WISC-IV²⁵ or -V,²⁶ Positive and Negative Syndrome Scale (PANSS),²⁷ Scale for the Assessment of Negative Symptoms (SANS)²⁸ Trail Making Test (TMT) A, TMT B and verbal fluency).
- ▶ Self-report questionnaires (Cloninger's Temperament and Character Inventory (TCI) 226 and^{29 30} Baron Cohen's Autism Quotient (AQ)³¹);
- ▶ Genetic analysis (including DNA testing for fragile-X syndrome, high-resolution karyotype, DNA CNVs from CGH array, DNA SNPs from exome sequencing).

Data collection process

Each investigator at study sites is responsible for collecting and entering data on the CRF paperwork at the time of the visit and for realising quality check of the data and regulation requirements. Results will be manually entered in the database by the clinical research assistants at the main investigator study centre. In order to avoid missing data or filling errors, a double-check will be carried out before freezing of the database. Data extraction and analysis of the database will be performed by the Department of Clinical Research and Innovation (DRCI) of the Nice University Hospital.

Statistical analysis

Statistical analysis will be conducted by the DRCI of the Nice University Hospital. A descriptive analysis of all collected parameters will be performed. For continuous variables (eg, age and weight), indicators such as the mean, SD and range values will be calculated. Categorical data will be presented by means of frequency (n, %). Changes in the parameters and in the distribution

of categorical variables from baseline to follow-up points will be tested with univariate analysis using appropriate testing according to the type of variable and distribution. In addition, multivariate analysis will be carried out to investigate putative relationships between variables. Level of significance for the p value was fixed at <0.05.

Outcomes

The main objective is to expand the repertoire of the genetic abnormalities associated to EOS phenotypes by performing:

- ▶ WES on trio (mother, father and child).
- ▶ Bioinformatics analysis and variant prioritisation using validated tools (RVIS and Polyphen2)^{32 33} and strategies in accordance with international guidelines.³⁴

The secondary objectives of the GenAuDiss study are the characterisation of the clinical and neurocognitive profile of patients and their first-degree relatives.

Clinical profile:

- ▶ K-SADS-PL for DSM IV-TR/DSM-5 comorbid psychiatric diagnosis of patients and minor siblings.
- ▶ MINI assessments for DSM IV-TR/DSM five psychiatric diagnosis of parents and major siblings.
- ▶ Evaluation of the intensity of positive and negative symptoms, general psychopathology and subtypes of schizophrenia using PANSS/SANS.
- ▶ Personality dimensional test using computerised version of TCI 226 (Cloninger) in parents.
- ▶ Autistic traits using AQ (Baron-Cohen) in siblings and parents.

Neurocognitive profile:

- ▶ Evaluation of executive and attentional functions of patients using Trail Making Test (TMT) A and B and verbal fluency.
- ▶ WISC-IV or WISC-V, full version in patient and WISC-IV abridged version in minor siblings; Wechsler Adult Intelligence Scale-III in a short form in adult siblings and parents.^{35 36}

Patient and public involvement

Results will systematically be transmitted with patients and legal caregivers. Results will also be discussed with the referent psychiatrist of child psychiatry care and/or medico-education structures. Patients have not been directly evolved in the study design. Nevertheless, our clinical experience and collaboration with families and care structures helped us to design this study, especially the clinical evaluation protocol.

DISCUSSION

This project is based on a translational cross-sectional study protocol that aims to gain further understanding of the pathogenic molecular mechanisms of EOS. In fact, even if genomics is more and more often used for the diagnosis of human diseases including psychiatry,³⁷ the pathophysiology of EOS still needs to be elucidated. Importantly, to date, contrary to other neuropsychiatric

pathologies (eg, Alzheimer's disease)³⁸ only two studies describe the WES genetic approach in patients with EOS.^{18 19}

Furthermore, the known genetic heterogeneity of adult onset schizophrenia spectrum disorders imposes to build up models that consider the interplay between several factors. In fact, the 'two-hit' hypothesis proposes to consider the impact of at least two genetic or clinical factors (CNV+CNV, CNV+SPN or CNV+epigenetic impact of trauma and so on).³⁹

Perspectives

Mutated genes will be classified according to their pathogenic level, and the most likely disease causing candidate mutations will be studied by a research team expert in neuroscience (Laboratory 'RNA Metabolism and Neurodevelopmental Disorders', Dr. Barbara Bardoni, IPMC, CNRS, France). Further study of gene expression levels and in vivo cell activity in animal models will help predict the impact of the mutation on the protein structure and function. Finally, better understanding of EOS pathophysiology should help to develop objective diagnosis tools, early intervention and targeted treatment strategies.

Limitations of the study

EOS is a rare (1/5000 to 1/20 000)^{1 2} and severe mental disorder with a major impact on patients, families and care givers. The follow-up as well as inclusions in research protocols are therefore very challenging and need an experienced multidisciplinary team. Social precariousness, geographical distance from the hospital and difficulties of reach both parents (eg, because of separation, conflict or death) might be significant obstacles limiting sample size.

ETHICS AND DISSEMINATION

The study protocol was authorised by the French National Agency for Medicines and Health Products Safety (ANSM 2013-A01699-36). All patients, their parents and siblings signed informed consent on enrolment in the study.

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Contributors AF, ST, FA, ED, BB: contributed to the conceptualisation of the GenAuDiss study and preparation of grant application. AF, ED, GL, FP, M-LM: clinical assessments of the patients. AF, BB, TM, MD: genetic analysis. AF and ST: drafting the first version of the manuscript. All authors have revised first version of the manuscript critically for important intellectual content and approved the final version; involved in the interpretation of results. .

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Competing interests None declared.

Patient consent Obtained.

Ethics approval 'Sud Méditerrané V' (number 14.002)

Provenance and peer review Not commissioned; peer reviewed for ethical and funding approval prior to submission.

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Publication 2



Childhood-Onset Schizophrenia: A Systematic Overview of Its Genetic Heterogeneity From Classical Studies to the Genomic Era

OPEN ACCESS

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Childhood-onset schizophrenia (COS), a very rare and severe chronic psychiatric condition, is defined by an onset of positive symptoms (delusions, hallucinations and disorganized speech or behavior) before the age of 13. COS is associated with other neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit and hyperactivity disorder. Copy number variations (CNVs) represent well documented neurodevelopmental disorder risk factors and, recently, *de novo* single nucleotide variations (SNVs) in genes involved in brain development have also been implicated in the complex genetic architecture of COS. Here, we aim to review the genetic changes (CNVs and SNVs) reported for COS, going from previous studies to the whole genome sequencing era. We carried out a systematic review search in PubMed using the keywords “childhood(early)-onset schizophrenia(psychosis)” and “genetic(s) or gene(s) or genomic(s)” without language and date limitations. The main inclusion criteria are COS (onset before 13 years old) and all changes/variations at the DNA level (CNVs or SNVs). Thirty-six studies out of 205 met the inclusion criteria. Cytogenetic abnormalities (n = 72, including 66 CNVs) were identified in 16 autosomes and 2 sex chromosomes (X, Y), some with a higher frequency and clinical significance than others (e.g., 2p16.3, 3q29, 15q13.3, 22q11.21 deletions; 2p25.3, 3p25.3 and 16p11.2 duplications). Thirty-one single nucleotide mutations in genes principally involved in brain development and/or function have been found in 12 autosomes and one sex chromosome (X). We also describe five SNVs in X-linked genes inherited from a healthy mother, arguing for the X-linked recessive inheritance hypothesis. Moreover, *ATP1A3* (19q13.2) is the only gene carrying more than one SNV in more than one patient, making it a strong candidate for COS. Mutations were distributed in various chromosomes illustrating the genetic heterogeneity of COS. More than 90% of CNVs involved in COS are also involved in ASD, supporting the idea that there may be genetic overlap between these disorders. Different mutations associated with COS are probably still unknown, and pathogenesis might also be explained by the

association of different genetic variations (two or more CNVs or CNVs and SNVs) as well as association with early acquired brain lesions such as infection, hypoxia, or early childhood trauma.

Keywords: childhood-onset schizophrenia, autism spectrum disorder, genetics, copy number variations, single nucleotide polymorphisms, exome sequencing

INTRODUCTION

Childhood-onset schizophrenia (COS) is a rare (< 1/40,000) and severe chronic psychiatric condition that shares with adult-onset schizophrenia (AOS) positive symptoms (delusions, hallucinations, and disorganized speech or behavior), but presents an early onset (before the age of 13) (Burd and Kerbeshian, 1987; Nicolson and Rapoport, 1999). It remains considered by many authors as an early and severe variant of AOS (Nicolson and Rapoport, 1999; Biswas et al., 2006). In COS, neurodevelopmental abnormalities (deficits in cognition, communication, or neuromotor impairments) and premorbid dysfunction are more frequent compared with AOS (Vourdas et al., 2003) and a clinical overlap exists with other neurodevelopmental disorders: 28% of patients with COS in the US cohort of the National Institute of Mental Health Child Psychiatry Branch met criteria for comorbid autism spectrum disorder (ASD) (Rapoport et al., 2009). In addition, more than 80% of children with schizophrenia or schizoaffective disorder present comorbid attention deficit and hyperactivity disorder (ADHD) (Ross et al., 2006). Few genetic studies of COS were reported, due to the very low prevalence (<1/40,000) (Burd and Kerbeshian, 1987) and to nosographic difficulties, which made it hard to obtain a consensual clinical definition of this disorder and to carry out etiological studies (Maier, 1999; Gochman et al., 2011). Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) classification provides recent clarification in this area with schizophrenia no longer excluding the diagnosis of ASD (Petty et al., 1984; American Psychiatric Association, 2013). Thus, clinical overlap between COS and ASD is now formally accepted. Surprisingly, DSM-5 still does not recognize the existence of COS, which therefore remains considered an adult clinical presentation (AOS) (American Psychiatric Association, 2013). Indeed, COS is a very rare complex disorder related to other neurodevelopmental disorders, and it represents a real challenge for clinical diagnosis with, to date, no objective test based on genetics (Petty et al., 1984). However, a high heritability rate of COS (> 80%) has been suggested in early adoption/twin studies (Kallmann and Roth, 1956) and has been confirmed by familial aggregation studies (Asarnow and Forsyth, 2013). To determine the etiology of COS, it is indispensable to start by reviewing the publications that have linked COS to DNA changes.

Macro-lesional cytogenetic abnormalities such as copy number variations (CNVs), including the 22q11.21 deletion, are more frequent in COS than in AOS [10.6% of patients with COS (DSM-III-R, onset <13 years) vs. 2–5%, in AOS, $p < 0.0001$]. These anomalies in the general population would concern only 0.86% of newborns (Nicolson et al., 1999).

Recently, Next Generation Sequencing (NGS) or “high throughput sequencing” allowed, with unprecedented scalability and speed, to determine the DNA sequence of a given individual. This tool opened up new perspectives to understand complex neurodevelopmental disorders, with particular attention to *de novo* single nucleotide variations (SNVs) occurring in genes involved in brain development (Veltman and Brunner, 2012). Only one study used whole exome sequencing (WES), a NGS method, in a cohort of patients with COS. This study identified 20 *de novo* variants in 17 COS probands (rate: 1.17) in genes previously linked to neuronal function or to psychiatric disorders (Ambalavanan et al., 2016). These arguments (phenotypic overlap with other neurodevelopmental disorders, high heritability, disease-related CNVs, and *de novo* SNV rates) strongly support the neurodevelopmental and genetic bases of COS (Rapoport et al., 2012). In this context, the main aim of this study is to describe the COS genomic variation (CNVs and SNVs) in the scientific literature to identify interesting genes or genetic pathways in both clinical practice and research.

METHODS

We carried out a systematic review of the MEDLINE database accessible *via* the search engine PubMed (www.ncbi.nlm.nih.gov/pubmed/) with the following key words: “childhood-onset schizophrenia” or “childhood-onset psychosis” or “early-onset schizophrenia” or “early-onset psychosis” and “genetics” or “genetic” or “gene” or “genes” or “genomic” or “genomics.” Our search terms were not limited by language or date of publication and were manually reviewed. According to inclusion criteria, we considered all genomic changes occurring in COS patients (age of onset before 13). We excluded all abnormalities at RNA or protein levels (regardless the age of onset). Genomic variations were classified based on cytogenetic position (**Table 1**) and candidate gene names (**Table 2**).

All CNVs were manually annotated using the University of California Santa Cruz (UCSC) Genome Browser (UCSC Mar. 2006 (NCBI36/hg18 or NCBI37/hg19) assembly; <http://genome.ucsc.edu/>). Regarding their type (gain or loss), their size, their genomic content, and making comparisons with external databases, we ranked each CNV as “pathogenic,” “uncertain clinical significance,” or “benign” (according to the American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants). For each CNV, we checked on the Simons Foundation Autism Research Initiative (SFARI) Gene database (autism/genetic database, <http://sfari.org>) which CNV involved in COS was also involved in ASD. For each gene, we checked on the Phenocarta Database (<https://gemma>).

TABLE 1 | Cytogenetic abnormalities (including copy number variations) in COS patients with their localization, length and type of inheritance.

CHR	CNV region and type (length)	Sex, age of onset	Neurodevelopmental disorders, Comorbidities	Inheritance	coordinates (hg18-hg19)	SFARI	Clinical significance	Bibliography
1	DUP 1q21.3 (248 kb)	Male, 12 y	–	inherited	chr1:151,514,380-151,762,871	+	UCS	Walsh et al., 2008
1 ; 7	t(1;7) (p22;q22)	Male, 9 y	ASD? - ADHD? - language, intellectual and motor impairments, dysmorphia, supraventricular tachycardia	inherited (father)	–	–	–	Gordon et al., 1994; Nicolson et al., 1999; Yan et al., 2000; Idol et al., 2008; Eckstrand et al., 2008; Addington and Rapoport, 2009* Walsh et al., 2008
2	DUP 2p14 (243 kb)	<13 y	–	inherited	chr2:65,637,097-65,879,935	+	UCS	Walsh et al., 2008
	DEL 2p16.3 (115 --> 112 kb) NRXN1	<13 y	–	not known	chr2:50,023,212-50,137,825 / chr2:50,025,162-50,136,989	+	P	Walsh et al., 2008; Addington and Rapoport, 2009*, Ahn et al., 2014
	DEL 2p16.3 (38 et 40kb) NRXN1	Male, 12 y	Motoric and verbal delay, IQ 82, macrocephalia and increase height (+3SD)	inherited (father)	chr2:51,151,955-51,190,352 / chr2:51,440,969-51,481,281	+		Duong et al., 2015
	DUP 2p25.3 (216 --> 245 kb) MYT1L	<13 y	–	inherited (mother)	chr2:1,618,945-1,835,426 / chr2:1,591,064-1,836,375	+	P	Walsh et al., 2008; Addington and Rapoport, 2009*, Ahn et al., 2014; Lee et al., 2012
	DUP 2p25.3 (143 --> 107kb) MYT1L	<13 y	–	not known	chr2:1,713,636-1,857,129 / chr2:1,720,133-1,827,317			
	DEL 2q31.2-31.3 (2,5 Mb)	Male, 11 y	–	de novo or germline mosaicism in one of the parents	chr2:179,643,864-182,145,339	-	UCS	Walsh et al., 2008; Addington and Rapoport, 2009* Rudd et al., 2015
3	DEL 3p12.2-p12.1 (2.2 Mb)	Female, 12 y	ASD, poor motor coordination, IQ 67	inherited (father)	–	-	UCS	
	DUP 3p21.31 (117 Kb)	<13 y	–	inherited	chr3:45,458,901-45,576,135	+	UCS	Walsh et al., 2008
	DUP 3p25.3 (120 --> 134 kb) SRGAP3	Male, 11 y	poor peer relationships, general anxiety disorder, panic disorder, agoraphobia, and depression	inherited (father)	chr3:9,100,744-9,220,529 / chr3:9,111,177–9,245,155	+	P	Walsh et al., 2008; Addington and Rapoport, 2009*, Wilson et al., 2011
	DEL 3q29 (1,58 Mb)	Male, 5 y	ASD, severe abnormal movements and tics	de novo	chr3:197,161,073-198,851,029	+	P	Sagar et al., 2013

(Continued)

TABLE 1 | Continued

CHR	CNV region and type (length)	sex, age of onset	Neurodevelopmental disorders, Comorbidities	Inheritance	coordinates (hg18-hg19)	SFARI	Clinical significance	Bibliography
5	DUP 5q12.3 (142 Kb)	Female, 10 y	–	inherited	chr5:64,795,287-64,937,409	+	UCS	Walsh et al., 2008
	Paternal segmental iUPD 5q32-qter (35 Mb)	Female, 9 y	MDD, inattention, learning disability, intellectual impairments	<i>de novo</i>	–	–	–	Eckstrand et al., 2008; Addington and Rapoport, 2009*, Seal et al., 2006
6	DEL 6p22.31 (144 kb)	Male, 9 y	OCD, expressive language disorder	inherited	chr6:119,596,633-119,740,850	+	UCS	Walsh et al., 2008
7	DUP 7p13 (120 Kb)	<13 y	–	not known	chr7:44,420,900-44,540,491	–	UCS	Walsh et al., 2008
	DUP 7q11.21-q11.22 (2,8 Mb)	<13 y	–	inherited	chr7:64,126,564-66,883,376	+	UCS	
8	DUP 8p22 (1,3 Mb)	Male, 11 y	–	inherited	chr8:13,400,795-14,679,483	+	UCS	Walsh et al., 2008
	DUP 8q11.23 (480 kb --> 493 kb)	<13 y	–	not known	chr8:53,563,161-54,043,063 / chr8:53,550,992-54,043,684	+	UCS	Walsh et al., 2008; Ahn et al., 2014
9	DUP 8q24.3 (369 Kb) PTK2	12 y	–	not known	chr8:142,025,432-142,393,948	+	UCS	Walsh et al., 2008
	DEL 9p24.2 (440 Kb)	11 y	–	–	chr9:3,104,250-3,544,339	+	UCS	
10	DUP 10p11.23 (176 Kb)	Male, 11 y	–	inherited	chr10:28,990,284-29,166,175	+	UCS	Walsh et al., 2008
	DUP 10p13 (145 Kb)	<13 y	–	inherited	chr10:15,688,654-15,833,865	+	UCS	
15	DEL 10q22.3 (173 kb)	Male, 12 y	–	<i>de novo</i>	chr10:81,415,378-81,588,866	+	UCS	Ahn et al., 2014
	DEL 15q11.2 (1386 kb)	<13 y	–	not known	chr15:18,818,086-20,203,694	+	UCS	Ahn et al., 2014
15	DEL 15q11.2 (575kb)	<13 y	–	inherited (mother)	chr15:20,203,694-20,778,963			
	DEL 15q13.3 (382kb)	<13 y	–	<i>de novo</i>	chr15:30,238,780-30,620,951	+	P	
	DEL 15q13.3 (475 kb)	<13 y	–	inherited (mother)	chr15:30,238,780-30,713,368			
	DUP 15q13.3 (503.5 Kb) CHRNA7	Female, 10 y	–	inherited (father)	chr15:32,012,361-32,515,849	+		Zhou et al., 2016
	DUP 15q13.3 (600,2 Kb) CHRNA7	Male, 12 y	ADHD	chr15:32,019,919–32,620,127				

(Continued)

TABLE 1 | Continued

CHR	CNV region and type (length)	sex, age of onset	Neurodevelopmental disorders, Comorbidities	Inheritance	coordinates (hg18-hg19)	SFARI	Clinical significance	Bibliography
16	DUP 15q26.2-q26.3 (687 Kb)	<13 y	–	not known	chr15:96,246,764-96,933,404	+	UCS	Walsh et al., 2008
	DUP 16p11.2 (433 --> 604 Kb)	8 y	PDD-NOS, poor social and motor development	inherited (father)	chr16:29,652,656-30,085,308 / chr16:29,502,984-30,107,306	+	P	Walsh et al., 2008; Addington and Rapoport, 2009*, Ahn et al., 2014; Rapoport et al., 2009
	DUP 16p11.2 (578 --> 445 kb)	10 y	Poor social and motor development	chr16:29,657,405-30,235,818 / chr16:29,782,436-30,227,808	father	+	UCS	Ahn et al., 2014
	DEL 16p12.1 (449kb)	–	–	–	chr16:21,498,074-21,946,841	+	UCS	Brownstein et al., 2016
	DEL 16p13.11 (15 à 131 kb)	Male, 6 y	Motor dyscoordination, langage impairments	Father or de novo	chr16:1,51,32,264–1,51,47,411 (min) to 1,50,48,733–1,51,79,946 (max)	+	UCS	
	DUP 16p13.11 (1,4 à 1,7 Mb)	Female, 4 y	ASD, Epilepsy, Chiari 1	father	chr16:1,48,97,761–1,62,76,117 to 1,47,80,303–1,64,58,270	–	UCS	
	DUP 16q22.2-ter (17 Mb)	Female, 11 y	Atypical Turner, motor, language and attention impairments	–	–	–	P	Eckstrand et al., 2008
DUP 16q22.3-q24.3 (16,7 Mb)	Female, 12 y	ASD, poor motor coordination, IQ 67	de novo	–	–	P	Rudd et al., 2015	
DUP 16q23.3 (1,5 Mb)	Female, 9.5 y	–	–	inherited	chr16:80,737,839-82,208,451	+	UCS	Walsh et al., 2008
DEL 16q24.1 (111 Kb)	<3 y	–	–	inherited	chr16:82,997,582-83,108,554	+	UCS	
DUP 17q21.31 (384 kb)	<13 y	–	–	father	chr17:41,321,621-41,706,070	+	UCS	Ahn et al., 2014
DUP 18p11.31-p11.23 (510 kb)	<13 y	–	–	inherited	chr18:7,067,237-7,576,777	+	UCS	Walsh et al., 2008
DUP 18q22.1 (768 Kb)	Male, 10 y	Asperger's disorder	–	inherited	chr18:61,907,915-62,675,869	+	UCS	Walsh et al., 2008
DEL 19p12 (397 Kb)	<13 y	–	–	not known	chr19:23,413,380-23,810,606	+	UCS	Walsh et al., 2008
DEL 20p12.1 (113 Kb)	10 y	Poor social and motor development	–	inherited	chr20:14,921,777-15,034,862	+	UCS	Walsh et al., 2008

(Continued)

TABLE 1 | Continued

CHR	CNV region and type (length)	sex, age of onset	Neurodevelopmental disorders, Comorbidities	Inheritance	coordinates (hg18-hg19)	SFARI	Clinical significance	Bibliography	
22	DEL 22q11.2 (3Mb) PRODH DGCR6	Male, 9 y	Language, motor and social impairments, generalized anxiety disorder, dysthymia and ADHD, craniofacial dysmorphism, hypospadias	de novo	–	+	P	Nicolson et al., 1999; Eckstrand et al., 2008; Addington and Rapoport, 2009*, Rapoport et al., 2009; Yan et al., 1998; Usiskin et al., 1999; Liu et al., 2002; Sporn et al., 2004	
		Female, 12 y	Language, motor and social impairments, craniofacial dysmorphism, celiac disease and ureteric reflux	de novo	–			Nicolson et al., 1999; Eckstrand et al., 2008; Addington and Rapoport, 2009*, Rapoport et al., 2009; Usiskin et al., 1999; Liu et al., 2002; Sporn et al., 2004	
		Female, 10 y	Language, motor and social impairments, craniofacial dysmorphism	de novo	–				
			<13 y	Craniofacial dysmorphism	–	–			Eckstrand et al., 2008; Addington and Rapoport, 2009*, Rapoport et al., 2009; Sporn et al., 2004
	DEL 22q11.21 (3 Mb)	<13 y	–	–	de novo	chr22:17,092,563-20,077,678	+		Ahn et al., 2014
	DEL 22q11.21 (2,6 Mb)	<13 y	–	–	de novo	chr22:17,224,632-19,842,333			
	DEL 22q11.21 (2,6 Mb)	<13 y	–	–	not known	chr22:17,257,787-19,855,248			
DEL 22q11.21 (2,7 Mb)	<13 y	–	–	de novo	chr22:17,257,787-19,963,350				
DEL 22q11.21 (2,9 Mb)	<13 y	–	–	de novo	chr22:17,269,794-20,128,199				
DUP 22q13.32 (1,6 Mb)	8 y		PDD-NOS, poor social and motor development	de novo	chr22:47,903,228-49,557,485	+	UCS	Ahn et al., 2014	

(Continued)

TABLE 1 | Continued

CHR	CNV region and type (length)	sex, age of onset	Neurodevelopmental disorders, Comorbidities	Inheritance	coordinates (hg18-hg19)	SFARI	Clinical significance	Bibliography
X	expansion CGG (1,5 Kb) FMR1	Female, 9 y	Dysmorphia, learning and social impairments, mild MR	mother	–	–	–	Vantalón et al., 2005
	47, XXX	Female, <13 y	–	–	–	–	–	Eckstrand et al., 2008; Addington and Rapoport, 2009*
	46,X,i(X)(q10) (22%)/45,X(78%) DEL Xq24-qter	Female, <13 y Female, 11 y	Mosaic Turner Atypical Turner, motor, language and attention impairments	<i>de novo</i> <i>de novo</i>	– –	– –	– P	Nicolson et al., 1999; Eckstrand et al., 2008; Addington and Rapoport, 2009*, Kumra et al., 1998 Walsh et al., 2008
	DUP Xp22.31 (342 Kb) DEL Xp22.31 (1.68 Mb) STS	<13 y Male, 11 y	– Congenital ichthyosis, microcephalia, epilepsy. Language, motor, social, learning impairment, IQ 57, ADHD, ASD	not known	chrX:8,384,117-8,726,291 chrX:6,456,036-8,139,238	+ –	UCS UCS	Walsh et al., 2008 Malik et al., 2017
	DUP Xq28 (6 a 35 Kb)	Female, 4 y	ASD, Epilepsy, Chiari 1	<i>de novo</i>	chrX:15,29,55,334–15,29,61,664 to 15,29,51,719–15,29,86,547	+	UCS	Brownstein et al., 2016
	DEL Xq23-q28 (43 Mb)	Female, 12 y	ASD, poor motor coordination, IQ 67	<i>de novo</i>	–	+	P	Rudd et al., 2015
Y	DUP Yq11.221 (183 Kb)	Male, 8 y	Generalized anxiety disorder	<i>de novo</i>	chrY:14,441,161-14,623,937	+	UCS	Walsh et al., 2008

ADHD, Attention Deficit Hyperactivity Disorder; ASD, Autism Spectrum Disorder; CNV, Copy number variation; COPD, Chronic Obstructive Pulmonary Disease; DEL, Deletion; DUP, Duplication; iUPD, Uniparental isodisomy; ID, Intellectual disability; Kb, Kilobases; Mb, Mégabases; MDD, Major Depressive Disorder; MR, Mental Retardation; OCD, Obsessive compulsive disorder; P, Pathogenic; PDD-NOS, Pervasive developmental disorder not otherwise specified; UCS, Uncertain Clinical Significance; VCF, Velocardiofacial; Y, Years; * Literature review.
When available, phenotypes (sex, age of onset, other neurodevelopmental disorders and comorbidities) are described.

TABLE 2 | Genomic microlesions (including single nucleotide variations) in COS patients with their localization, length, and type of inheritance.

Gene name	Gene localization	SNV ID number/ mutation (protein level)	Population / Phenotypes	Inheritance	p-value	Bibliography
FAMILY BASED ASSOCIATION STUDY						
DAOA (G72) / DAOA-AS1 (G30)	13q33.2	rs1935058, rs3916967, rs2391191	n=64 (53 COS trios, 11 COS dyads)	–	0.015 to 0.5	Addington et al., 2004
DTNBP1	6p22.3	rs11558324	n=92 (73 COS + PDD-NOS trios, 19 COS + PDD-NOS dyads)	–	0.014	Gornick et al., 2005
GAD1	2q31.1	rs3749034, rs2270335, rs2241165	n=66 (55 COS + PDD-NOS trios, 11 COS + PDD-NOS dyads)	–	0.005	Addington et al., 2005
NRG1	8p12	rs35753505, rs2881272, rs327417	n=70 (59 COS + PDD-NOS trios, 11 COS + PDD-NOS dyads)	–	0.009 to 0.05	Addington et al., 2007
CASE CONTROL STUDY						
BDNF	11p13	val66met	65 patients (10.5 ± 3.7 y) vs 111 controls	–	0.03	Pakhomova et al., 2010
COMT	22q11.21	val158met	83 patients (<13 y) vs 208 controls	–	–	Raznahan et al., 2011
TPH1	11p15.3-p14	ala218cys	51 patients (<16 y) vs 148 controls	–	0.0058	Sekizawa et al., 2004
GENES SEQUENCING (candidate genes or full exome)						
ATP1A3	19q13.2	val129met	Male, onset: 6 y, motor delay	<i>de novo</i>	–	Smedemark- Margulies et al., 2016
		asp801asn	Male, onset: 10 y, ASD, dysmorphia, motor, intellectual and learning delays. Recurrent MDD		–	Chaumette et al., 2018
		glu815lys	Male, onset: 12 y, motor and communication impairments, dysmorphia, ASD		–	
		ala813val	Male, onset: 10 y, ASD, motor and intellectual delays	mother	–	
FXYP1	19q13.12	arg90cys	Male, onset: 7 y, Asperger's disorder	inherited	–	
FXYP6-FXYP2	11q23.3	val101ala	Male, onset: 13 y		–	
FXYP6	11q23.3	gly73arg	Female, onset: 12 y		–	
GPR153	1p36.31	arg73cys	Male, onset: 12 y	<i>de novo</i>	–	Ambalavanan et al., 2016
GTF2IRD1	7q11.23	arg357cys	Female, onset: 12 y		–	
ITGA6	2q31.1	glu1063del	Female, onset: 12 y		–	
LUZP4	Xq23	arg278fs*10	–	mother	–	Ambalavanan et al., 2019
OPHN1	Xq12	met461val	IQ 88, PDDNOS		–	
PCDH19	Xq22.1	leu1022ile	–		–	
RPS6KA3	Xp22.12	arg723his	IQ 64, PDDNOS		–	
RYR2	1q43	glu746tyr	Male, onset: 8 y, PDDNOS, separation anxiety disorder, Asperger's disorder	<i>de novo</i>	–	Ambalavanan et al., 2016
SEZ6	17q11.2	thr229_thr231del	Male, onset: 11 y		–	
TTBK1	6p21.1	arg258gln	Male, onset: 7 y, Asperger's disorder		–	
UPF3B	Xq24	gln228fsX18	Male, 10 y, ADHD, PDDNOS, ASD	mother	–	Addington et al., 2011

ADHD, Attention Deficit Hyperactivity Disorder; ASD, Autism Spectrum Disorder; CNV, Copy number variation; COS, Childhood Onset Schizophrenia; DEL, Deletion; DUP, Duplication; MDD, Major Depressive Disorder; PDD-NOS, Pervasive developmental disorder not otherwise specified; SNV, Single Nucleotide Variation; Y, Years. When available, phenotypes (sex, age of onset, other neurodevelopmental disorders, and comorbidities) are described.

msl.ubc.ca/home.html) the evidence linking genes to phenotypes of neurodevelopmental disorders (**Figure 1**, Venn diagram). Phenotypes were systematically described, if available.

The selection took place before September 2018. At this time, 36 articles (1994 to 2018) out of 205 (1982 to 2018) met the inclusion criteria. Article reviewing process, including selection and exclusion, is summarized in a PRISMA flow

diagram (**Figure 2**). Two articles were added after the freezing of the inclusion process (41; 52). Mutations were identified in 21 chromosomes. The results were ranked either in ascending order of their chromosomal position for structural variants (cytogenetic abnormalities) (**Table 1**) or in alphabetical order according to their gene name for genetic variants (lesions at gene level) (**Table 2**).

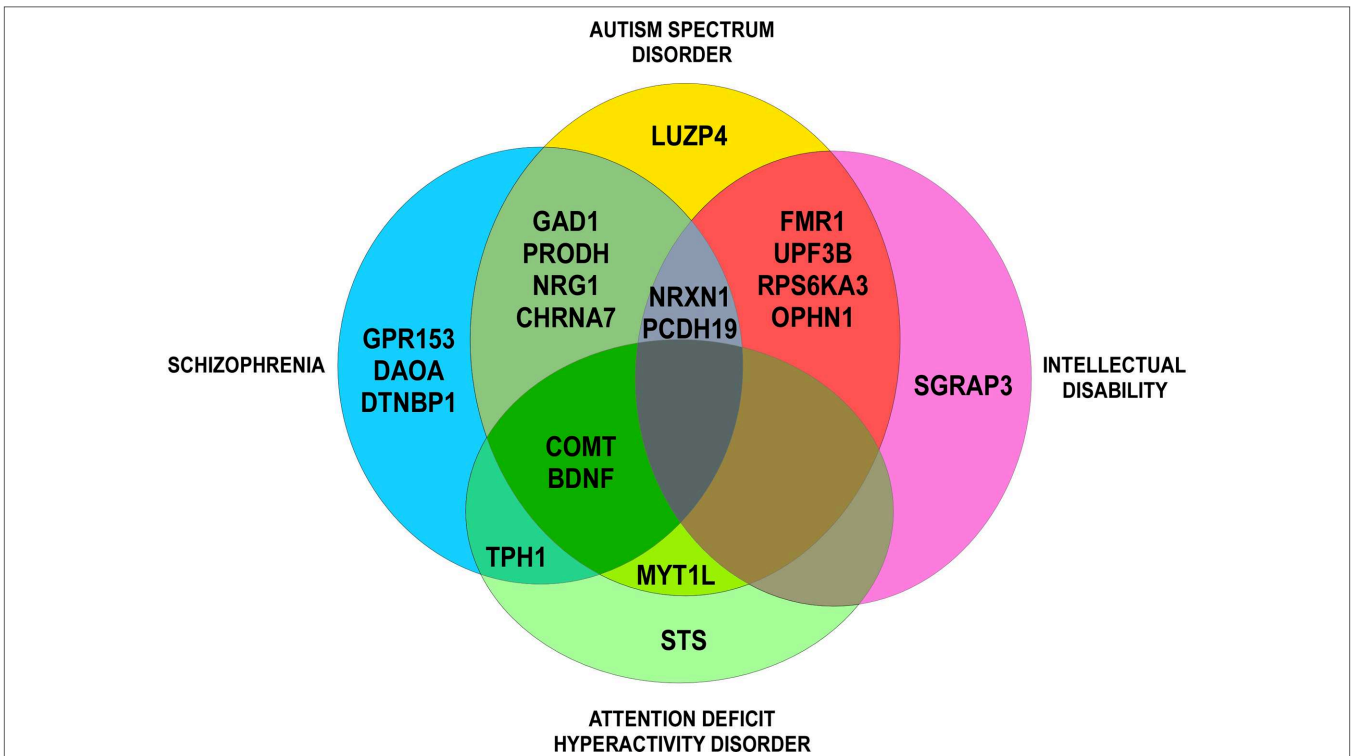


FIGURE 1 | Venn diagram: evidence for genetic overlap in neurodevelopmental disorders (Phenocarta Database).

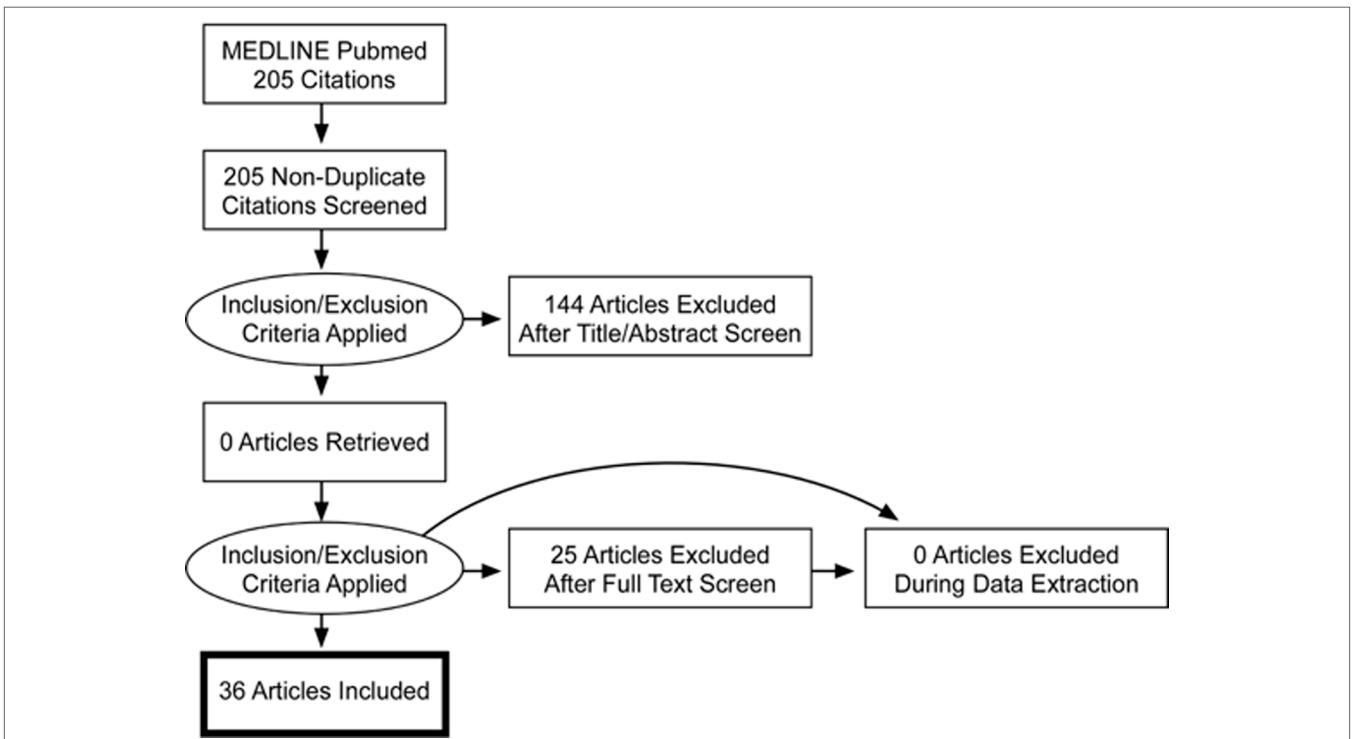


FIGURE 2 | PRISMA Flowchart diagram.

RESULTS

I) Cytogenetic Abnormalities Associated With COS

The following cytogenetic abnormalities ($n = 72$, including 66 CNVs) were identified in 16 autosomes (1, 2, 3, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, 20, and 22) and two sex chromosomes (X and Y) of 46 patients (17–40). The results were ranked in ascending order of their chromosomal position (**Table 1**) and were summarized in a genomic map (**Figure 3**).

1) CNVs

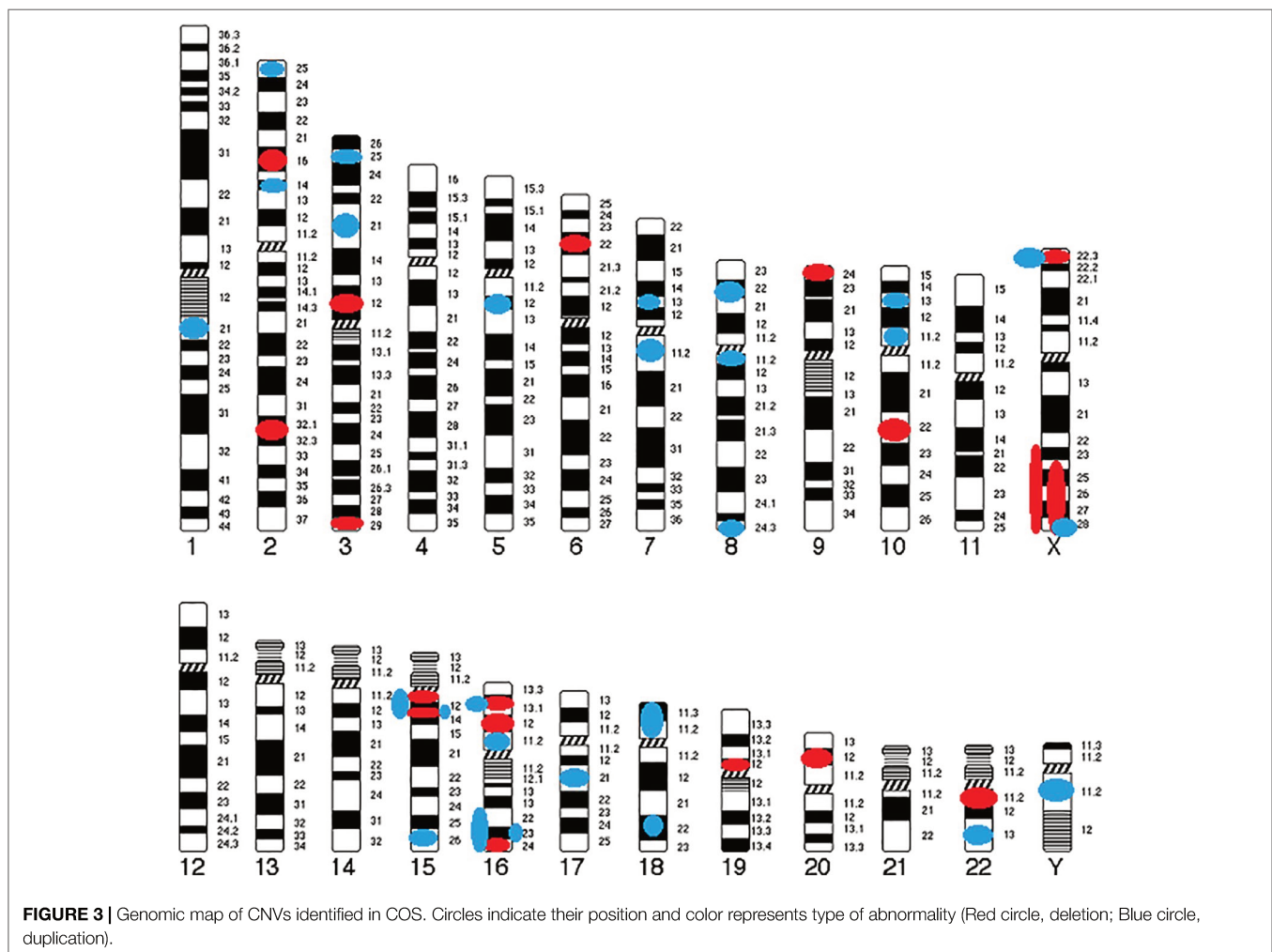
- Deletions (CNVs) from 15Kb to 43Mb: 2p16.3, 2q31.2-q31.3, 3p12.2-p12.1, 3q29, 6p22.31, 9p24.2, 10q22.3, 15q11.2, 15q13.3, 16p12.1, 16p13.11, 16q24.1, 19p12, 20p12.1, 22q11.21, Xp22.31, Xq24-ter, and Xq23-q28;
- Duplications (CNVs) from 120Kb to 17Mb: 1q21.3, 2p14, 2p25.3, 3p21.31, 3p25.3, 5q12.3, 7p13, 7q11.21-q11.22, 8p22, 8q11.23, 8q24.3, 10p11.23, 10p13, 15q11-q13,

15q13.3, 15q26.2–26.3, 16p11.2, 16p13.11, 16q22.2-ter, 16q22.3-q24.3, 16q23.3, 17q21.31, 18p11.31-p11.23, 18q22.1, 22q13.32, Xp22.31, Xq28, and Yq11.221.

Only six CNVs (9%) of our study are described in COS only: del2q31.2-q31.3 (smaller than the CNV described in ASD), del3p12.2-p12.1 (described in ASD as a duplication), delXq24-ter (larger than the CNV described in ASD), dup7p13 (described in ASD as a deletion), dup16q22.2-ter (larger than the CNV described in ASD), and dup16q22.3-q24.3 (larger than the CNV described in ASD).

In an on-site case series and literature review focusing on Childhood-Onset Schizophrenia Spectrum Disorders (SSDs; larger spectrum than COS), Giannitelli et al. (2018) showed that some CNVs, previously un-described in COS patients, are associated to childhood psychosis: 1q21.1 deletion, 1q21.1 duplication, Williams-Beuren region (7q11.23) duplication and 16p11.2 deletion (Giannitelli et al., 2018).

The phenotypes of only 15 out of the 46 patients were fully described (33%). The neurodevelopmental disorders that have been



presented are: motor impairments (fine or growth milestones delay, coordination disability, or tics) in 11/15 patients, language retardation in 7/15 patients, intellectual disability (IQ < 70) in two patients, and ASD in five patients [(including 1 Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS)]. Social impairment was present in six other patients with COS and ADHD in three patients. Inattention impairment was specified in only one patient. The psychiatric comorbidities that have been highlighted are: two cases with mood disorders (major depressive disorder or dysthymia) and two cases with anxiety disorders. The somatic comorbidities detected are: dysmorphia in four cases and epilepsy in two cases. Sporadic cases of hypospadias, ureteric reflux, congenital ichthyosis, Chiari type 1, or celiac disease were also described.

2) Other DNA Lesions

a) Aneuploidy

One case of Trisomy X (47XXX) and two cases of Turner syndrome (45X0), one atypical form (46,X,del(X)(q24-ter) and one with mosaicism 46,X,i(X)(q10)(22%)/45,X(78%) (Eckstrand et al., 2008; Addington and Rapoport, 2009);

b) Uniparental and Segmental isoDisomy (iUSD)

In isodisomy, both copies of a chromosomal set are inherited from one parent (the mother or the father). An iUSD on 5q32-ter (35Mb) was described in a patient with COS (Eckstrand et al., 2008; Addington and Rapoport, 2009; Seal et al., 2006);

c) Translocation 1, 7: p22q22 (Gordon et al., 1994; Nicolson et al., 1999; Yan et al., 2000; Eckstrand et al., 2008; Idol et al., 2008; Addington and Rapoport, 2009)

d) Trinucleotide Repeat Expansions

i) CGG Expansions: Although a link between Fragile X syndrome (FXS) and COS has never been reported, Vantalon et al. (2005) described a 1.5 Kb expansion and complete methylation of the CpG island upstream of FMR1 in a 9 years-old girl with COS, dysmorphia, learning and social impairments, and mild mental retardation (Vantalon et al., 2005). This abnormality is inherited from the mother who carries an FXS premutation. Interestingly, instead of being unaffected or weakly affected as most patients carrying a premutation, the mother presents schizotypal personality. It seems that the severity of the schizophrenic spectrum disorder, which affects both mother and daughter with gradual severity, is linked to the CGG expansion degree. Effect of the hazard could not be excluded in this case (Vantalon et al., 2005).

ii) CAG/CTG Expansion: a longer repeat expansion on chromosome 18 was found in a COS cohort and in a male subclass, with a significant p-value especially for the males (0.036 and 0.002, respectively; Wilcoxon–Mann–Whitney U test) (Burgess et al., 1998).

e) Genetic Syndromes

In their recent review, Giannitelli et al. (2018) showed that some genetic syndromes, previously un-described

in COS, are associated to childhood-onset SSDs: juvenile Huntington disease, Prader-Willi syndrome, Steinert myotonia, Ondine syndrome, Rubinstein-Taybi syndrome, and GLUT1 deficiency syndrome (Giannitelli et al., 2018).

II) Genes Associated With COS

In total, 32 candidate genes have been described on 12 autosomes (1, 2, 3, 6, 7, 8, 11, 13, 15, 17, 19, and 22) and 1 sex chromosome (X) (Tables 1 and 2) through the following studies (Addington et al., 2004; Sekizawa et al., 2004; Gornick et al., 2005; Addington et al., 2005; Addington et al., 2007; Pakhomova et al., 2010; Addington et al., 2011; Raznahan et al., 2011; Smedemark-Margulies et al., 2016; Chaumette et al., 2018; Ambalavanan et al., 2019):

1) Genetic Association Studies (Table 2)

a. Family-Based “Transmission Disequilibrium Test” Studies (From 2004 to 2007)

Transmission disequilibrium test, a family-based association test for the presence of genetic linkage between a genetic marker and a trait, was used to describe the following candidate genes: *DAOA*, *DAOA-ASI*, *DTNBPI*, *GADI*, and *NRG1*. The Linkage Disequilibrium Analyses for Quantitative and Discrete Traits (QTDT) program was used to make statistical analysis (p-values).

i) *DAOA* and *DAOA-ASI* (Addington et al., 2004): three Single Nucleotide Polymorphisms (SNPs) are associated with COS (rs1935058, rs3916967, and rs2391191 (p = 0.5, 0.015, 0.3, respectively)). The most significant SNP (p = 0.015) is rs3916967 (genomic sequence reference: NG_012694.1:g.4133T > C);

ii) *DTNBPI* (Gornick et al., 2005): one SNP, rs11558324 (NG_009309.1:g.5154A > G), is associated with COS (p = 0.014) and two two-marker haplotypes (containing rs11558324) are also associated with COS (p = 0.021, 0.008),

iii) *GADI* (Addington et al., 2005): three four-marker haplotypes are associated with COS (p = 0.005);

iv) *NRG1* (Addington et al., 2007): individual markers show association with COS (rs35753505, rs2881272, 420M9-1395 (microsatellite), and rs327417, with p-value between 0.009 and 0.05). The most significant SNP (p = 0.009) is rs327417 (NG_012005.2:g.341913G > A). Further, several novel four-marker haplotypes are associated with COS (lowest p = 0.0004).

b. Population-Based “Case vs. Control” Studies (From 2004 to 2011)

A polymorphism (VAL66MET) in the *BDNF* (11p13) gene was associated with COS in a 65 patient cohort (10.5 ± 3.7 years old at onset) vs. 111 controls (p = 0.03; χ^2 test) (Pakhomova et al., 2010). A mutation (VAL158MET) in *COMT* (22q11.21) that increases protein activity levels in the brain accelerated adolescent cortical thinning (MRI findings) in both schizophrenia probands and their siblings (with resolution after a certain age for siblings), illustrating

the influence of dopaminergic disruption on brain cortical maturation. Authors analyzed data from an 83 COS patient cohort vs. 208 healthy controls (Raznahan et al., 2011). A mutation (A218C) in the *TPH1* (11p15.3-p14) gene ($p = 0.0058$; χ^2 test) is described in 51 patients (early adolescent onset cohort before 16 years old) vs. 148 controls (Sekizawa et al., 2004).

2) Gene Sequencing Studies (2011 to Present)

Finally, five studies by DNA sequencing (candidate genes or whole exome sequencing) have allowed identifying the following 18 mutations in 11 genes (Addington et al., 2011; Ambalavanan et al., 2016; Smedemark-Margulies et al., 2016; Chaumette et al., 2018; Ambalavanan et al., 2019).

i. Six SNVs inherited from a healthy mother:

- Five X-linked recessive variants: *LUZP4* (arg278fs*10), *OPHN1* (met461val), *PCDH19* (leu1022ile), *RPS6KA3* (arg723his), and *UPF3B* gene (gln228fsX18) that also segregates in the sibling.
- One SNV (ala813val) in *ATP1A3* (a boy with onset at 10 years old and co-morbid ASD).

ii. Three SNVs inherited (missenses variants) in the FXYP gene family. *FXYP1* (arg90cys), *FXYP6-FXYP2* (val101ala), and *FXYP6* (gly73arg) genes. Only the patient with the mutation in *FXYP1* presents a co-morbid ASD (Asperger's disorder).

iii. Nine *de novo* SNVs in the following genes: *ATP1A3* (val129met; asp801asn; glu815lys), *GPR153* (arg73cys), *GTF2IRD1* (arg357cys), *ITGA6* (glu1063del), *RYR2* (glu746tyr), *SEZ6* (thr229_thr231del), and *TTBK1* (arg258gln).

The ATPase Na⁺/K⁺ transporting Alpha-3 Polypeptide (*ATP1A3* gene) encodes the alpha-3 catalytic subunit of the Na⁺/K⁺-ATPase transmembrane ion pump mapping at 19q12-q13.2 (Harley et al., 1988). The *ATP1A3* isoform is exclusively expressed in neurons of various brain regions, including the basal ganglia, hippocampus, and cerebellum (summary by Rosewich et al., 2012). Mutations in this gene have been associated with a spectrum of disorders depending on the domain they affect in the corresponding protein. The majority of mutations associated with rapid-onset dystonia parkinsonism, or dystonia-12 (*DYT12*), were located in exons 8 and 14 whereas those with alternating hemiplegia of childhood-2 (*AHC2*) were located in exons 17 and 18 and in general they seem to affect transmembrane and functional domains, being the most severe dysfunctions. By genetic analysis of clinical data from 155 patients with *AHC2*, 132 confirmed to have *ATP1A3* mutations. Among those with *AHC2*, the most frequent mutations were D801N (in 43%), E815K (in 16%) and G947R (in 11%). E815K was associated with a severe phenotype, with greater intellectual and motor disability; D801N appeared to confer a milder phenotype and G947R correlated with the most favorable prognosis. For those with epilepsy, the age at seizure onset was earlier for patients with the E815K or G947R mutations than for those with the D801N mutation (Panagiotakaki et al., 2015). In 10 patients

from three unrelated families with cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS; 601338) (Demos et al., 2014), the same heterozygous missense mutation in the *ATP1A3* gene was identified (E818K; OMIM 182350.0014).

The G Protein-coupled Receptor 153 (*GPR153*) gene, located on 1p36.31, belongs to the large rhodopsin (RHO; OMIM 180380) family of GPCRs (Gloriam et al., 2005) and shows a highest similarity to serotonin receptors, (Gloriam et al., 2005). Furthermore, knockdown of *GPR153* in mice showed reduction in food intake and increased anxiety according to the elevated plus Maze test (Sreedharan et al., 2011).

The InTeGrin Alpha-6 (*ITGA6*) gene is located on 2q31.1 (Hogervorst et al., 1991). While functional absence of *ITGA6* has been associated with epidermolysis bullosa (Hogervorst et al., 1991; Georges-Labouesse et al., 1996), a few works addressed the role of *ITGA6* in neurons. Alpha-6 integrin was initially reported to be involved in neural migration (Yao et al., 2018). In addition, recent data suggested that $\alpha 6$ and $\beta 1$ integrins may play a role in mediating Schwann cell interactions with axons and promote axonal regeneration (Chang et al., 2018).

The Ryanodine Receptor 2 (*RYR2*) gene—located on chromosome 1 between q42.1 et q43—encodes a calcium channel that is located in the sarcoplasmic reticulum and is the major source of calcium required for cardiac muscle excitation-contraction coupling. (Bhuiyan et al., 2007). *Ryr2*^{-/-} mice die at approximately embryonic day 10 with morphologic abnormalities in the heart tube. Ca²⁺ signaling has been associated with ASD (Kabir et al., 2016; Stephenson et al., 2017; Castagnola et al., 2018) and with other psychiatric and neurological diseases (Heyes et al., 2015). It is not surprising that *RYR2* was linked to ASD by genetic studies (Lu and Cantor, 2012; Soueid et al., 2016; Chen et al., 2017). However, it is very interesting to underline that an SNP in this gene was associated with ASD in families with only affected males in contrast with those with affected females (Lu and Cantor, 2012) suggesting that *RYR2* is a sex-related genetic factor for ASD.

The SeiZure-related 6 (*SEZ6*) gene is located on the 17q11.2 chromosome. *Sez6* types 1 and 2 have an N-terminal signal sequence, followed by a threonine-rich region, a Short Consensus Repeat (SCR), a CUB-like domain, a second SCR, a second CUB-like domain, three tandem SCRs, a transmembrane domain, and a cytoplasmic C-terminal tail. They differ only in the region between the last SCR and the transmembrane domain. *SEZ6* was predicted to be involved in neuronal maturation and plasticity (Miyazaki et al., 2006). Recently mutations and altered expression of this gene have been associated with Alzheimer's and Niemann-Pick disease (Causevic et al., 2018; Paracchini et al., 2018).

The Tau Tubulin Kinase 2 (*TTBK2*) gene, located on 15q15.2, encodes a member of the casein kinase (CK1) group of eukaryotic protein kinases. *TTBK1* has been implicated in Alzheimer's disease (OMIM 104300) and in neurofibrillary tangles formation (Sato et al., 2006). Mutations in this gene also cause spinocerebellar ataxia 11 (SCA11; 604432). SCA11 is a pure progressive cerebellar ataxia that has been linked to 15q14-q21 (Worth et al., 1999; Houlden et al., 2007). In an

8-generation English family they found a one-base insertion in the *TTBK2* gene creating a premature stop codon and a truncation of the normal protein (OMIM 611695.0001). In a second family of Pakistani ancestry, a different mutation was found (OMIM 611695.0002). Goetz et al. (2012) concluded that *TTBK2* is required for removal of CP110 for the initiation of ciliogenesis (Goetz et al., 2012).

3) Candidate Genes From Cytogenetic Studies (Table 1)

Interesting candidate genes deleted, duplicated, or truncated by the CNVs have also been found in cytogenetic studies (see above). These genes have brain expression and are mostly described in other neurodevelopmental or psychiatric disorders (Figure 1). Nine genes are described as putative COS-causing genes: *CHRNA7* (15q13.3), *DGCR6* (22q11.2), *FMR1* (Xq27.3), *MYT1L* (2p25.3), *NRXN1* (2p16.3), *PRODH* (22q11.2), *PTK2* (8q24.3), *STS* (Xp22.31), and *SRGAP3* (3p25.3) (Yan et al., 1998; Nicolson et al., 1999; Usiskin et al., 1999; Liu et al., 2002; Sporn et al., 2004; Vantalón et al., 2005; Eckstrand et al., 2008; Walsh et al., 2008; Addington and Rapoport, 2009; Rapoport et al., 2009; Wilson et al., 2011; Lee et al., 2012; Ahn et al., 2014; Duong et al., 2015; Zhou et al., 2016; Malik et al., 2017).

CONCLUSIONS

COS is a neurodevelopmental disorder with several degrees of complexity (clinical and genetic heterogeneity). Clinically, getting the diagnosis is very challenging (severe disorder, comorbidities, and association with other neurodevelopmental disorders) (Gochman et al., 2011). The clinical overlap with ASD is well documented and in our study we found a co-morbidity rate (33%) nearly equal to the National Institute of Mental Health (NIMH) COS cohort rate (28%) (Rapoport et al., 2009). The genetic overlap with ASD is also well documented and we show that 91% of described CNVs are also described in ASD (*SFARI*). In the literature, we found only 20% of COS patients with co-morbid ADHD vs. 84% according to Ross et al. and we hypothesize that this trouble was under-diagnosed in schizophrenia studies (Ross et al., 2006). All intellectual, motor, communication, and learning impairments are also frequently observed in COS (Ross et al., 2006; Nicolson et al., 1999). Psychiatric comorbidities were rarely described (two cases of mood disorders and two cases of anxiety disorders), which was an unexpected outcome given the published literature (Ross et al., 2006). Here, we highlight that only one-third of the full phenotypes associated with the mutations published in the literature are described, which constitutes a significant loss of information for researchers. Therefore, it appears fundamental to carry out preliminary work before genetic testing: perform a rigorous and homogeneous phenotypic characterization using International Classification of Disease (*ICD-10* and *DSM-5*) with standardized and internationally validated psychiatric categorical assessments and in accordance with medical history (including perinatology), biography (with significant

life event and trauma), and environmental factors (such as toxic exposure).

COS is characterized by a complex genetic architecture with both inherited and *de novo* mutations distributed in almost all chromosomes. Most of the genes causing COS are unknown yet. It is interesting that, the few that have been already proposed (see before) are involved both in neurodevelopmental and neurodegenerative disorders such as Parkinson, Alzheimer, or ataxia. Moreover, schizophrenia has been shown to have complex genetic traits with high polygenic risk (Ahn et al., 2016). Thus, a second hit (or more), in addition to CNV, is probably essential to explain the phenotypes. It includes *de novo* SNVs, other CNVs and/or environmental factors (e.g., trauma at early childhood, central nervous system infections or injuries) (Davis et al., 2016). At the interplay between genetic and environmental factors, epigenetics opens new perspectives to understand biological mechanisms of psychosis. In fact, recent findings suggest that pangenomic methylation changes during adolescence accompany conversion to psychosis (Kebir et al., 2018). In clinical practice, as suggested by Szego et al. for ASD (Szego and Zawati, 2016), it would seem useful to propose to COS patients genetic sequencing instead or in addition to microarrays (Anagnostou et al., 2014; Soden et al., 2014) to improve genetic testing and to allow *de novo* SNV detection.

In research, the major challenge of the upcoming years will be the analysis of big data from NGS (prioritization and interpretation of DNA variations) (Richards et al., 2015) and the experimental validation of putative mutations. Sharing data with other teams around the world will be helpful to unravel the molecular pathology of COS and its underlying causes, paving the way for an early therapeutic intervention.

AUTHOR CONTRIBUTIONS

AF, FA, and BB: contributed to the conceptualization of the study and drafted the first version of the manuscript. All other authors MD, ST, ED, and MC have revised first version of the manuscript critically for important intellectual content and approved the final version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publication 3

Reduction of *Fmr1* mRNA Levels Rescues Pathological Features in Cortical Neurons in a Model of FXTAS

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Fragile X-associated tremor ataxia syndrome (FXTAS) is a rare disorder associated to the presence of the fragile X premutation, a 55–200 CGG repeat expansion in the 5' UTR of the *FMRI* gene. Two main neurological phenotypes have been described in carriers of the CGG premutation: (1) neurodevelopmental disorders characterized by anxiety, attention deficit hyperactivity disorder (ADHD), social deficits, or autism spectrum disorder (ASD); and (2) after 50 years old, the FXTAS phenotype. This neurodegenerative disorder is characterized by ataxia and a form of parkinsonism. The molecular pathology of this disorder is characterized by the presence of elevated levels of *Fragile X Mental Retardation 1 (FMRI)* mRNA, presence of a repeat-associated non-AUG (RAN) translated peptide, and *FMRI* mRNA-containing nuclear inclusions. Whereas in the past FXTAS was mainly considered as a late-onset disorder, some phenotypes of patients and altered learning and memory behavior of a mouse model of FXTAS suggested that this disorder involves neurodevelopment. To better understand the physiopathological role of the increased levels of *Fmr1* mRNA during neuronal differentiation, we used a small interfering RNA (siRNA) approach to reduce the abundance of this mRNA in cultured cortical neurons from the FXTAS mouse model. Morphological alterations of neurons were rescued by this approach. This cellular phenotype is associated to differentially expressed proteins that we identified by mass spectrometry analysis. Interestingly, phenotype rescue is also associated to the rescue of the abundance of 29 proteins that are involved in various pathways, which represent putative targets for early therapeutic approaches.

INTRODUCTION

The *Fragile X Mental Retardation 1 (FMRI)* gene encodes the fragile X mental retardation protein (FMRP), an RNA binding protein whose functional absence causes fragile X syndrome (FXS), the most common form of intellectual disability (ID) and autism spectrum disorder (ASD). The mutation in the *FMRI* gene causing FXS is the presence of a repeated sequence encompassing >200 CGG repeats in its 5' UTR. Hypermethylation of this sequence determines

gene promoter inactivation, causing the silencing of the *FMRI* gene.¹ Although 6–54 CGG repeats in the 5' UTR of *FMRI* is a polymorphism in normal individuals, a repeat sequence of variable length (55–200 CGG repeats) represents the premutation¹ that can cause fragile X tremor ataxia syndrome (FXTAS) in patients over 50 years of age.^{2,3} This is an adult-onset progressive neurodegenerative disorder leading to a variable combination of ataxia, essential tremor, gait imbalance, parkinsonism, peripheral neuropathy, anxiety, and cognitive decline, occurring predominantly in older men carrying the premutation. It is known that people carrying the premutation have a reduced hippocampal volume that correlates with impaired performance in standardized tests of memory.³ At the cellular level, this disorder is characterized by the presence of eosinophilic, ubiquitin-positive nuclear inclusions, which have been observed throughout the brain, with a high percentage being located in the hippocampus of patients, as well as in the animal model of the disease.^{3,4} Inclusions are negative for tau isoforms, alpha-synuclein, or polyglutamine peptides, reflecting a new class of inclusion disorder.³ At the molecular level, FXTAS is characterized by an elevated (2- to 8-fold) level of *FMRI* mRNA, whereas the level of FMRP is normal or slightly reduced in patients, as well in the CGG-KI mouse model.^{2–4} The *FMRI* mRNA is a component of nuclear inclusions³. The product of repeat-associated non-ATG (RAN) translation of the *FMRI* mRNA was also reported to be involved in the generation of inclusions when overexpressed.^{5,6} Although FXTAS is a late-onset disorder, it is also characterized by a set of developmental hallmarks, such as self-reported memory problems, autism-related traits, attention deficit hyperactivity disorder (ADHD), executive functioning, and psychopathology.⁷ Knockin (KI) mouse models have been generated displaying both neurodegenerative and neurodevelopmental

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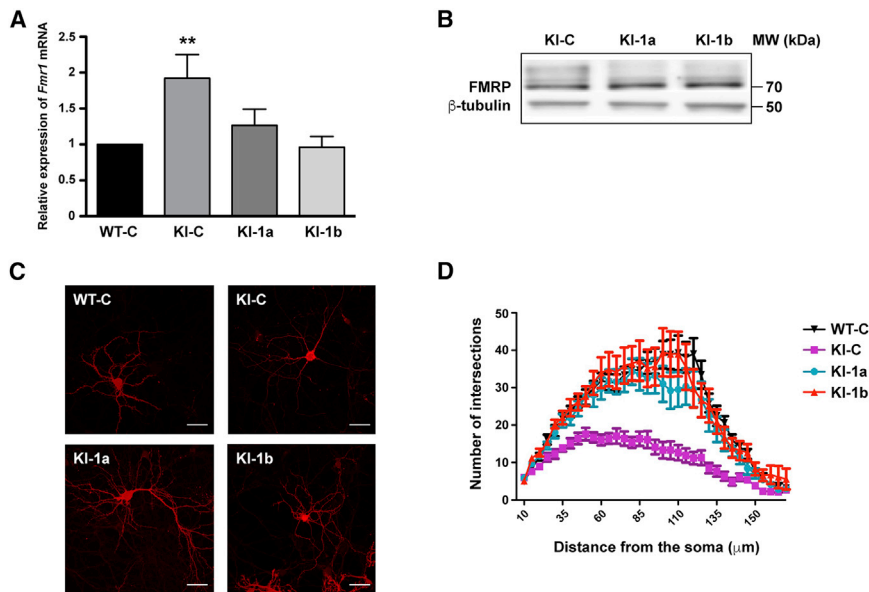


Figure 1. Role of *Fmr1* mRNA Levels in Dendritic Arborization

(A) RNA was prepared from cultured WT and knockin-CGG (KI-CGG) neurons transduced with the control (C) shRNA or two different shRNAs directed against *Fmr1* mRNA (1a and 1b). The level of *Fmr1* mRNA was measured by qRT-PCR using specific primers. Ten different experiments have been carried out for each transduced lentivirus, and a reduction of *Fmr1* levels was observed by using both shRNAs. Results are presented as mean \pm SEM; one-way ANOVA with Tukey's multiple comparisons test, ** $p < 0.01$. (B) Representative western blot analysis of cell cultures of cortical neurons transduced with C, 1a, or 1b shRNAs. FMRP (70 kDa) and β -tubulin (50 kDa) were revealed with specific antibodies. (C) Image of WT and KI-CGG cortical neurons transduced with lentiviruses expressing C, 1a, or 1b shRNAs. Scale bars: 20 μ m. (D) Sholl analysis of WT and KI cultured mouse cortical neurons transduced with C or 1a or 1b shRNAs. Reduced arborization of KI-CGG neurons is rescued by *Fmr1* knockdown. Two-way ANOVA was used to compare KI-C and 1a treatments: genotype F(2; 1,527) = 227.9, $p < 0.0001$; treatment (34; 1,527) = 34.12, $p < 0.0001$; interaction F(68; 1,527) = 3.067, $p < 0.0001$; two-way ANOVA was used to compare KI-C and 1b treatments: genotype F(2; 1,318) = 293.6, **** $p < 0.0001$; treatment F(34; 1,318) = 36.21, **** $p < 0.0001$; interaction F(68; 1,318) = 3,803, $p < 0.0001$.

phenotypes. It was reported that neuronal abnormalities and behavioral alterations in the animal model are present before the appearance of neuronal inclusions. Indeed, cultured hippocampal neurons obtained from a CGG-KI display shorter dendritic length and reduced dendritic complexity.^{4,8-10} In CGG-KI mice, cortical migration is also affected. Indeed, at embryonic day (E) 17, a 2-fold higher percentage of migrating neurons was detected to be oriented toward the ventricle in wild-type (WT) compared with CGG-KI mice.¹¹ All of these abnormalities were observed in inclusion-free cells, because CGG-KI mice display ubiquitin-positive intranuclear inclusions in neurons and astrocytes of the hippocampus and cerebellar internal cell layer starting at 12 weeks of age.^{4,8,9,11} Here we investigated the impact of normalization of *Fmr1* mRNA levels on the morphology and proteomics of cortical cultured neurons obtained from a FXTAS mouse model (CGG-KI)^{8,9} before the generation of nuclear inclusions. By this analysis we have obtained a deeper insight into the physiopathological role of *Fmr1* mRNA levels in FXTAS and identified putative targetable pathways for early treatments.

RESULTS

Hippocampal neurons were isolated from knockin-CGG (KI-CGG) E15.5 mice harboring the CGG premutated allele. It has been shown that these mice display abnormal cortical neuron migration patterns *in utero*.^{10,11} Furthermore, abnormal dendritic architecture and reduced cellular viability have been observed in hippocampal primary neurons of KI-CGG mice, where increased expression of *Fmr1* is already present even if nuclear inclusions are not detected.¹⁰ Indeed, nuclear inclusions appear first in the hippocampus of these mice at the age of 3 months, whereas the same hallmark appears later in the parietal neocortex.^{8,9} We decided to explore whether cultured cortical neurons obtained from these mice have an abnormal

morphology of dendrites and axons during development before the appearance of nuclear inclusions. We studied the dendritic arborization of wild-type (WT) and KI-CGG cortical neurons by Sholl analysis, as previously described,¹² and observed that KI-CGG neurons have a reduced arborization compared with controls (Figures S1A and S1B). To assess whether *Fmr1* mRNA levels impact the morphology of these cultured neurons, we produced lentiviruses expressing two different shRNAs selectively reducing the expression of *Fmr1* mRNA (sh-1a and sh-1b) and one shRNA control (sh-C),¹³ and we used them to transduce cortical cultured neurons obtained from WT and from KI-CGG mice. The infection was performed at 5 DIV (days *in vitro*), and RNA and proteins were prepared from these cultures at 20 DIV. As expected,^{4,13} *Fmr1* mRNA levels were elevated 2-fold in cultured KI cortical neurons compared with WT. sh-1a reduced *Fmr1* transcript levels by 30%, whereas sh-1b reduced them by 50%, (Figure 1A). FMRP levels are not changed in KI cultured neurons compared with WT,^{10,13} and as previously shown in patients.^{2,3} Similarly, the expression level of FMRP was not affected by *Fmr1* knockdown in KI neurons (Figure 1B), as we already observed by transfecting fibroblasts obtained from FXTAS patients with the same shRNAs.¹³ We then analyzed the arborization of WT and KI cortical neurons transduced with *Fmr1* shRNAs. We confirmed that KI neurons transduced with the lentivirus expressing the control shRNA (KI-C) are less arborized than WT neurons transduced with the same lentivirus (Figures 1C and 1D). However, KI neurons transduced with lentivirus expressing either sh-1a or sh-1b displayed a normal dendritic arborization (Figures 1C and 1D).

In 2 DIV neurons we measured the axon length, and we found that they are significantly shorter in *Fmr1*-KI neurons compared with

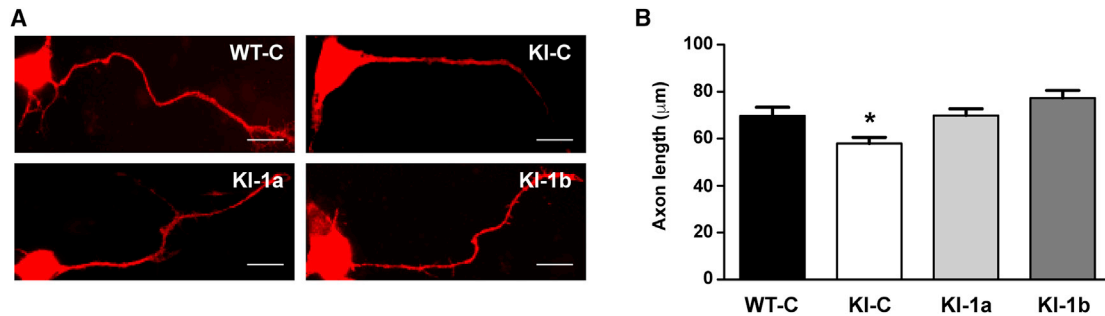


Figure 2. *Fmr1* mRNA Levels Are Associated to Axon Growth

(A) Representative pictures of 2 days *in vitro* cultured WT-C, KI-C, KI-1a, and KI-1b primary cortical neurons. Scale bars: 10 μm. (B) Histogram of axon length of WT and KI cultured neurons transfected with C, 1a, or 1b shRNAs. Results show mean axon length ± SEM of 150 randomly selected cells for each condition from three independent cultures. One-way ANOVA with Tukey's multiple comparisons test, * $p < 0.05$.

WT (Figure S2). We confirmed that KI-C neurons have shorter axons than WT-C, but the length of these axons was normalized in KI-1a and KI-1b neurons (Figures 2A and 2B).

We then focused on dendritic spine abnormalities. The absence of FMRP causes a peculiar morphology of dendritic spines that has been described in FXS patients and in the mouse model, where it is particularly evident in young animals.^{14,15} Conversely, the study of dendritic spines in the brain of FXTAS patients was never reported, and in *Fmr1* KI mice it was studied only in hippocampi and in the visual cortex from adult animals, displaying an increased length of spines.^{4,16} Here we studied the morphology of dendritic spines of 20 DIV cultured cortical neurons. KI-C spines are longer, denser, and larger than controls. All of these hallmarks are rescued in KI-1a and KI-1b neurons (Figures 3A–3D), indicating that the elevated level of *Fmr1* mRNA causes a set of morphological alterations in KI-CGG neurons. However, the percentage of the different types of spines (thin, stubby, and mushroom) is not significantly different in the two genotypes (Figures 3E–3G).

To get deeper insight into the molecular pathology of FXTAS cultured neurons, we performed a proteomic analysis of neurons that have been transduced with control shRNA or with the shRNAs targeting the *Fmr1* mRNA. Protein extracts of three replicates for each condition were analyzed by nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) after tryptic digestion.¹⁷ A total of 487 proteins were identified (Table S1), among which 251 have a ratio >2 (upregulation) or <0.5 (downregulation) for at least one of the three comparisons (WT-C versus KI-C, WT-C versus KI-1a, and WT-C versus KI-1b) with a p value <0.10. Interestingly, the abundance of 29 differentially expressed proteins is rescued after reduction of *Fmr1* mRNA by using both specific shRNAs (Table 1). Gene Ontology analysis of these proteins reveals a significant enrichment of proteins involved in different biochemical pathways, among which are GTP binding and RNA binding proteins (Figure 4; Table S2).

DISCUSSION

Two main neurological phenotypes have been described in carriers of the CGG premutation: those exhibiting neurodevelopmental disorders characterized by anxiety, ADHD, social deficits, or ASD, and after 50 years old, FXTAS, a neurodegenerative disorder.³ For some time, nuclear inclusions have been considered as the cause of neurodegeneration, whereas more recent studies suggest that nuclear inclusions may represent a mechanism used by neurons to protect themselves from toxic events.^{4,18–20} So far, two other main physiopathological elements are known to underpin the FXTAS phenotype: the elevated abundance of *FMR1* mRNA² and the presence of a RAN polypeptide.^{5,6} We considered that it is crucial to unravel the role of each element in the molecular pathology of FXTAS to understand the progression of the disorder and its role in pathophysiology. Indeed, it is interesting to remind that neuronal abnormal dendritic morphology (e.g., reduced length and number of dendrites that display longer spines) has been observed in FXTAS neurons at a time of development when nuclear inclusions are not detectable.^{3,4,8–11} These findings suggest that some developmental abnormalities may contribute to the late manifestation of neurodegenerative process and/or that the disease appears, with subtle phenotypes, earlier than predicted up to date. So far, no specific treatment is available for FXTAS patients. A therapy based on allopregnanolone was shown to improve cognitive functioning in patients with FXTAS and to partially alleviate some aspects of neurodegeneration,²¹ opening the possibility to search for new targetable pathways in young patients. In this study, we used a murine model of FXTAS to investigate the impact that the reduced level of *Fmr1* mRNA, but not of its encoded protein, has on the morphological and molecular phenotypes of FXTAS cultured neurons. Certainly, by reducing *Fmr1* mRNA levels, we are also supposed to reduce RAN levels, which is possibly involved in the pathophysiology of FXTAS.⁶ However, that peptide was never detected at endogenous levels in neurons so far.^{3,22}

First, we defined phenotypes that were never considered before in cultured cortical neurons obtained from the FXTAS mouse

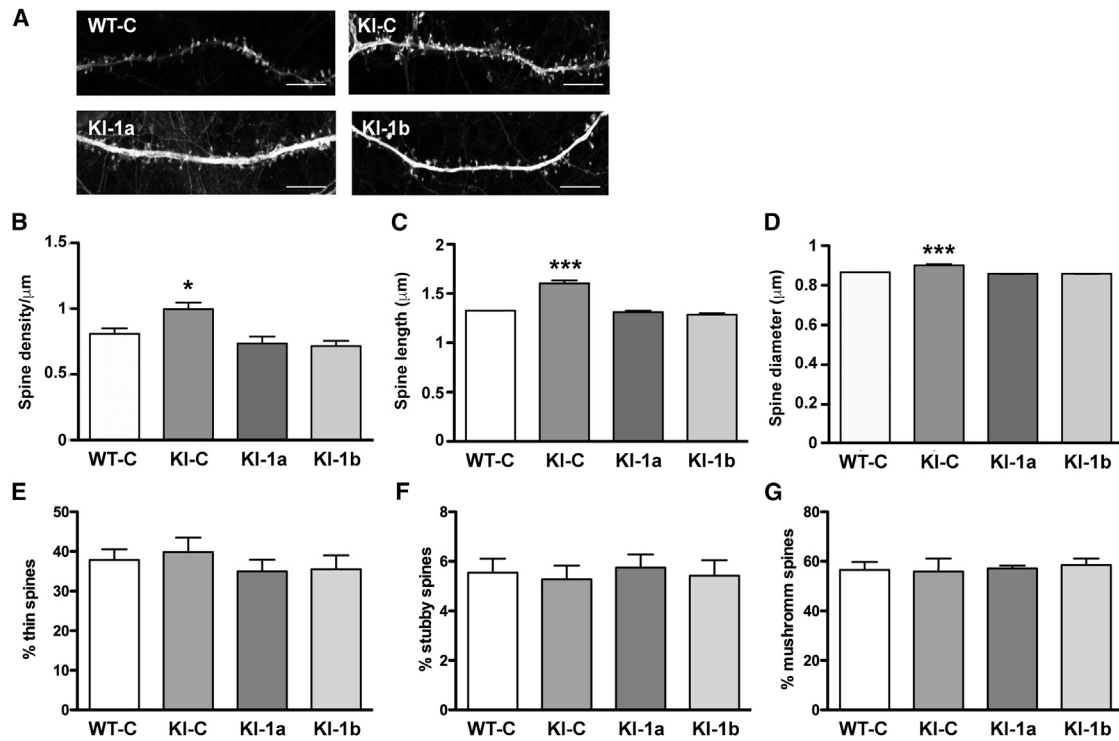


Figure 3. Reduction of *Fmr1* mRNA Levels Normalizes Dendritic Spine Morphology in KI Cortical Neurons

(A) Representative high-resolution confocal images showing dendritic spine morphology were assessed using NeuronStudio software and compared with the measurement obtained from control transduced neurons. Scale bars: 2 μm . All histograms present mean \pm SEM values. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons test. (B–G) Histograms showing the mean \pm SEM values of (B) protrusion frequency in the various cultures, * $p < 0.05$; (C) spine length, *** $p < 0.001$; (D) spine head size, *** $p < 0.001$; (E) percentage of thin spines; (F) percentage of stubby spines; and (G) percentage of mushroom spines.

model: axon length and dendrite morphology. All of these new phenotypes are detected in cells not yet displaying nuclear inclusions, which are known to first appear in hippocampal neurons of 12-week-old KI-CGG mice.⁹ Interestingly, these animals have mild learning and memory deficits,^{4,9} and this behavioral phenotype is consistent with the phenotype that we observed in cultured cortical neurons. Importantly, we confirm that these phenotypes are associated with the levels of *Fmr1* mRNA because they are rescued after the expression of shRNAs specifically targeting this mRNA. We can deduce that an elevated abundance of *Fmr1* mRNA may interfere with normal RNA metabolism. We can predict that the overexpression of *Fmr1* mRNA may also likely interfere with the normal activity of RNA binding proteins or microRNAs (miRNAs) in neuronal soma, and at the synapse by competing for their binding at specific sites or at miRNA response elements (MREs).¹³ All of these considerations suggest that *Fmr1* mRNA metabolism can be regulated by a large number of RNA binding proteins and can be co-regulated with a plethora of other RNAs. For this reason, as the second step of our study, we performed a differential proteomic analysis of neurons expressing control or *Fmr1*-specific shRNAs, and we observed that a set of proteins, whose expression is deregulated in KI-CGG neurons, is normalized after treatment. Consistently, several proteins rescued by *Fmr1*-specific shRNAs are RNA binding proteins (e.g., Tial1,

Hnrnp11, and ROAA). In addition, some proteins whose levels were rescued by the reduction of *Fmr1* mRNA abundance are involved in the regulation of actin cytoskeleton dynamics, which is known to modulate the morphology of neurons and, in particular, of dendritic spines. Defective synaptic actin regulation seems to be involved in different neurodevelopmental and psychiatric disorders.²³ Interestingly, we have shown here that in KI-CGG neurons, dendritic spines are more numerous, and they appear overall longer and with a larger head, but the percentage of the various types of spines is unchanged. These results are consistent with a previous study displaying longer, but not immature, spines in the CGG-KI visual cortex.¹⁶ In addition, our findings suggest that, at least at 20 DIV, the elevated levels of *Fmr1* mRNA do not interfere with the spine maturation process but cause subtle abnormalities of their features, which may have an impact on synaptic transmission. Other rescued proteins belong to the Rab-GTPase family, a sub-class of the RAS superfamily, which spatially and temporally orchestrates specific vesicular trafficking that is critical for synaptic function in neurons in brain developmental disorders,²⁴ as well as in Parkinson's disease.²⁵ Along the same direction, another interesting protein is CLIP2, a cytoplasmic linker factor that is considered as a mediator between organelles and the cytoskeleton.²⁶ Consistent with their role in neurons, mutants of this class of proteins are associated with impaired cognitive

Table 1. Proteins Differentially Expressed in the Different Samples Studied

Name	UniProt Accession Number	WT-C	KI-C	WT-C/ Ki-C	Log (Fold Change)	p Value	KI-1a	WT-C/ Ki-1a	Log (Fold Change)	p Value	KI-1b	WT-C/ Ki-1b	Log (Fold Change)	p Value
sp O89023 TPP1_MOUSE	O89023	0.00	0.93	0.00	NA	0.00001	0.00	NA	NA	1.00000	0.00	NA	NA	1.00000
sp P05132 KAPCA_MOUSE	P05132	5.16	10.17	0.51	-0.29	0.00132	5.83	0.89	-0.05	0.81905	4.79	1.08	0.03	0.81905
sp P21126 UBL4A_MOUSE	P21126	0.33	1.53	0.21	-0.67	0.04762	0.66	0.49	-0.31	0.51266	0.38	0.85	-0.07	0.51266
sp P21278 GNA11_MOUSE	P21278	7.43	4.06	1.83	0.26	0.03470	6.49	1.14	0.06	0.69780	7.98	0.93	-0.03	0.69780
sp P24369 PPIB_MOUSE	P24369	8.43	12.31	0.68	-0.16	0.03540	6.73	1.25	0.10	0.68137	9.91	0.85	-0.07	0.68137
sp P30677 GNA14_MOUSE	P30677	2.57	0.93	2.77	0.44	0.04928	2.40	1.07	0.03	0.88974	3.24	0.79	-0.10	0.88974
sp P35283 RAB12_MOUSE	P35283	5.82	3.08	1.89	0.28	0.00979	5.87	0.99	0.00	0.95886	5.51	1.06	0.02	0.95886
sp P52912 TIA1_MOUSE	P52912	0.00	0.93	0.00	NA	0.00001	0.00	NA	NA	1.00000	0.32	0.00	NA	1.00000
sp P56371 RAB4A_MOUSE	P56371	5.81	3.08	1.89	0.28	0.00560	5.87	0.99	0.00	0.94159	5.19	1.12	0.05	0.94159
sp Q80TS3 AGRL3_MOUSE	Q80TS3	0.97	2.16	0.45	-0.35	0.01678	0.37	2.64	0.42	0.17665	0.38	2.53	0.40	0.17665
sp Q8BGH2 SAM50_MOUSE	Q8BGH2	2.28	0.29	7.74	0.89	0.01329	2.07	1.10	0.04	0.79778	2.13	1.07	0.03	0.79778
sp Q8BHC4 DCAKD_MOUSE	Q8BHC4	0.00	0.93	0.00	NA	0.00001	0.31	0.00	NA	0.37390	0.00	NA	NA	0.37390
sp Q8CHT0 AL4A1_MOUSE	Q8CHT0	2.94	0.63	4.64	0.67	0.02926	2.43	1.21	0.08	0.54007	1.95	1.51	0.18	0.54007
sp Q91ZR1 RAB4B_MOUSE	Q91ZR1	5.82	3.68	1.58	0.20	0.02923	5.51	1.05	0.02	0.71442	5.19	1.12	0.05	0.71442
sp Q921F4 HNRL1_MOUSE	Q921F4	0.97	0.00	∞	NA	0.00000	0.98	0.99	0.00	0.98875	0.78	1.24	0.09	0.98875
sp Q922D8 C1TC_MOUSE	Q922D8	1.28	2.78	0.46	-0.34	0.00809	0.92	1.40	0.15	0.72353	1.29	1.00	0.00	0.72353
sp Q99020 ROAA_MOUSE	Q99020	1.63	0.31	5.34	0.73	0.04589	1.69	0.97	-0.02	0.90543	2.19	0.74	-0.13	0.90543
sp Q99PU5 ACBG1_MOUSE	Q99PU5	2.92	1.55	1.88	0.27	0.01399	2.73	1.07	0.03	0.62466	2.61	1.12	0.05	0.62466
sp Q9CPY7 AMPL_MOUSE	Q9CPY7	1.61	3.38	0.48	-0.32	0.00921	2.00	0.81	-0.09	0.53465	1.43	1.13	0.05	0.53465
sp Q9CW03 SMC3_MOUSE	Q9CW03	0.33	1.53	0.21	-0.67	0.04762	0.71	0.46	-0.34	0.64890	0.38	0.85	-0.07	0.64890
sp Q9CYR6 AGM1_MOUSE	Q9CYR6	0.33	1.86	0.18	-0.75	0.01091	0.00	∞	∞	0.37390	0.32	1.04	0.02	0.37390
sp Q9DCN2 NB5R3_MOUSE	Q9DCN2	4.53	6.79	0.67	-0.18	0.00385	4.79	0.95	-0.02	0.62926	4.48	1.01	0.00	0.62926
sp Q9WTX2 PRKRA_MOUSE	Q9WTX2	0.33	1.53	0.21	-0.67	0.04762	0.00	∞	∞	0.37390	0.00	∞	∞	0.37390
sp Q9Z0H8 CLIP2_MOUSE	Q9Z0H8	4.51	8.09	0.56	-0.25	0.04003	4.68	0.96	-0.02	0.88427	5.62	0.80	-0.10	0.88427
tr A0A087WPM2 A0A087WPM2_MOUSE	A0A087WPM2	1.94	2.78	0.70	-0.16	0.00099	1.64	1.18	0.07	0.61624	1.12	1.73	0.24	0.61624
tr A0AUM9 A0AUM9_MOUSE	A0AUM9	0.00	0.93	0.00	NA	0.00001	0.00	NA	NA	1.00000	0.00	NA	NA	1.00000
tr D3YZ68 D3YZ68_MOUSE	D3YZ68	39.80	47.10	0.84	-0.07	0.03447	38.80	1.03	0.01	0.89853	36.50	1.09	0.04	0.89853
tr E9Q7C9 E9Q7C9_MOUSE	E9Q7C9	7.46	9.91	0.75	-0.12	0.03009	5.75	1.30	0.11	0.10120	7.97	0.94	-0.03	0.10120
tr S4R232 S4R232_MOUSE	S4R232	5.50	3.04	1.81	0.26	0.03279	5.50	1.00	0.00	0.99389	5.17	1.06	0.03	0.99389

NA, not applicable.

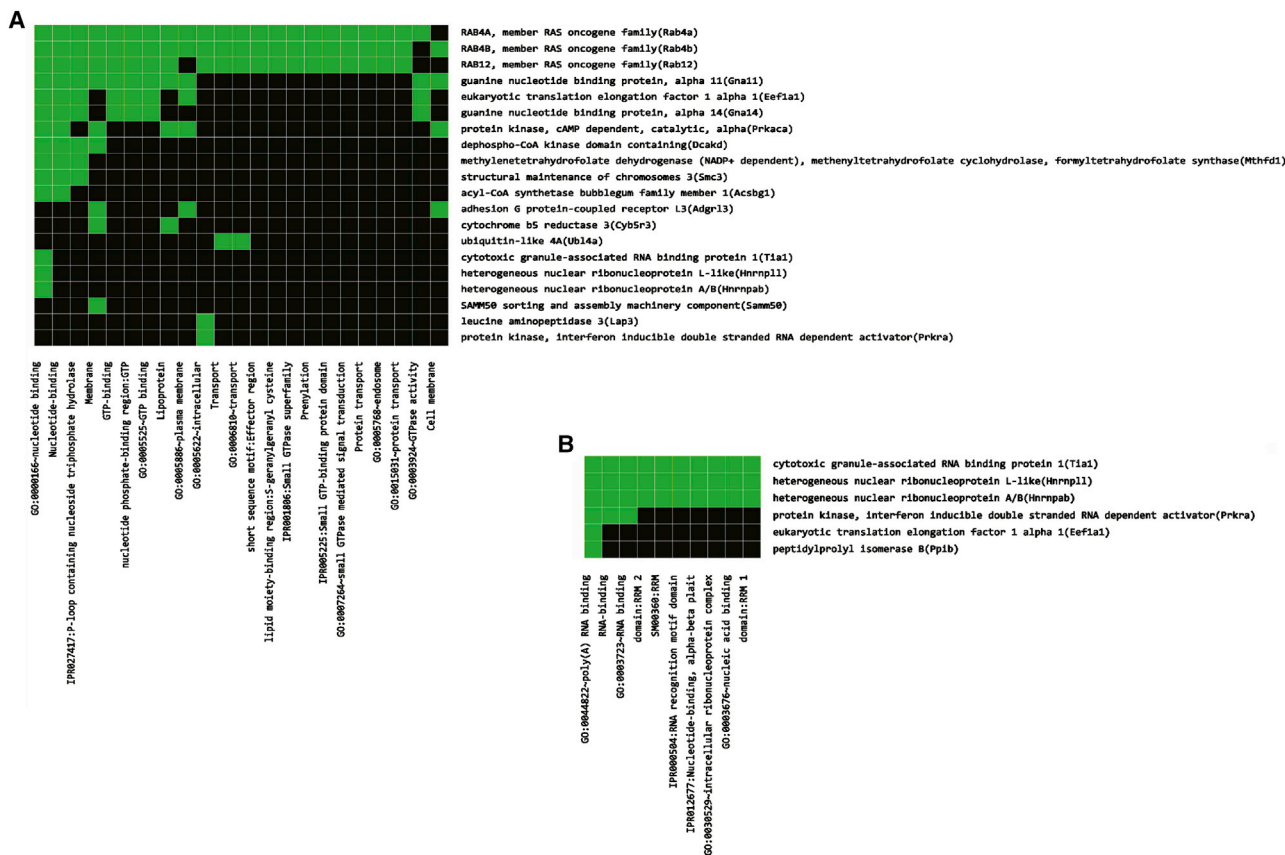


Figure 4. Gene Ontology Classification of Rescued Proteins in KI-CGG Neurons

(A and B) The DAVID Gene Ontology Functional Annotation Clustering tool⁴⁰ was used to show functional classification of rescued proteins in KI-CGG neurons involved in (A) nucleotide-GTP binding and (B) RNA binding.

functions.^{21,24,27} Importantly, we also found mitochondrial proteins (e.g., pyrroline-5-carboxylate dehydrogenase [Aldh4a1/P5CDH] and Samm50) that are deregulated in CGG-KI neurons, confirming the importance of mitochondria in the pathophysiology of FXTAS, as previously reported by independent studies.^{28,29} This result suggests that an altered mitochondrial function is probably involved in early phenotypes of this disorder. Remarkably, the levels of those proteins were normalized by the reduction of *Fmr1* mRNA abundance. Thus, our data show molecular alterations that may contribute to explain the neurodevelopmental phenotypes of the mouse model and patients carrying the CGG premutation, thus providing an indication for future early therapies to treat CGG-premutation carriers. For instance, Aldh4a1/P5CDH is a mitochondrial matrix NAD(+)-dependent dehydrogenase that catalyzes the second step of the proline degradation pathway, converting pyrroline-5-carboxylate to glutamate. Altered function of this enzyme can result in a deregulation of the glutamate signaling, which is at the basis of various forms of brain disorders, as well as of Parkinson’s disease.^{30,31} Thus, Aldh4a1/P5CDH could be a therapeutic target for FXTAS patients during different periods of life and phases of the disorder associated with the CGG premutation. Other targets may be small GTPases by

using, in this case, a strategy based on the modulation of their regulators, as recently proposed.^{32,33} In conclusion, by our approach, we defined altered pathways in FXTAS neurons that are due to the increased levels of *Fmr1* mRNA and impact on neuronal morphology. They represent the molecular pathology underpinning the late FXTAS phenotype. In particular, differentially expressed proteins between WT and CGG-KI neurons are promising pharmacological targetable molecules for early therapeutic intervention for FXTAS. On the other side, future gene therapies could also target the CGG-repeat by excising it, as already shown in an induced pluripotent stem cell (iPSC) line.³⁴

MATERIALS AND METHODS

Animals

The experiments were performed following the Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines.³⁵ *Fmr1* knockin (KI) and wild-type (WT) mice on a C57BL/6J congenic background were produced as described previously.³⁶ All animals were generated and housed in groups of four in standard laboratory conditions (22°C, 55% ± 10% humidity, and 12-h light/12-h dark diurnal cycles) with food and water provided *ad libitum*. Animal care was

conducted in accordance with the European Community Directive 2010/63/EU. All experiments were approved by the local ethics committee (Comité d’Ethique en Expérimentation Animale CIEPAL-AZUR N. 00788.01).

Lentivirus Generation

Lentivirus particles were produced as previously described.³⁷

Axon Length Measurements

Dissociated neurons were transfected with lentivirus plasmids by using Nucleofector as previously described.¹³ Neurons were cultured for 48 h and then fixed. Axons were labeled with anti-Tuj1 antibody, and the size of individual growing axons (distance from the soma to the tip of the axon) was manually measured using the ImageJ software.¹⁵

Neuronal Culture and Dendritic Spine Morphology Analysis

Primary neurons were prepared from E15.5 pregnant C57BL/6 *Fmr1*^{CGG/ly} and WT mice as previously described.^{12,15} Neurons (5 days *in vitro*) were transduced with lentivirus as previously described.¹² Transduced neurons (20 days *in vitro*) were rinsed twice in PBS at room temperature (RT) after 19 h of transduction and then fixed with 4% paraformaldehyde and Triton-permeabilized. Sequential confocal images (512 × 220 pixels; zoom 3.0; average 4; speed 7) of GFP-expressing neurons were acquired with a 63× oil-immersion lens (NA 1.4) on an inverted Zeiss LSM780 confocal microscope. z series of seven to eight images of randomly selected secondary dendrites were analyzed using the NeuronStudio software, which allows for the automated detection and quantification of dendrite parameters and morphological classification, as previously performed.¹⁵

Protein Extraction and Western Blot Analysis

Immunoblotting was performed as follows: cells or grinded tissues were homogenized in lysis buffer, and debris was removed by centrifugation (20,000 × g, 10 min, 4°C).³⁸ Protein content in the supernatant was measured using the Bradford assay (Bio-Rad), and samples were separated on NuPAGE Bis-Tris 4%–12% gels in MOPS buffer. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with PBS-Tween (0.05%) and milk (5%), and incubated with primary antibodies overnight.

Antibodies

The monoclonal 1C3 anti-FMRP antibody³⁹ was used at a 1:1,000 dilution, the anti-Tuj1 (BioLegend; TUJ1 1-15-56) antibody was used following the manufacturer’s instructions, and the monoclonal anti-β-tubulin antibody (Sigma) was used at a 1:10,000 dilution.

qRT-PCR

qPCR was performed on a LightCycler 480 (Roche) with MasterMix SYBRGreen (Roche) as previously described.¹³ Primers used to amplify *Fmr1* mRNA were previously reported.³⁷

Protein Identification and Analysis

Proteomic analysis was performed as previously described¹⁷ and resulted in the identification of around 2,000 proteins for each condition. These proteins were then compared to highlight the proteins identified under only one condition or under several conditions (“on/off” effect) and to highlight proteins predominantly identified in one condition relative to another (up if ratio >2, down if ratio <0.5). In order to compile a short list of differentially regulated proteins, a Student’s t test was performed after normalization of the spectral count quantification data. This allowed us to obtain a p value used to make this short list: from the global alignment table of the 2,888 different proteins, we focused on proteins with a p value threshold less than 0.10.

Statistical Analysis

Results are expressed as mean ± SEM. All statistical analyses were based on biological replicates. Appropriate statistical tests used for each experiment are described in the corresponding figure legends. All statistical analyses were carried out using GraphPad Prism version 6.0e.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.09.018>.

AUTHOR CONTRIBUTIONS

M.D., S.D., T.M., S.M., M.G., F.B., M.J., and M.C. performed the experiments; R.W. and R.K.H. provided material; T.M., E.L., and B.B. designed the experiments; M.C., E.L., R.K.H., and B.B. wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Publication 4



Modeling Fragile X Syndrome in *Drosophila*

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Intellectual disability (ID) and autism are hallmarks of Fragile X Syndrome (FXS), a hereditary neurodevelopmental disorder. The gene responsible for FXS is Fragile X Mental Retardation gene 1 (*FMR1*) encoding the Fragile X Mental Retardation Protein (FMRP), an RNA-binding protein involved in RNA metabolism and modulating the expression level of many targets. Most cases of FXS are caused by silencing of *FMR1* due to CGG expansions in the 5'-UTR of the gene. Humans also carry the FXR1 and FXR2 paralogs of *FMR1* while flies have only one *FMR1* gene, here called *dFMR1*, sharing the same level of sequence homology with all three human genes, but functionally most similar to *FMR1*. This enables a much easier approach for *FMR1* genetic studies. *Drosophila* has been widely used to investigate *FMR1* functions at genetic, cellular, and molecular levels since *dFMR1* mutants have many phenotypes in common with the wide spectrum of *FMR1* functions that underlay the disease. In this review, we present very recent *Drosophila* studies investigating FMRP functions at genetic, cellular, molecular, and electrophysiological levels in addition to research on pharmacological treatments in the fly model. These studies have the potential to aid the discovery of pharmacological therapies for FXS.

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INTRODUCTION

The Fragile X Syndrome (FXS), previously known as Martin-Bell syndrome or marker X syndrome or FRAXA, is the first X-linked intellectual disability (ID) syndrome described involving a DNA alteration and the most frequent heritable monogenic form of ID (reviewed in Penagarikano et al., 2007; Santoro et al., 2012; Hayward et al., 2017). Human FXS patients present severe ID often accompanied by an increase in Autism Spectrum Disorder (ASD) traits and other phenotypes like delayed development, hyperactivity, attention deficit, hypersensitivity to sensorial stimuli, anxiety, aggression, sleep, cardiac disorders, and epileptic seizures (reviewed in Hagerman, 2002; Garber et al., 2008; Utari et al., 2010; Santoro et al., 2012; Hagerman et al., 2014; Kidd et al., 2014; Maurin et al., 2014; Schaefer et al., 2015; Dahlhaus, 2018). These abnormalities can be explained by defects in neuronal development and maturation. Some patients also present characteristic morphological facial traits, macrocephaly, flat feet, and male macroorchidism. The first morphological phenotype observed in FXS patients was the presence of abnormalities in the spines (Comery et al., 1997; Irwin et al., 2001). More recently, ElectroEncephaloGraphy (EEG) and Magnetic Resonance Imaging (MRI) have evidenced volume and Event Related Potential (ERP) defects in FXS patients (Devitt et al., 2015).

FXS was initially associated with an X-chromosome fragile site (an isochromatid gap in metaphase chromosomes) in position Xq27.3 (Harrison et al., 1983). In 1991, this site was mapped to a CGG trinucleotide expansion in the 5' non-coding region of a gene named *Fragile X Mental Retardation 1 (FMR1)*, the first gene associated with an X-linked ID (Verkerk et al., 1991). *FMR1* is 38 kb long and transcribed in a 4.4 kb full length mRNA that encodes a 632 aa protein called Fragile X Mental Retardation Protein (FMRP). Through alternative splicing, at least 12 different isoforms of 67–80 kD are produced. The CGG repeats are polymorphic in the population ranging from 5 to 54 repeats in normal individuals to more than 200 (full mutation) in severely affected patients (reviewed in Hayward et al., 2017). The repeat expansion results in hypermethylation of the CGG repeat, of a 5' CpG island, and of flanking promoter sequences causing the reduction or absence of *FMR1* expression through an epigenetic mechanism involving *FMR1* mRNA (Colak et al., 2014). Several deletions and point mutations leading to the production of non-functional proteins have also been described (Okroy et al., 2015 and references therein). Individuals with 55–200 CGG repetitions (premutation) do not present FXS symptoms, but may develop two other disorders: Fragile-X Primary Ovarian Insufficiency (FXPOI) (reviewed in Sherman et al., 2014) or Fragile X Associated Tremor/Ataxia Syndrome (FXTAS) (reviewed in Hall et al., 2016; Dahlhaus, 2018). FXTAS has been modeled in *Drosophila* by overexpressing 90 rCGG repeats alone fused to GFP, which causes a neuron-specific degeneration and the formation of inclusions (Jin et al., 2003; Qurashi et al., 2012).

In mammals, FMRP is nearly ubiquitous, present mainly in neurons (particularly in the cortex, hippocampus, and Purkinje cells) and in testes and absent from muscles and the heart (Devys et al., 1993). FMRP has two paralogs: Fragile X Related 1 (*FXR1*) and Fragile X Related 2 (*FXR2*). While *FXR2* has a distribution comparable to that of FMRP, some isoforms of *FXR1* display a specific expression in brain while other isoforms are only present in muscle and heart (Khandjian et al., 1998; Bechara et al., 2007). These three proteins are members of the same family, namely the Related Fragile X Protein family, and are RNA-binding proteins mainly localized in the cytoplasm, although they carry a Nuclear Localization Signal (NLS) and a Nuclear Exportation Signal (NES) (Bardoni et al., 2000). Indeed, some isoforms of FMRP have also been localized in the nucleus (Eberhart et al., 1996; Bardoni et al., 1997). Collectively, these results have suggested that the three FXR proteins are able to shuttle between nucleus and cytoplasm to export their target mRNAs.

Three RNA-binding sequence motifs are the hallmarks of FMRP that may explain its function, *i.e.*, two K homology (KH) domains and one Arginine-Glycine-Glycine (RGG) box. The main function of FMRP is to regulate translation and indeed it has been found associated with polyribosomes in different cell lines and, importantly, in the brain (Khandjian et al., 2004). Although FMRP mainly acts as a repressor, an activator function has been observed (reviewed in Maurin et al., 2014). Many methods have been used to identify FMRP targets (reviewed in Maurin et al., 2014; Davis and Broadie, 2017; Hayward et al., 2017): binding to biotinylated RNAs (Ashley

et al., 1993), Cross-Link Immuno Precipitation (CLIP) (Darnell et al., 2011; Ascano et al., 2012; Anderson et al., 2016), Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Chen et al., 2003), yeast two-hybrid system (Ma et al., 2016), yeast three-hybrid system (Dolzanskaya et al., 2003), and Antibody-Positioned RNA Amplification (APRA) (Miyashiro et al., 2003). Many of the identified targets have been involved in autism, other neuronal pathologies or gonadal development and many of them encode synaptic proteins (reviewed in Maurin et al., 2014). Finally, FMRP has been linked to the microRNA (miRNA) and Piwi-interacting RNA (piRNA) pathways in Mammals, *Drosophila*, and zebrafish (reviewed in Kelley et al., 2012; Specchia et al., 2017).

FUNCTIONAL INSIGHTS ON FXS FROM DROSOPHILA STUDIES

The first model of FXS was the mammalian mouse model (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006), which recapitulates some major patients' phenotypes (Dahlhaus, 2018 and references therein). However, ever since then, also research on *Drosophila melanogaster* has brought important knowledge on the basic mechanisms underlying FMRP function. The *Drosophila* homolog of *FMR1* was first identified in 2000 (Wan et al., 2000) and named *dfmr1*. Over the years, it has been called by many different names that are listed in the *Drosophila* database FlyBase (<http://flybase.org/reports/FBfrf0174476.html>). It is now named *Fmr1* with a capital F, meaning that it has been identified through the human homolog *FMR1*. Here, we will call it *dFMR1* to distinguish it from the mouse gene (*Fmr1*). FlyBase names the protein *Fmr1*, but here we will call it *dFMRP* with the "d" indicating *Drosophila*. The *dFMR1* gene exhibits high sequence homology with all three human genes (FMRP, *FXR1*, and *FXR2*; Zhang et al., 2001; Coffee et al., 2010), but is most functionally related to *FMR1* (Coffee et al., 2010; see below). *dFMR1* is 8.7 kb long and transcribed in many different mRNAs of 2–4 kb encoding many different proteins of different sizes (<http://flybase.org/reports/FBgn0028734.html>). All functional domains are highly conserved with the two KH domains being 75% identical and 85% similar between *dFMR1* and *hFMR1* (Wan et al., 2000).

The gene expression of *dFMR1* in embryos was explored soon after its cloning and observed in the Central Nervous System (CNS), in the somatic musculature, in pole cells, in the gut and in the gonads (Wan et al., 2000; Zhang et al., 2001; Schenck et al., 2002). In **Figure 1**, we show the expression of *dFMR1* at stage 14 by *in situ* hybridization with a full-length probe using the Tyramide Signal Amplification (TSA) (Tevy et al., 2014). High levels of expression are found in the brain (**Figure 1A**, arrowhead), in the CNS (**Figure 1A**, arrow) and in muscle precursors (**Figure 1B**), confirming the previously described pattern of *dFMR1* expression at this stage through a sensitive method.

In embryos, *dFMRP* has been localized in the brain, ventral nerve cord, and mesoderm (Zhang et al., 2001; Schenck et al., 2002; Dolzanskaya et al., 2003; Ma et al., 2016), muscle

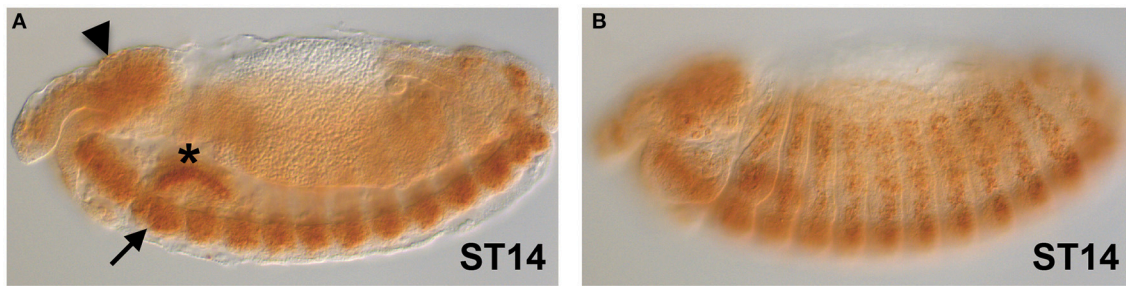


FIGURE 1 | *dFMR1* expression in stage 14 *Drosophila* embryos. **(A)** Lateral view of a stage 14 embryo (middle focus) showing expression in the brain (arrowhead) and in the CNS (arrow). The salivary gland (asterisk) is non-specific background. **(B)** Lateral view of the same stage 14 embryo (surface focus) showing expression in several muscle precursors. The *dFMR1* anti-sense probe was synthesized from the full length EST-clone LD09557 (Drosophila Genomics Resource Center, Bloomington, IN, USA) linearized with EcoRI and transcribed with the T7 RNA polymerase using the Riboprobe Combination System kit (Promega, Madison, WI) and the DIG RNA Labeling Mix (Roche, Indianapolis, IN). *In situ* hybridization was performed as in Tevy et al. (2014) except that SA-HRP and TSA were diluted at 1:250. Images were acquired at the SPIBOC imaging platform of the Institut Sophia Agrobiotech (Sophia Antipolis, France) on an Axioplan II microscope using the ZEN software (Carl Zeiss, Germany).

attachment sites and dendritic tips of chordotonal organs, dendrites of the trachea-innervating neurons (Schenck et al., 2002), developing egg chambers (Zarnescu et al., 2005; Pepper et al., 2009), and in punctate cytoplasmic structures in cleavage-stage embryos in association with cytoplasmic RNP bodies (Monzo et al., 2006; Papoulas et al., 2010). In larvae, dFMRP has been detected in the CNS, the PNS, the eye disk, the testis, at low levels in muscles, in the Mushroom Bodies (MBs, the *Drosophila* learning and memory center; Schenck et al., 2002) and in dendritic arborization (DA) neurons (Lee et al., 2003). dFMRP is found exclusively in the cytoplasm of the soma of neurons (Zhang et al., 2001). Finally, in adults dFMRP has been found in the central brain and eyes (Zhang et al., 2001), in pupal and adult brain neurons (Morales et al., 2002), in specific cells of the adult brain (Morales et al., 2002), in antennal lobe projection neurons (Sudhakaran et al., 2014), and in the cell bodies of specific neurons of the MBs (Pan et al., 2004). Although the main tissue in which dFMRP is present is the CNS, these studies suggest that this protein also has non-neuronal functions, most of which still have to be dissected. Indeed, *dFMR1* has been involved by FlyBase (<http://flybase.org/reports/FBgn0028734.html>) in 58 biological processes, which are summarized in **Table 1**.

It is interesting to note that the phenotypes of *dFMR1* mutants largely recall the pathological symptoms of FXS patients. For instance, some FXS patients have delayed motor development, which can be compared to uncoordinated behavior of flies measured by flight or climbing assays. Moreover, olfactory learning and courtship conditioning of *Drosophila* can serve to test learning and memory behaviors that are often impaired in FXS patients. Also the changes in neuron structure observed in FXS patients are mimicked in the fly model (reviewed in van Alphen and van Swinderen, 2013). The first *dFMR1* mutants were generated soon after its cloning (Zhang et al., 2001) and resulted viable and fertile as in humans. Nevertheless, mutant viability is highly sensitive to the genetic background, such that *dFMR1* mutants can become fully lethal in some backgrounds (see Morales et al., 2002) in a generation-dependent manner, a

phenomenon that requires further study. These original alleles were loss-of-function excisions of hypomorphic EP insertions (producing over-expression of the gene in which they are inserted) presenting phenotypes upon over-expression in the eye (Zhang et al., 2001). Other null mutants were obtained by excision of other EP elements (Dockendorff et al., 2002; Inoue et al., 2002) or by EMS mutagenesis (Lee et al., 2003). Altogether, *dFMR1* mutants show defects in many different biological functions listed in **Table 1**. Over-expression studies in flies have also provided evidence for understating the mis-functions of mutant human FMRP as in the case of the assessment of a neomorphic function for a frameshift FMRP mutant (Okray et al., 2015). In summary, *dFMR1* mainly plays a crucial role in synaptic plasticity and this affects many neuronal processes that are important for fly behavior.

RNA-mediated interference (RNAi) enables the knockdown of a gene of interest at the post-transcriptional stage (Fire et al., 1998). Combining the RNAi with the UAS/GAL4 system (Brand and Perrimon, 1993) in flies makes it possible to down-regulate gene expression in certain tissues and/or at a desired stage of development. Using this combined mechanism, the promoter-GAL4 fusion drives the expression of the RNAi hairpin fragment under the control of UAS sequences (Piccin et al., 2001). Tissue- or stage-specific expression is achieved by the use of specific GAL4 drivers. For instance, *elav-GAL4* is usually used to trigger pan-neuronal expression, *mef2-GAL4* for pan-muscular expression and *Act5c-GAL4* for ubiquitous expression. Since 2007, the Vienna Drosophila Resource Center (VDRC; <http://stockcenter.vdrc.at/control/main>; Dietzl et al., 2007) has established many RNAi lines of *D. melanogaster*. These comprise around 12,671 (91%) of *Drosophila* protein-coding genes, making it the largest collection of RNAi lines for all model systems. Currently, there are three different types of UAS-RNAi stocks available: GD and KK with long hairpins and shRNA with short hairpin micro RNAs. The existence of such a broad array of RNAi lines provides many experimental benefits for biological studies, including those focusing on FMRP.

TABLE 1 | Main phenotypes of loss of function *dFMR1* mutants.

Phenotype	References
BEHAVIOR	
Adult locomotion/Climbing	Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Martinez et al., 2007; Banerjee et al., 2010; Adewoye et al., 2015; Novak et al., 2015; Monyak et al., 2016
Circadian rhythm	Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Banerjee et al., 2007, 2010; Gatto and Broadie, 2009; Siller and Broadie, 2011; Xu et al., 2012; Adewoye et al., 2015; Monyak et al., 2016
Courtship	Dockendorff et al., 2002; McBride et al., 2005; Banerjee et al., 2007, 2010; Chang et al., 2008; Choi et al., 2010; Tauber et al., 2011; Gross et al., 2015
Grooming	Tauber et al., 2011
Larval crawling	Xu et al., 2004; Coyne et al., 2015; Günther et al., 2016; Kashima et al., 2017
Olfactory learning and memory	Morales et al., 2002; Bolduc et al., 2008; Kanellopoulos et al., 2012; Andlauer et al., 2014; Sudhakaran et al., 2014; Choi et al., 2015; Monyak et al., 2016
Social behavior	Bolduc et al., 2010
Sleep	Bushey et al., 2009; van Alphen et al., 2013
Touch perception	Cvetkovska et al., 2013
NEURAL PHYSIOLOGY/STRUCTURE	
Bouton area	Pan et al., 2004; Gatto and Broadie, 2008; Reeve et al., 2008; Coffee et al., 2010; Friedman et al., 2013; Cavolo et al., 2016; Doll et al., 2017
Bouton/synapse number	Zhang et al., 2001; Zarnescu et al., 2005; Banerjee et al., 2007, 2010; Gatto and Broadie, 2008; Xu et al., 2008; Pepper et al., 2009; Coffee et al., 2010; Beerman and Jongens, 2011; Bhogal et al., 2011; Siller and Broadie, 2011; Friedman et al., 2013; Kashima et al., 2016, 2017; Mansilla et al., 2017
Calcium signaling	Tessier and Broadie, 2011; Gatto et al., 2014; Sudhakaran et al., 2014; Doll and Broadie, 2016
M B β -lobe crossing	Michel et al., 2004; McBride et al., 2005; Banerjee et al., 2007; Bolduc et al., 2008; Chang et al., 2008; Beerman and Jongens, 2011; Gross et al., 2015
Neural branching	Morales et al., 2002; Lee et al., 2003; Pan et al., 2004; Reeve et al., 2005, 2008; Gatto and Broadie, 2008; Tessier and Broadie, 2008; Xu et al., 2008; Cziko et al., 2009; Pepper et al., 2009; Coffee et al., 2010; Bhogal et al., 2011; Siller and Broadie, 2011; Friedman et al., 2013; Kim et al., 2013; Doll and Broadie, 2015; Myrick et al., 2015; Kashima et al., 2016
Neurite extension	Morales et al., 2002; Michel et al., 2004; Pan et al., 2004; Reeve et al., 2005; Gatto et al., 2014
Neural fasciculation	Schenck et al., 2003; Reeve et al., 2005
Neurotransmission	Zhang et al., 2001; Martinez et al., 2007; Gatto and Broadie, 2008; Friedman et al., 2013; Doll et al., 2017; Franco et al., 2017
Synaptic growth	Zhang et al., 2001; Morales et al., 2002; Schenck et al., 2003; Abekhoukh et al., 2017; Doll et al., 2017
Branch/Neurite/NMJ/Synaptic length	Lee et al., 2003; Schenck et al., 2003; Reeve et al., 2008; Tessier and Broadie, 2008; Bhogal et al., 2011; Siller and Broadie, 2011; Cvetkovska et al., 2013; Kim et al., 2013; Gatto et al., 2014; Bozzetti et al., 2015; Myrick et al., 2015; Sterne et al., 2015; Abekhoukh et al., 2017; Doll et al., 2017; Kennedy and Broadie, 2017
Synapse structure	Zhang et al., 2001; Morales et al., 2002; Lee et al., 2003; Schenck et al., 2003; Michel et al., 2004; Pan et al., 2004; McBride et al., 2005; Reeve et al., 2005, 2008; Zarnescu et al., 2005; Banerjee et al., 2007, 2010; Bolduc et al., 2008; Chang et al., 2008; Gatto and Broadie, 2008, 2009; Tessier and Broadie, 2008; Xu et al., 2008; Cziko et al., 2009; Pepper et al., 2009; Coffee et al., 2010; Beerman and Jongens, 2011; Bhogal et al., 2011; Siller and Broadie, 2011; Cvetkovska et al., 2013; Friedman et al., 2013; Kim et al., 2013; Gatto et al., 2014; Bozzetti et al., 2015; Doll and Broadie, 2015; Gross et al., 2015; Myrick et al., 2015; Sterne et al., 2015; Cavolo et al., 2016; Kashima et al., 2016, 2017; Abekhoukh et al., 2017; Doll et al., 2017; Kennedy and Broadie, 2017; Mansilla et al., 2017
Synapse volume	Mansilla et al., 2017
OTHERS	
Adult eclosion	Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002
Aging	Martinez et al., 2007; Bushey et al., 2009
Apoptosis	Gatto and Broadie, 2011
Blastoderm cellularization	Deshpande et al., 2006; Monzo et al., 2006; Papoulas et al., 2010
Cell cycle	Deshpande et al., 2006; Monzo et al., 2006; Epstein et al., 2009; Callan et al., 2010, 2012; Papoulas et al., 2010
Germline development	Zhang et al., 2004, 2014; Costa et al., 2005; Deshpande et al., 2006; Megosh et al., 2006; Epstein et al., 2009; Yang et al., 2009; Bozzetti et al., 2015; Jiang et al., 2016
Heart rate	Novak et al., 2015
Oviposition	Kacsoh et al., 2015a,b; Jiang et al., 2016
Phagocytosis	O'Connor et al., 2017

For example, tissue-specific RNAi studies uncovered the role of dFMRP in early developmental stages of fly larvae. It was shown that dFMRP regulates glial-dependent proper timing of

neuroblast reactivation during brain development (Callan et al., 2012). In another study, RNAi knockdown of *Torsin* revealed its involvement in locomotion. *Torsin* probably works together with

dFMR1 to regulate synaptic plasticity and *dFMR1* expression is altered in *Torsin* mutant flies (Nguyen et al., 2016). By creating the cardiac-specific *dFMR1* RNAi knockdown, *dFmr1* was also shown to be involved in regulating heart rate during development using the climbing assay. In this simple assay, adults are placed in a vial and tapped down. The time by which they reach the height of 5 cm is then measured (Novak et al., 2015). Some FMR1 patients present cardiac defects (Kidd et al., 2014) and changes in FMRP levels have been associated with structural and functional defects in zebrafish and mice (Mientjes et al., 2004; Van't Padje et al., 2009). On the contrary, in *Drosophila* no structural defects have been found, suggesting that *dFMR1* and *FMR1* regulate distinct targets. In a different study, hemocyte-specific *dFMR1* knockdown by RNAi causes a defect in immune cell phagocytosis of bacteria and increases *Drosophila* sensitivity to bacterial infections, suggesting that *dFMR1* is involved in the regulation of phagocytosis (O'Connor et al., 2017; reviewed in Logan, 2017). This study can provide further insights into the engagement of the immune system in FXS pathogenesis, especially considering the fact that some FXS patients exhibit defects in this system (O'Connor et al., 2017 and references therein). Aberrations in calcium homeostasis are connected with changes in neuron structure that probably cause the learning and memory deficits seen in FXS patients (Tessier and Broadie, 2011 and references therein). This was supported by RNAi knockdown of *dFMR1* during a critical period of development, which proved the importance of the dFMRP role in regulating calcium signaling in the learning and memory circuitry (Doll and Broadie, 2016). These studies highlight the value of the *Drosophila* system in detailed phenotypic analyses of FMRP function.

NeuroMuscular Junctions (NMJs) of *Drosophila* are simple synapses that resemble those present in the Vertebrate CNS. Thus, they are a good model for the study of synapses (reviewed in Menon et al., 2013). The neuromuscular system of *Drosophila* contains 32 motor neurons in each abdominal hemisegment. NMJ synapses show developmental and functional plasticity. They are large and individually specified, enabling their visualization. NMJs are composed of branches and three types of boutons (I, II, and III) that are oval structures hosting synapses differing in size, shape, physiology, and in the amount of sub-synaptic reticulum surrounding them. Type I boutons are glutamatergic and have been the focus of FXS studies. Immunohistological staining can be used to visualize these structures and observe different NMJ phenotypes. *dFMR1* is expressed pre-synaptically in motor neurons (Zhang et al., 2001), but post-synaptically in muscles (Zhang et al., 2001; Schenck et al., 2002). In *dFMR1* mutants, NMJs display increased synapse arborization and branching, increased synaptic bouton numbers, and elevated neurotransmission, whereas larvae over-expressing *dFMR1* show the opposite phenotypes (Zhang et al., 2001). These phenotypes recall the dendritic spine over-growth observed in mammalian mutants and in FXS patients (Irwin et al., 2001).

In addition to defects in NMJ synaptic architecture in neurons, *dFMR1* mutants also exhibit fecundity and testes dysfunctions, which can be used to evaluate non-neuronal requirements (Zhang et al., 2004). Coffee et al. examined the evolutionary conservation of *FMR1* and its paralogs in the *Drosophila* FXS

model at the neuronal and non-neuronal levels (Coffee et al., 2010). In this study, out of the three human genes, only *FMR1* turned out to be able to restore the normal number of boutons in *dFMR1* null mutants. On the other hand, all three homologs rescued the sterility and testicular phenotypes. These results indicate that in neurons *FMR1* has a unique evolutionarily conserved role. In contrast, in non-neuronal tissues *FMR1*, *hFXR1*, and *hFXR2* are able to substitute each other (Coffee et al., 2010).

The larval NMJ has proved a powerful system to study genetic interactions occurring in the actin remodeling pathway that is altered in mammalian FMRP-null neurons (Castets et al., 2005; Pyronneau et al., 2017). FMRP has been shown to interact with Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) *in vitro* (Schenck et al., 2001, 2003) and also in both mammals (Schenck et al., 2001) and *Drosophila* (Schenck et al., 2003; Abekhouk et al., 2017). CYFIP1 is part of the WAVE regulatory complex (WRC) along with five other proteins involved in actin polymerization (reviewed in Cory and Ridley, 2002; Takenawa and Suetsugu, 2007). Human CYFIP1 has been linked to neurodevelopmental disorders such as ID, autism, schizophrenia, epilepsy, and Burnside-Butler (15q11.2 BP1-BP2 micro-deletion) syndrome (Madrigal et al., 2012; Waltes et al., 2014; Huang, 2015; Wang et al., 2015). Abekhouk et al. (2017) utilized the fine genetic tools of *Drosophila* to investigate the genetic interactions between dFMRP and dCYFIP1. Through loss- and gain-of-functions studies, the authors showed that *dFMR1* and dCYFIP1 have opposing functions on larval NMJ length: dFMRP represses while dCYFIP1 promotes synaptic growth at the NMJ in gene dosage studies using the presynaptic *elav-Gal4* driver. A rescue of the reciprocal NMJ length phenotypes is observed in double homozygous mutant animals. It should be noted that double homozygous mice are lethal, thus preventing a similar epistatic genetic analysis in the mouse model. Here, the advantage of *Drosophila* for synaptic plasticity and actin studies is that specific parameters can easily be monitored. Since *dFMR1* and *CYFIP1* are candidates for ID and autism, these studies on the fly pave the way to deeper and more refined studies in mice and in humans.

The first *Drosophila* target of dFMRP was the gene *futsch*, identified by RNA immunoprecipitation (Zhang et al., 2001). This gene encodes a microtubule-associated protein with homology to mammalian MAP1B. *futsch* and *dFMR1* have opposite phenotypes (undergrowth and overgrowth of synaptic boutons, respectively) and dFMRP binds *futsch* mRNA, negatively regulating its translation (Zhang et al., 2001). Binding of dFMRP was also found for the mRNA of the actin monomer binding protein *profilin* (encoded by *chickadee*; Reeve et al., 2005) and of the small GTPase Rac1 (Lee et al., 2003), whose loss- and gain-of-functions also have opposite phenotypes to those of *dFMRP* that are rescued by over-expression of both genes. dFMRP also binds the mRNAs of BMPR2 (Kashima et al., 2016), DSCAM (Cvetkovska et al., 2013), and the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) mRNA together with Ataxin-2 (Sudhakaran et al., 2014), confirming the involvement of *dFMR1* in the Ca²⁺ pathway. Nevertheless, there is still a need for high throughput studies to identify novel dFMRP targets.

MBs consist of bilateral clusters of ~2,500 neurons in the fly brain. In MBs, there are three types of intrinsic neurons: $\alpha\beta$, $\alpha'\beta'$, and γ (Crittenden et al., 1998). $\alpha'\beta'$ are a prerequisite for gaining olfactory memory, whereas $\alpha\beta$ are required to retrieve memory (Krashes et al., 2007). MBs play a major role in olfactory learning and memory in *Drosophila*. Odor and courtship-based tests are frequently used to assess memory dysfunctions in this system (reviewed in Weisz et al., 2015). MBs are also involved in visual context generalization, information processing, locomotion, sleep, courtship conditioning, and choice behavior. The MB axons in *dFMR1* null mutants show architectural defects such as an increase in volume and branching, as well as abnormalities in synapse formation (Pan et al., 2004; Chang et al., 2008). *dFMR1* null mutants show morphological MB defects in the lobes, the most frequent of which is the failure of β -lobe arrest at the brain midline (Michel et al., 2004 and see **Table 1**), whereas over-expression of *dFMR1* causes the opposite phenotype (Reeve et al., 2005). These MB malformations can be restored through pharmacological treatment, but they are not indispensable for restoring memory (McBride et al., 2005). It has been shown that *dFMR1* expression requirements differ between lobe types in MBs. Simultaneous expression of *dFMR1* in α , β , and γ lobes is essential for learning skills. The lack of *dFMR1* expression in α and β lobes is sufficient to impair associative olfactory learning and memory, whereas the knock-down of *dFMR1* only in γ lobes does not exhibit detrimental effects on learning (Kanellopoulos et al., 2012). The MBs have also been the focus of calcium-signaling studies using a transgenic GCaMP calcium sensor (Doll and Broadie, 2016 and references therein). The MB neurons have been shown to be involved in activity-dependent processes during critical period development thanks to their ability to respond when illuminated by a blue light (see below; reviewed in Doll and Broadie, 2014). All the *dFMR1* phenotypes in the MBs correlate with learning and memory dysfunctions and make the FXS *Drosophila* model very appealing because of its easy *in vivo* analysis and the wide range of tools that have been developed (Ugur et al., 2016; Chow and Reiter, 2017).

Electrophysiological techniques have been used in *Drosophila* to study the effect of *dFMR1* loss-of-function and over-expression on synaptic transmission. Two-Electrode Voltage Clamp (TEVC) studies at NMJs in *Drosophila* showed that FMRP has clear pre-synaptic functions (Zhang et al., 2001), although photoreceptor synaptic transmission is normal in the mutants (Morales et al., 2002). Through TEVC recordings, Gatto and Broadie (2008) showed that presynaptic *dFMR1* expression in *dFMR1* mutants rescues the defects in NMJ structure, but not in neurotransmission, suggesting that dFMRP also has post-synaptic functions. On the other hand, in mice, FMRP was first shown to act post-synaptically (Huber et al., 2002) and only recently has also been found to play a pre-synaptic role in signal transmission (Koga et al., 2015; Myrick et al., 2015; Zhang et al., 2015) as in the fly. The possibility to target gene expression to specific cells through the UAS/GAL4 system (Brand and Perrimon, 1993), the wide availability of genetic mutants and the precise spatial and temporal resolutions make electrophysiology a very informative technique for FXS modeling in flies, and a useful complement to the advanced imaging studies described below.

Optogenetics is an *in vivo* technique that uses light to measure neuronal activity in living tissues and has also been exploited in the FXS *Drosophila* model. For example, dFMRP has been shown to play a cell-specific role in the regulation of activity-dependent calcium transients that is restricted to the early critical period (Tessier and Broadie, 2011; Doll and Broadie, 2016). Using optogenetic stimulation, it was recently established that *dFMR1* mutants show increased circuit excitability (probably due to reduced GABAergic lateral inhibition; Franco et al., 2017) and that dFMRP is required for the activity-dependent regulation of synaptic connectivity (Doll et al., 2017). These data may explain the deficits in olfactory behaviors and the hyper-excitation found in FXS patients. *Drosophila* has been used to study even sleep patterns in *dFMR1* mutants by electrophysiology and optogenetics. *dFMR1* mutants show deeper night-like sleep during the day (van Alphen et al., 2013), which is likely because of the FMRP function in synaptic remodeling. It is clear that, through *Drosophila*, the cellular and physiological processes involved in FXS pathology can be studied at a deeper level than in any other model system. In addition, flies have been used for pharmacological studies on FXS through their complex behaviors like for example olfactory learning and memory, courtship, circadian rhythm, crawling and sleep (see **Table 1** and below).

FXS IN OTHER MODEL SYSTEMS

Drosophila is not the only animal model used to study FXS. Here, we report some examples of other animal models that can help in FXS studies. Mammalian mouse and rat models have been predominantly used for FXS studies. Two different mouse (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006) and rat (Hamilton et al., 2014; Tian et al., 2017) KO models have been generated. It should be noted that, in mice, the full CGG expansion does not cause methylation and *Fmr1* silencing (Brouwer et al., 2007) as it does in humans. Thus, all FXS studies in mice have been carried out using the KO animal (The Dutch-Belgian Fragile X Consortium, 1994; Mientjes et al., 2006). In general, the *Fmr1*-KO mouse is considered to be a good model for this disorder since it recapitulates most FXS phenotypes (deficits in learning and memory, hyperactivity, altered volumes of some brain regions, altered morphology of dendritic spines, and increased size of testis) and because it allows genetic experimentation. Also KO mutant rats present behavioral abnormalities related to ASD-like altered patterns of social interaction (Tian et al., 2017) and social play behavior (Hamilton et al., 2014), defects in visual attention (Berzhanskaya et al., 2016), and speech and auditory dysfunctions (Engineer et al., 2014). Rats have provided an excellent model for neuroscience and pharmacology as they have bigger brains than mice, are easier to train, can learn sophisticated behaviors and have an elaborated social repertoire; however, they are more expensive and much less genetically amenable than mice and flies. These recent studies on *Fmr1* KO mice and rats show that both of these models are useful to study the complex phenotypes of FXS patients. Nevertheless, although Vertebrate model organisms provide more precise insights into the human disease pathogenesis,

they are much more difficult than flies to maintain, more time-consuming and incur considerable expenses. In addition, performing experiments on Vertebrate models is much more restrictive in the context of animal laws and often triggers ethical issues.

Other animal models have been generated to understand the physiopathology of FXS, such as the zebrafish, a small fresh water fish endogenous to South-East Asia, the frog *Xenopus laevis* and the marine mollusk *Aplysia californica*. Although zebrafish *fmr1* mutants generated by morpholino knock-down showed gross morphological defects in neurons (Tucker et al., 2006), true genetic null alleles later obtained by random mutagenesis resulted viable, fertile, and of normal morphology (den Broeder et al., 2009) likely due to off-targeting effects of the morpholino technology. Importantly, mutant zebrafish exhibit hyperactivity, learning deficits, impaired anxiety, and increased social behaviors like shoaling (Ng et al., 2013; Kim et al., 2014; Wu et al., 2017) maybe because of hyperactivity as in mice (Sorensen et al., 2015). The frog *fmr1* mutant was also obtained by morpholino KO and showed behavioral (Truskowski et al., 2016) and FMRP level-sensitive neuronal defects (Faulkner et al., 2015). Finally, in *Aplysia*, basic neurobiological studies have evidenced the pre- and post-synaptic control of plasticity regulating long-term memory and a functional interaction with the Na⁺-activated K⁺ channel (K_{Na}) Slack (reviewed in Abrams, 2012). Overall, these simpler systems have also facilitated further insights into the mechanisms of FXS pathology.

In comparison to vertebrate models, the more complex human behaviors do not always correspond to those of flies and many neurological diseases can only be modeled in certain aspects. This is mainly due to the fact that *Drosophila* and humans differ in anatomy and despite of having many orthologs in common their pathways exhibit many differences. In addition, although drug administration is much simpler in *Drosophila*, the potential toxicity is much tougher to predict in humans because of significant metabolic differences and complexities (Pandey and Nichols, 2011).

SUCCESSFUL PHARMACOLOGICAL TREATMENTS OF FXS PHENOTYPES IN DROSOPHILA

Since the *Drosophila dFMR1* phenotypes recapitulate the patients' symptoms, this model has been used to develop pharmacological treatments for the disease. One reason is that drugs can easily be administered in the standard fly food in which larvae feed and grow. This food is composed of cornmeal, agar and yeast and requires boiling. Recently, the Formula 4–24 (Carolina Biological Supply Company) medium has been exploited because it does not require heating and can simply be dissolved in room temperature water so that even heat-sensitive drugs can be tested (Kashima et al., 2017). Simple feeding has been used in the articles reported below, but many other methods to feed embryos, larvae or adults have been developed (reviewed in Pandey and Nichols, 2011). Another reason is that *Drosophila* does not carry vessels, but all of its organs bathe in hemolymph, which circulates thanks to a

tubular heart in an open circulatory system. The fly blood/brain barrier is only made of a thin layer of glial cells presenting septate and gap junctions contrary to the Vertebrate one that is composed of glial cells and of endothelial cells forming tight junctions. Thus, the fly blood/brain barrier is simpler and allows for a better pharmacokinetic penetration (Pinsonneault et al., 2011; Limmer et al., 2014). Here, we discuss some examples of drugs successfully tested on flies to cure *dFMR1* phenotypes that affect the different pathways listed below.

Glutamate is the main excitatory neurotransmitter in the CNS. Metabotropic glutamate receptors (mGluRs) at synapses control Long Term Depression (LTD), which mediates synaptic plasticity, thus weakening the synaptic response to stimuli. mGluR-dependent LTD was initially found to be enhanced in *FMR1* mutant mice (Huber et al., 2002) and later in *Drosophila* (McBride et al., 2005). After the establishment of the “mGluR theory” of FXS by Bear et al. (2004), it was in *Drosophila* that inhibition of mGluR signaling was first shown to alleviate the behavioral fly phenotypes mimicking human FXS symptoms (McBride et al., 2005). In addition, a rescue of the defects in the fibers crossing the β -lobe of the MBs was observed in the treated *dFMR1* mutants. Interestingly, adult therapy is sufficient to restore normal courtship behavior and short-term memory, but not β -lobe crossing, suggesting that other morphological defects are responsible for the memory defects (McBride et al., 2005). Since then, much research has been carried out to pharmacologically correct this defect both in mice (reviewed in Hagerman et al., 2014) and in flies (Choi et al., 2010; Kanellopoulos et al., 2012). *dFMR1* mutants have less vigorous courtship behavior, learn normally, but forget. Treatments of larvae and adults either with the non-competitive mGluR antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP), three competitive mGluR antagonists or LiCl rescue naive courtship behavior, immediate recall memory and short-term memory of *dFMR1* mutants (Choi et al., 2010). Subsequently, Choi et al. (2010) compared naive courtship, locomotion, olfactory capabilities, learning, and memory between 20 day-old flies and 5 day-old flies. They found that the inhibitors of the Glutamate receptor pathway and Lithium rescue these phenotypes of *dFMR1* mutants to different degrees. The best rescue is obtained when the treatment is applied at both larval and adult stages. Learning is defective only in old flies, without the involvement of cell death. Treatment with four different mGluR inhibitors or LiCl exclusively during development rescues the learning, but not the courtship defects. Despite these successful treatments in pre-clinical trials, all clinical trials carried out so far have failed, although others are currently underway (<https://clinicaltrials.gov>). One reason could be the timing of the treatment or the need to combine different drugs since many other molecular mechanisms have been evoked as causative aspects of this disease. Importantly, in addition to FXS, many other neurodevelopmental disorders are correlated with defects in mGluR signaling.

As opposed to glutamate, GABA is the main inhibitory neurotransmitter in the brain. It functions through Gamma-AminoButyric Acid (GABA)_A and GABA_B receptors (R) that are fast-acting inotropic and metabotropic receptors, respectively.

A decrease in GABA_A R expression and defects in GABAergic signaling were found in the *FMRI* Knock Out (KO) mouse (Idrissi et al., 2005; D'Hulst et al., 2006; Gantois et al., 2006; Sabanov et al., 2017). GABA_B R has been linked to mGluR signaling and its reduction associated with autism (reviewed in Hagerman et al., 2014). Many clinical treatments targeted to the GABAergic pathway have been attempted or are underway (reviewed in Braat and Kooy, 2015). Altered GABAergic circuitry, like depressed glutamic acid decarboxylase (GAD) levels, was also found in the *Drosophila* FXS model, particularly in neurons expressing *dFMRI* (D'Hulst et al., 2006; Gatto et al., 2014). In this system, through a fine mapping method of Mosaic Analysis with a Repressible Cell Marker (MARCM), Gatto et al. (2014) showed that *dFMRI* is not required for GABAergic neuron survival, but it regulates the architecture of GABAergic neurons innervating the MBs. In addition, *dFMRI* GABAergic neurons display elevated calcium signaling (Gatto et al., 2014). To further underline the critical role of the GABA pathway in defining the FXR phenotype in the fly, it is worth mentioning that the down regulation of the GABA receptors in projection neurons of the antennal lobe observed in *dFMRI* mutants is sufficient to produce olfactory behavioral defects (Franco et al., 2017).

Thanks to the toxicity of glutamate for the *dFMRI* mutant, Chang et al. (2008) were able to carry out an unbiased screen for small molecules that can rescue the lethality of glutamate-treated *dFMRI* mutants, using the mGluR5 non-competitive antagonist MPEP as a positive control. The active compounds belong to biochemical pathways not targeting mGluR signaling, namely the GABAergic, the muscarinic, the serotonin, and hormone-related pathways. Notably, GABA and MPEP treatments were also able to rescue the β -lobe crossing in the MBs and the courtship behavior phenotypes. Indeed, a decrease in GABA_A R expression and defects in GABAergic signaling were found in the *FMRI* KO mouse (Sabanov et al., 2017 and references therein) and in the *Drosophila* FXS model, particularly in neurons expressing *dFMRI* (Gatto et al., 2014). This is the first chemical screen for FXS in flies and was made possible by the easily scorable phenotype of lethality, which revealed novel pathways implicated in FXS (Chang et al., 2008). GABA_B R has been linked to mGluR signaling and its reduction associated with autism (reviewed in Hagerman et al., 2014). Many clinical treatments targeting the GABAergic pathway have been attempted or are underway (reviewed in Braat and Kooy, 2015).

Another pathway that has been shown to be involved in FXS alterations is the Bone Morphogenetic Protein (BMP) signal transduction pathway (Kashima et al., 2016), controlling a number of developmental processes including nervous system development (Liu and Niswander, 2005). This pathway has been linked to anxiety and object exploration in mice (McBrayer et al., 2015). It was previously established that the LIM domain kinase 1 (LIMK1) co-localizes with the BMP type II receptor (BMPR2) in the neuronal terminals (Lee-Hoeflich et al., 2004). These two proteins bind to each other leading to activation of the LIMK1 catalytic activity. Once activated by BMPR2, LIMK1 promotes neural growth and dendritogenesis through phosphorylation and inhibition of cofilin (Meng et al., 2002; Lee-Hoeflich et al., 2004). The C-terminal domain (CTD) of

BMPR2 plays a role as a repressor of BMPR2 translation and as an activator of LIMK1 function. Kashima et al. (2016) found that FMRP binds to the CTD of the BMPR2 and inhibits the translation of full-length BMPR2 in humans and mice. These data have been genetically confirmed in the *Drosophila* model. In the NMJs of *dFMRI* mutants, synaptic boutons are overgrown, whereas they are under-grown in mutants for the BMPR2 homolog *wishful thinking (wit)* (see also Eaton and Davis, 2005). Double *wit*, *dFMRI* mutants show a number of boutons as in single *wit* mutants, indicating that *wit* is negatively regulated by *dFMRI*. These results obtained in the fly encouraged the authors to carry out pharmacological studies in mice that revealed the *Fmr1* rescue potential of a LIMK1 inhibitor (Kashima et al., 2016). These cellular phenotypes correlate with the crawling behavior of *Drosophila* larvae, which has been used as a simple genetic and pharmacological screening tool for FXS treatment (Kashima et al., 2017). *dFMRI* mutant larvae crawl faster than wild-type larvae: heterozygotes for three different *dFMRI* alleles show increased larval locomotor activity correlated with an increased bouton number at the NMJ of muscle 6/7 in A3. This phenotype is rescued by a loss-of-function allele of *wit* in heterozygosis suggesting that it is due to an up-regulation of the BMPR2 homolog. The oral treatment with a pharmacological inhibitor of LIMK1 (downstream target of BMPR2, see above) restores the number of boutons in *dFMRI* mutants. Using a newly developed larval crawling assay and a sophisticated algorithm (LarvaTrack) for drug screening, the locomotion defects (distance and velocity) of *dFMRI* larvae were used as a readout of the larval bouton number phenotype (Kashima et al., 2017). In this case, LIMK1 inhibitors and puromycin rescue the *dFMRI* locomotion phenotypes in correlation with the bouton number. Hyperactivity and anxiety of *Fmr1*-KO mice are also ameliorated by treatment with a LIMK inhibitor, showing that this specific and simple assay developed in flies has considerable potential in the assessment of new drug therapies. Indeed, all of these results can be applied to the human condition because an increase in BMPR2 is observed in the prefrontal cortexes of FXS patients (Kashima et al., 2016), paving the way to drug trials in humans.

Neuronal Calcium Sensor 1 (NCS-1) and its *Drosophila* homolog *Frequenin (Frq) 2* are Ca²⁺-binding proteins that play a role in the control of the synapse number: loss-of-function mutations increase the synapse number and over-expression decreases it. Human NCS-1 is involved in schizophrenia (Koh et al., 2003; Torres et al., 2009) and autism (Piton et al., 2008). Frq2 physically interacts with the guanine nucleotide-exchange factor *Ric8a* and the structure of their interaction has been resolved (Romero-Pozuelo et al., 2014). The *Ric8a* protein is localized at larval motor neuron terminals and is particularly abundant in boutons. *Ric8a* knockdown causes a reduction in the number of synapses, thus producing a phenotype opposite to that of Frq2 with Frq2 acting as a negative regulator of Ric8a in the control of the synapse number (Romero-Pozuelo et al., 2014). On the other hand, as in humans and mice (Irwin et al., 2001; Hutsler and Zhang, 2010), loss of *dFMRI* causes an increase in the synapse number and neuron volume (see **Table 1**). Mansilla et al. (2017) identified the aminophenothiazine derivative FD44

as a potent inhibitor of the *Drosophila* NCS-1/Ric8a interaction and showed that treatment of *dFMR1* mutant adults and larvae with this drug reduces their synapse number and volume at the glutamatergic NMJ of larval muscle fibers 6/7 of A3. In addition, FD44 restores normal associative learning. This indicates that, in the *dFMR1* mutant, the interaction between NCS-1 and Ric8a is unbalanced, probably because dFMRP controls *Frq2* transcription (Tessier and Broadie, 2011). Consistently, over-expression of both NCS-1 and *Ric8a* restores a normal number of synapses (Romero-Pozuelo et al., 2014). FD44 is an interesting drug because it is small and able to cross the blood/brain barrier and because the structure of the interaction with its target proteins has been clearly illustrated (Mansilla et al., 2017).

The Down Syndrome Cell Adhesion Molecule (DSCAM) is a conserved protein the levels of which have been found to be elevated in many brain disorders including FXS. This has been confirmed in the mouse and *Drosophila* models where it has been shown that FMRP binds the *Dscam* mRNA and down-regulates its translation (Darnell et al., 2001; Cvetkovska et al., 2013; Kim et al., 2013). *Dscam* encodes a transmembrane protein that is a member of the immunoglobulin superfamily of cell adhesion molecules involved in self-avoidance, synaptic target selection, and axon guidance. In pre-synaptic terminals, *Dscam* has been shown to interact with *Abelson tyrosine kinase* (*Abl*), which mediates the exaggerated presynaptic terminal growth followed by *Dscam* over-expression probably because it is activated by DSCAM (Sterne et al., 2015). As multiple FDA-approved *Abl* inhibitors were available, Sterne et al. (2015) tested whether *ABL* inhibition could restore normal presynaptic terminal growth in larvae over-expressing *Dscam* or mutant for *dFMR1*. The nilotinib inhibitor was found to have this property, which suggests that *ABL* is as a promising therapeutic target for FXS.

Insulin and insulin-like growth factors (IGF) are evolutionarily highly conserved proteins that play an important role in growth and metabolism, but also in neurogenesis through their role in neuronal stem-cell homeostasis (reviewed in Lee et al., 2016; Ali et al., 2018). In *Drosophila*, expression of *dFMR1* specifically in the insulin-producing cells (IPs) or in the whole nervous system is able to rescue the defective free-running locomotor rhythmicity of *dFMR1* mutants, as well as short-term and long-term memory defects in olfactory learning tests (Monyak et al., 2016). Protein levels of the major insulin-like peptide DILP2 are elevated in the IPs of *dFMR1* mutants, suggesting a post-transcriptional control, and markers of the insulin pathway are up-regulated. Through fine genetic manipulations aimed at reducing insulin signaling (IS), the authors were also able to obtain a rescue of the circadian rhythmicity of *dFMR1* flies. These data are consistent with the fact that *FMRI* is expressed in the IPs of the mammalian pancreas and that insulin and IGFs have been involved in many neurological events, including synaptogenesis and progenitor cell growth (reviewed in Fernandez and Torres-Alemán, 2012). Metformin is a drug used to cure type 2 diabetes that acts as a sensitizer of IS signaling by increasing Phosphatase and TENsin homolog protein (PTEN) expression, AMP-activated protein Kinase (AMPK) activation and decreasing Target Of

Rapamycin (TOR) signaling. Treatment of *dFMR1* mutants with metformin restores normal short-term courtship memory and normal olfactory long-term memory (Monyak et al., 2016). Using GAL80, the temperature-sensitive repressor of GAL4, Monyak et al. (2016) also tested the precise stage at which reduction of IS is necessary to rescue the different behavioral phenotypes of *dFMR1* mutants. While lowering IS during adulthood is sufficient to rescue learning and memory in the olfactory-based paradigm, reduction during the pupal stages is indispensable to rescue circadian behavior. This may mean that dFMRP is required at different developmental steps to temporally modulate IS. The weight of KO *Fmr1* mice is significantly increased compared to wild-type (Dölen et al., 2007) and some FXS patients are obese (Nowicki et al., 2007) and show elevated IS through the mTor pathway in blood and brain (Hoeffler et al., 2012), implying that mis-regulation of the insulin pathway is likely to be one cause of the disease and a promising target for therapy (reviewed in Castagnola et al., 2017).

CONCLUSIONS

Individuals affected by FXS show a broad spectrum of clinical presentations, with a great variability of signs, symptoms and severity levels (Hagerman, 2002). Indeed, due to the high number of *FMRI* targets, FXS can be considered as a multifactorial disorder. The possibility to study null *dFMR1* flies and mice brought significant advantages for understanding the functional roles of FMRP. These systems made it possible to decrypt several pathways responsible for phenotypes recalling human symptoms, such as cognitive and learning deficits. In comparison to the other animal models, *Drosophila* is less expensive and easier to maintain, lays many eggs that enable to perform genetic screens, has a shorter lifespan and generation time, a smaller entirely sequenced genome (Adams et al., 2000) and sophisticated genetic tools to study human diseases like the easiness to make transgenics or to carry out *in vivo* functional studies (reviewed in Wangler et al., 2015; Ugur et al., 2016; Chow and Reiter, 2017). The fly genome has many orthologs displaying similar roles to human disease genes (Fortini et al., 2000; Doronkin and Reiter, 2008) that can be potential targets for functional and therapeutic studies. *dFMR1* mutants exhibit defects that resemble those observed in human FXS patients, and flies and humans share many pathways that are altered in the disease and likely responsible for a specific set of phenotypes. Studies on FXS in *Drosophila* have set a paradigm to validate drug targets and gain a deeper insight into their molecular mechanisms for future research on FXS and other neurodevelopmental diseases. This is also because the dissection of the correlation between pathways and genotypes can be more easily realized in the fly than in any other model system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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