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Characterization of the pathological role of the cerebellum in a mouse model of autism

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ABBREVIATIONS

ADHD: Attention deficit hyperactivity disorder
AP: Anteroposterior
APA: American Psycological Association
ASD: Autism Spectrum Disorder
C: Cortical component
CDC: Center for Disease Control and Prevention
CDFE : Cortical dysplasia and focal epilepsy
CF: Climbing fiber
cKO: Conditional Knock-out
CNTNAP2: Contactin-associated protein like 2
CNVs: Copy number variations
CS: Complex spike
CV: Coefficient of variation
CV2: Coefficient of variation 2
DC: Direct current
DSM: Diagnostic and Statistical Manual of Mental Disorders
E/I: Excitation/Inhibition balance
ERP: Event-related potential
FF: Firing frequency
fMRI: Functional magnetic resonance imaging
FPE: Female protective effect
FR: Firing rate
FXS: Fragile X Syndrome
GWAS: Genome-wide association studies
ISI: Inter-spike interval
KO: Knock-out
Kv+: Voltage –gated potassium channels
L 2/3: Cortical layers 2 and 3
MDS: MECP2 duplication syndrome
MF: Mossy fiber
MMR: Vaccine against measles, mumps, and rubella
mPFC : Medial prefrontal cortex
P7 : Postnatal day 7
PC: Purkinje cell
PF: Parallel fiber
PFC : Prefrontal cortex
pSTS: Posterior superior temporal sulcus
PV+ : Pavalbumin-positive interneurons
S.E.M: Standard error of mean
S1: Somatosensory cortex
SEP: Sensory evoked potential
SS: Simple spike
T: Trigeminal component
tACS: Transcranial alternanting stimulation
tDCS: Transcranial direct current stimulation
tES: Transcranial electrical stimulation
TSC: Tuberos sclerosis complex
UsV: Ultrasonic vocalizations
WGS: Whole-genome sequencing
WT: Wild type

1. Introduction

Autism spectrum disorder (ASD) represents a group of heterogeneous neurodevelopmental conditions characterized by deficits in social cognition, together with the presence of restricted and/or repetitive patterns of behaviors, activities, or interests (APA, 2013). Social abilities are affected in ASD patients and often, they find issues facing the challenges of living in a social environment or group. Successful and functional social cognition implies the coordination of several domains of behaviors, including attention, memory, emotion, and motivation. This last one is especially important to be able to understand the actions, social hierarchy, and emotional status of a conspecific and therefore guide the appropriate behavioral response (Adolphs, 2001). The failure in processing social cues in individuals with autism are observed at multiple levels: response to social interactions, lack of interest in social situations, abnormal social approach, difficulties expressing and understanding verbal and nonverbal communication (i.e., body language and facial expressions), and problems adjusting behavior to different social situations, among others (Baio, Wiggins, Christensen, & et al, 2018). Inside the spectrum, ASD is often associated with other conditions in addition to the main symptoms, such as hyperactivity, epilepsy, aggression, irritability, sleep problems, gastrintestinal symptoms, and sensory processing abnormalities (Geschwind, 2009).

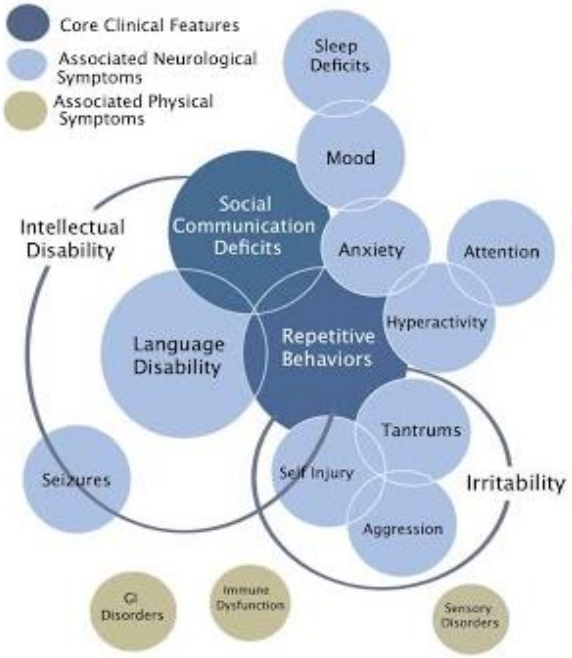
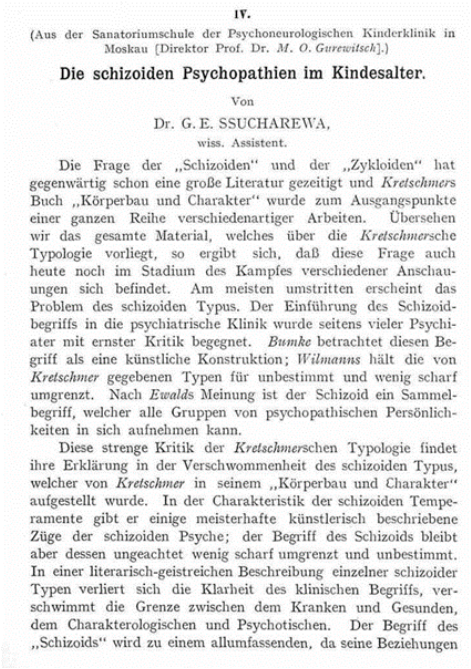


Figure 1. Symptoms present in ASD patients. The main core (dark blue) are composed by social communications deficits and repetitive behaviors. Besides, other symptoms could be associated inside de spectrum. Source: autismag.org

A recent study published by CDC estimate the prevalence of autism in US in 1 in 54 children (Maenner, Shaw, Baio, & et al., 2020) with a clear differentiation by sex: 1/34 boys are diagnoses with autism vs. 1/144 girls. Besides, the same report identified that even if autism affects all ethnic and socioeconomics groups, the minority groups tend to report fewer cases or have a later diagnosis. The high prevalence, together with the fact that there is not an effective treatment for the core symptoms of autism, make Autism one of the most important mental health issues worldwide.

Autism History

The word "autism" is derived from the Greek word "autos" which means "self". It was firstly used by the Swiss psychiatric Dr. Eugen Bleuler, who also coined the term "schizophrenia" in 1911 in his paper "*Dementia praecox oder Gruppe der Schizophrenien*". Originally, he included autism as one of what he calls "the four schizophrenias" (Bleuler, 1911). To Bleuler, autism was not a pathology, but a mode of processing information referring to a group of children who exhibit a withdrawal from other people and the external word. Few years later, in 1926, Dr. Grunya Efimovna Sukhareva, a Soviet psychiatrist, published her works, which are considered the first clinical accounts of autism. Although at the beginning of her research she used the term "autistic" in the same way Bleuler did (as a kind of schizophrenia), she started soon to observe other children with this trait and made the effort to characterize it in more detail. In her work "Die schizoiden Psychopathien im Kindesalter" she perfectly described some of the symptoms that we consider nowadays to be necessary to get autism diagnosis. Sukhareva described autism as a "flattened affective life," "lack of facial expressiveness and expressive movements" and a desire to be "keeping apart from their peers" (Sukhareva, 1926).



IV.

(Aus der Sanatoriumschule der Psychoneurologischen Kinderklinik in Moskau [Direktor Prof. Dr. *M. O. Gurewitsch*].)

Die schizoiden Psychopathien im Kindesalter.

Von

Dr. G. E. SSUCHAREWA,

wiss. Assistent.

Die Frage der „Schizoiden" und der „Zykloiden" hat gegenwärtig schon eine große Literatur gezeitigt und *Kretschmers* Buch „Körperbau und Charakter" wurde zum Ausgangspunkte einer ganzen Reihe verschiedenartiger Arbeiten. Übersehen wir das gesamte Material, welches über die *Kretschmer*sche Typologie vorliegt, so ergibt sich, daß diese Frage auch heute noch im Stadium des Kampfes verschiedener Anschauungen sich befindet. Am meisten umstritten erscheint das Problem des schizoiden Typus. Der Einführung des Schizoidbegriffs in die psychiatrische Klinik wurde seitens vieler Psychiater mit ernster Kritik begegnet. *Bumke* betrachtet diesen Begriff als eine künstliche Konstruktion; *Wilmanns* hält die von *Kretschmer* gegebenen Typen für unbestimmt und wenig scharf umgrenzt. Nach *Ewalds* Meinung ist der Schizoid ein Sammelbegriff, welcher alle Gruppen von psychopathischen Persönlichkeiten in sich aufnehmen kann.

Diese strenge Kritik der *Kretschmer*schen Typologie findet ihre Erklärung in der Verschwommenheit des schizoiden Typus, welcher von *Kretschmer* in seinem „Körperbau und Charakter" aufgestellt wurde. In der Charakteristik der schizoiden Temperamente gibt er einige meisterhafte künstlerisch beschriebene Züge der schizoiden Psyche; der Begriff des Schizoids bleibt aber dessen ungeachtet wenig scharf umgrenzt und unbestimmt. In einer literarisch-geistreichen Beschreibung einzelner schizoider Typen verliert sich die Klarheit des klinischen Begriffs, verschwimmt die Grenze zwischen dem Kranken und Gesunden, dem Charakterologischen und Psychotischen. Der Begriff des „Schizoids" wird zu einem allumfassenden, da seine Beziehungen

Figure 2. Extract from "Die schizoiden Psychopathien im Kindesalter" written by Dr. Grunya Efimovna in 1926 where she described the symptoms observed in children with "autism".

In 1943, Leo Kanner, a child psychiatrist in US, borrowing the term from Bleuler, published his famous paper "Autistic disturbances of affective contact" (Kanner, 1943). In this manuscript, he described 11 cases of children with autism diagnosis, listing the symptoms as an "extreme autistic aloneness"; abnormal speech with echolalia, pronominal reversal and inability to use language for communication and monotonous repetitive behaviors with "anxiously obsessive desire for the maintenance of sameness". From the beginning, he noticed the differences in the sex ratio, only 3 of the 11 children he observed in his study were girls. Contemporary to Leo Kanner, the Austrian pediatrician Hans Asperger described an account of children who presented similarities to Kanner's autism description but he also included the presence of cognitive abilities, including grammatical language in the average or even in a superior range (Asperger 1944). After this description, the name of Asperger's syndrome was used. The notably difference with Kanner's description is the presence of normal language in children diagnosed with Asperger's syndrome. At that point, Kanner stressed the contributory effects of parental lack of warmth or affection as constitutionally predisposing the children to develop autism. The theories about parental care and autism continued during the following years. In 1967 the phycologist Bruno Bettelheim popularized the theory and the term "refrigerator mothers", blaming the mothers as the cause of their children's conditions by not loving them enough (Bettelheim, 1967).

Stella Chess in 1971, a psychiatrist in the University of New York, published a report where she pointed out for the first time the possible relation between infections during pregnancy and autism development in children (Chess 1971). Lorna Wing, an English psychiatrist, was pioneer in the autism field research, treatment and in the understanding of autism. She also

took part in founding the National Autistic Society (NAS) in the UK and participated in the birth of parent's organizations, by developing literature on autism for parents and families and contributing to the diagnosis, treatment and care of affected people and their families, besides she also put in the maps Asperger's syndrome again (Wing, 1981). In 1979 Lorna Wing, together with Judith Gould, examined the prevalence of autism among children with different conditions. They found a prevalence of ASD of nearly 5 per 10.000 in children who presented an IQ under 70. Nevertheless, they also found a larger group of children (about 15 per 10.000) who had difficulties with social interaction, communication, and imagination, as well as repetitive stereotyped patterns of activities. Although those children did not fit the full picture of early childhood autism (or typical autism) as described by Kanner, they were on the broader 'autism spectrum' (Wing & Gould, 1979). Considering the description made by Wing and Gould about autism spectrum, in 1987 the DSM replaces the term "infantile autism" with a more expansive definition of "autism disorders", including a list of symptoms for diagnosis criteria.

In 1998, Andrew Wakefield and 12 of his colleagues published a case series in the Lancet (Wakefield et al., 1998), which suggested that the measles, mumps, and rubella (MMR) vaccine might predispose to behavioral regression and pervasive developmental disorder in children. Despite the small sample size (n=12), the uncontrolled design, and the speculative nature of the conclusions, the paper received wide publicity, and MMR vaccination rates began to drop because parents were concerned about the risk of autism after vaccination. This article was rapidly spread around the society, creating a strong anti-vaccine movement and many parents refused to get their children vaccinated. In 2004, 10 of 12 authors published a retraction claiming that "no causal link was established between MMR vaccine and autism as the data were insufficient" (Murch et al., 2004). Finally, twelve years after the publications, The Lancet published a small and anonymous paragraph in the journal, on behalf of the editors, retracting completely the original article published by Wakefield, admitting that several elements in the paper were incorrect (The Editors of The Lancet, 2010). In fact, Wakefield aimed at discrediting the MMR vaccine used at that time, as he was involved in the development of a new one. These episodes created a huge damage in the ASD field, and the consequences can be observed nowadays in the anti-vaccine groups.

Autism nowadays

The autism definition have been change along the time. The more recent changes in the autism concept was induce by the DSM V publication in 2015. This new publication redefine the diagnoses since combines four independent diagnoses (autistic disorders, Asperger syndrome, pervasive developmental disorders-not otherwise specified and childhood disintegrative disorder) into a single label of Autism Spectrum Disorder (ASD). Even the changes were made following a rationale argument (the DSM-V working group argued that

there are not consistent biological features that can distinguish Asperger syndrome from autism) the decision from separate Asperger from autism has been controversial for families and professionals. In previous volume for the autism diagnosis, the symptoms were divided into three domains: deficits in social behaviors, language deficits and repetitive and restrictive behaviors. In this new publication, only two domains were defined for the diagnosis, combined the social and language deficits into a single measure. To summarize, to the diagnosed with autism spectrum disorder, an individual must present deficits in social cognition, together with the presence of restricted and/or repetitive patterns of behaviors, activities, or interests (APA, 2013).

Etiology of ASD

The etiology underlying ASD is very complex and heterogonous, since there is not a unique cause for most of autism diagnosis. Different studies described the implication of genetic variants and mutations, epigenetic factors or environmental factors, and all of them could be affecting isolated or be associated one another. The identification of candidate genes has been a powerful tool in order to understand the ASD etiology. Besides, animal models with similar genetic mutations or alterations than humans have become an incalculable source of information for the autism research field (Möhrle et al., 2020). Focusing on the DNA mutations, the advance of new research tools have made possible to identify neuronal alterations and their implication in ASD development.

Classically autism have been classified into two groups, syndromic and no syndromic autism. Is necessary remark that this classification is exclusively based on clinical criteria.

- Syndromic autism: refers to condition in which ASD occurs in conjunction with additional phenotypes and/or dysmorphic features. In most of the cases the etiology is known and involve mutations in a specific gene, such fragile X syndrome (FXS), Rett syndrome (RTT), MECP2 duplication syndrome (MDS), tuberos sclerosis complex (TSC) Cortical dysplasia focal epilepsy (CDFE) and PTEN (Sztainberg & Zoghbi, 2016).

- Non syndromic autism: it refers at “classical autism” described by Kanner in which no -additional dysmorphic features, apart from the core-symptoms are present. In these cases of non-syndromic ASD, the etiology is unknown and probably due to a combination between environmental factors and genetic risk.

Genetic Factors

Copy number variations (CNVs) are micro-duplications or micro-deletions resulting from insertions, deletions, or translocations in the human genome. Those alterations are

present in the general population and are more common in patients with neuropsychiatric disorders, such ASD (Levy et al., 2011). Using cytogenetic techniques, large deletions or duplications, such as duplication of 15q (Bundey, Hardy, Vickers, & et al., 1994) deletion of 22q11.2 (Fine et al., 2005), deletion of Xp22.3 (Thomas et al., 1999), and duplication or deletion of 16p11.2 (Weiss et al., 2008), were identified as genetic risk factors for ASD. De novo CNVs exhibit vast heterogeneity in size – some are small enough to affect a single gene or large enough to encompass many genes – and clinical presentation; patients with multiple de novo CNVs usually have more severe phenotypes (Marshall et al., 2008; Pinto et al., 2010). Also, copy number variations (CNVs) have been found in between 5.6- 7% of families where only one person has an autism diagnosis ("simplex families") (Marshall et al., 2008; Pinto et al., 2010), suggesting that the disorder may frequently result not from an inherited mutation but from a spontaneous, or *de novo*, mutation in a sperm or egg cell.

Common variants are defined as genetic polymorphisms present in at least 5% of the population. These variants have been estimated to account for a major part of ASD liability (Gaugler et al., 2014), as has been observed for other common neuropsychiatric disorders. However, the effect size of each individual variant is quite small (Anney et al., 2012), so it is necessary a large sample sizes to achieve sufficient detection power to identify specific risk alleles. In a recent study performed in 2019 were using more than 18,000 individuals with ASD the authors were capable to identify common risk variants robustly associated with ASD (Grove et al., 2019). The contribution of the common variants contribution to ASD has been studying using genome-wide association studies (GWAS). Using GWAS studies, Grove et al identify up to 88 loci variations associated with ASD in genes such as CADPS, SOX7 or KCNN2 (Grove et al., 2019).

Rare inherited alleles, as we mentioned before, based on clinical criteria the autism can be distinguish in two type; the idiopathic and the syndromic autism. Although the idiopathic ASD present a strong male bias prevalence, this difference is not present in the ASD cases, where we can find a 1:1 male-to-female ratio (Yoo, 2015). Classic neurodevelopmental syndromes such fragile X or Rett syndrome are due to a rare inherited alleles. Rare alleles are polymorphic alleles with <1% frequency presence in genome and, although these rare alleles can be found in 3-5% of ASD cases, the genetic of inherited are poorly understood (Ramaswami & Geschwind, 2018). Mutation analysis in multiplex families, families in which than one member is affected wit ASD, has become a good strategy to identify genes underlying syndromic ASD. Rare X-linked mutations in two members of the neuroligin family NLGN3 and NLGN4 were identified in males diagnosed with ASD and mental retardation from multiple families (Jamain et al., 2003). Using linkage analysis or homozygosity mapping in consanguineous families, rare recessive mutations were identified in CNTNAP2 from Amish families as well as in SLC9A9 and BCKDK from Middle Eastern families in individuals with ASD and epilepsy (Morrow et al., 2008; Novarino et al., 2012; Strauss et al., 2006). More recent study, using whole-genome sequencing (WGS) in a family with three children affected

with ASD, identified inherited DNA variants in ASD-associated genes and pathways such RELN, SHANK2, DLG1, SCN10A, KMT2C (Dhaliwal et al., 2021).

CNTNAP2 gene and Autism spectrum disorder

In 2006, Straus et al discover a recessive mutation in the contactin-associated protein like 2 (CNTNAP2) gene present in an Amish family which was causative of a syndromic form of ASD called cortical dysplasia-focal epilepsy (CDFE) syndrome (Strauss et al., 2006). At the pathological level, CDFE is associated with an aberrant neuronal migration in the cortex and results in epileptic seizures, language regression, intellectual disability, hyperactivity and, in nearly two-thirds of the patients, ASD. Since then, several reports have linked this gene to an increased risk of autism or autism-related endophenotypes (Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Vernes et al., 2008). The CNTNAP2 protein (also known as CASPR2) shows a wide expression in the human brain, especially in cortical layers in the temporal lobe, amygdala, dorsal thalamus, putamen and basal ganglia (Abrahams et al., 2007; Alarcón et al., 2008; Bakkaloglu et al., 2008).

Considering the genetic characteristic of the CNTNAP2 has to be remark that CNTNAP2 is one of the largest genes in the human genome and it is located on the long arm of chromosome 7 and expands more than 3.3mb in the q35-q36.1 region. The CNTNAP2 protein encodes a protein which present different functions, in the nervous system, on one hand Cntnap2 protein is a cell adhesion molecule involved in cell-cell adhesions, including neuron-glia and extracellular matrix interactions (Rodenas-Cuadrado et al., 2016) and in the other hand, CNTNAP2 protein as a member of the neuroxin family, is involved in nerve excitation and conduction, and participate in the neurotransmitter release as well. Besides, also play a role in the stability of myelinated axons partly depends on proteins members of this family, comprising Caspr1-5, as these proteins are located to the juxtaparanode regions of the nodes of Ranvier in myelinated nerves (Bralten et al., 2010; Poliak et al., 1999; Poliak et al., 2003).

Epigenetic

Epigenetics studies heritable effects on the phenotype without modifications in the DNA sequence. Epigenetic mechanisms acts on chromatin accessibility to transcriptional regulation, therefore, those changes alter the DNA structure, and the genes expression are modified. Different process such DNA methylation, histone metilation or microRNA

dysregulation has been associated in the pathophysiology of the ASD (Table ; (Eshraghi et al., 2018).

Relations between epigenetics changes and ASD have been described in genes and chromosomes related to ASD such: 15q11-13, UBE3A or Mecp2 (Mbadiwe & Millis, 2013).

Epigenetic mechanism	Observation	Species	Tissue type	References
DNA methylation	Lower methylation of Proline-rich transmembrane protein 1 (*PRRT1*) gene.	Human	Brain Tissue	Ladd-Acosta et al., 2014
DNA methylation	Higher methylation in placenta from ASD subjects by placental methylome analysis.	Human	Placenta	Schroeder et al., 2016
DNA methylation associated with maternal asthma	Hypermethylation of *FAM181A*, *CHFR*, and *AURKA* genes.	Human	Blood	Gunawardhana et al., 2014
DNA methylation associated with maternal asthma	Hypomethylation in *MAP8KIP3* and *NALP1L5*.	Human	Blood	Gunawardhana et al., 2014
Epigenetic proteins	Increased expression of Tet methylcytosine dioxygenases (*TETs*)-1,-2, and-3.	Human	Brain	Zhubi et al., 2017
Epigenetic proteins	Decreased expression of DNA methyltransferase 1 (*DNMT1*).	Human	Brain	Zhubi et al., 2017.
Transgenerational inheritance	Valproic acid (VPA) exposure leading to autistic-like phenotypes in male offspring.	Rodent	Live animals	Choi et al., 2016
Gene polymorphisms associated with variation in diet	Association of methylenetetrahydrofolate reductase (*MTHFR*) C677T polymorphism having ASD in children from countries without folic acid food fortification.	Human	Blood	Pu et al., 2013
Histone modifications	A common acetylome signature at >5,000 cis-regulatory elements observed in greater than 68% of syndromic and idiopathic ASD cases.	Human	Brain	Sun et al., 2016
microRNA (miRNA) dysregulation	Upregulation of hsa-miR-21-3p miRNA that targets neuronal genes downregulated in ASD.	Human	Brain	Wu et al., 2016
microRNA (miRNA) dysregulation	Downregulation of hsa_can_1002-m that regulates the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) signaling pathways involved in neural development and immune function.	Human	Brain	Wu et al., 2016

Table X. Summarize of different epigenetics changes associated with ASD, and the species and the tissue where the changes were found (Eshraghi et al., 2018).

Environmental Factors

Although the role of genetic alterations in ASD is clear, genetics alone explains about 40% of cases. The rest harbor common genetic variants that, together with environmental factors, result in higher risk for ASD development (Hollander et al., 2020).

Different events occurring during conception and pregnancy have been linked to a higher risk of developing autism. One of them is the advanced parental age, it has been reported that every 10-years increase in maternal and paternal age, the risk of developing ASD in the off-spring is increased up to 18 and 21% respectively (Wu et al., 2017). The fetus development during pregnancy is a critical period where different factors could disrupt the appropriate brain development. The use of certain medications during the pregnancy (e.g., valproic acid) or mother smoking habits (Modabbernia, Velthorst, & Reichenberg, 2017), are associated with an increased risk of ASD. Also, autism has been associated with an altered (i.e. hyperactive) immune system and an active neuroinflammatory process (Modabbernia et al., 2017). Some pro-inflammatory cytokines are increased in ASD patients (Masi et al., 2015)

and this fact has been linked to alterations during pregnancy where the mother presented autoimmune diseases (Croen et al., 2008; Dalton et al., 2003). In the same way, activation of maternal immune system during pregnancy after viral infection is also linked to a higher risk of autism development (Lombardo et al., 2018).

Also, a lot of studies have been performed in other to assess a clear linked between environmental toxins and ASD, there is not a clear relevance of this association, showing a high variability between the studies (Modabbernia et al., 2017).

Sex differences in ASD

Most of the neurodevelopmental disorders have a male bias, including attention deficit hyperactivity disorder (ADHD) and intellectual disorders (Werling & Geschwind, 2013). Despite the huge heterogeneity characteristic of the ASD phenotype, one of the most remarkable finding is the male predominance in the diagnosis; the male/female ratio of idiopathic ASD has been reported to be 4.5:1 (Maenner et al., 2020).

One of the theories that attempts to explain these differences is the Female Protective Effect (FPE). The theory proposes that females require greater environmental and/or genetic risk than males to express the same level of phenotype symptoms, assuming that the females have a "protective factor" from autistic characteristics (Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013). Several studies revealed that female patients present more genes with de novo CNVs mutations and higher rates of de novo deletions than males. The median number of genes per CNS identified in autistic females is 10-15, compared to 2-3 in males with ASD (Ferri, Abel, & Brodkin, 2018). Besides, it seems that the CNVs in female patients are more likely to involve genes that are central to the gene network functionality, suggesting that a more serious interruption of the biological pathways is needed to provoke an ASD phenotype in females (Gilman et al., 2011).

On the other hand, it can not be ignored the fact that the origin of the many behavioral and anatomical differences that exist between males and females is due to sex chromosomes. In addition to a maternally inheritance of X chromosomes, there is a paternal inheritance, Y in case of males and X in case of females. An existing mechanism of compensation corrects the gene dosage in females. One of the X chromosomes is partially silenced, in a process known as X-inactivation. Nevertheless, some of those genes (10-15%) escape from this inactivation and these genes could potentially represent a mechanism for male bias in ASD (Ferri et al., 2018).

Secondary to sex chromosomes, the implication of sex hormones during development has been proposed as causative of the differences in the preferred male prevalence. Testosterone release during the fetus development is critical for the expression of proteins involved in synapse formation, maintenance, cell adhesion and scaffolding and spine formation (Ferri et

al., 2018), therefore, since mutations in these proteins are linked to ASD, the implication of the differential testosterone release between males and females, could explain the male bias observed in autism.

1.1 Neuropathology of ASD

Investigating the neuropathology of ASD allow the identification of its impact in the subtle characteristics of the human brain that cannot be assed using imaging studies. This approach provide the insight to study neuronal morphology, cytoarchitectural alterations or migration processes.

Whole cortical areas changes

In general developmental abnormalities has been found in ASD patients, more concretely alterations manifested in the archicortex, brainstem and other subcortical structures were found in post-mortem brain of autistic subjects (Wegiel et al., 2014; Wegiel et al., 2015). In addition differences in the head circumference and brain size are founded in autistic patients, presenting increase in size comparing with neurotypical subjects (Sacco, Gabriele, & Persico, 2015).

Regarding the microarchitecture of cortical areas is observe a disorganization of gray and white matter, such as cortical dysplasia and heterotopia, nodules of misplaced cortical neurons (Varghese et al., 2017). These alterations are not the only alterations in cortical layer, but problems in the migration and neuronal maturation are also found in patients with ASD as studies of Reelin, protein implicated in normal cortical migration, expression shown (Camacho, Ejaz, Ariza, Noctor, & Martínez-Cerdeño, 2014). These defects in in neuronal development and maturation could explain the differences seeing in the volume of neuronal nucleus and soma cytoplasm in children with ASD. It seen like post-mortem studies using children with autism diagnosis and neurotypical samples show a decreased in the volumes of cytoplasma and neuronal size in the majority of the areas compared, such striatum and amygdala, compared to age-matched controls (Wegiel et al., 2014; Wegiel et al., 2015). In the same way cortical alteration in the neuronal dendritic arbor and spines has been found in ASD patients, for example, in the hippocampus neurons of ASD patients present a reduced dendritic branching (Raymond, Bauman, & Kemper, 1995) and fewer dendrites number (Mukaetova-Ladinska, Arnold, Jaros, Perry, & Perry, 2004).

The Cerebellum

The cerebellum (form the Latin, little brain) is a structure located in the posterior cranial fossa. Classically, this structure has been linked to motor behaviors and equilibrium. Nevertheless, an increasing number of reports provide evidence of the role of the cerebellum in higher cognitive functions such cognition, language, and social and affective behaviors (Fernández, Sierra-Arregui, & Peñagarikano, 2019).

Cerebellar anatomy and morphology

Cerebellar anatomy is really circumvallated and divided into two hemispheres separated by a midline called vermis. The cerebellar circumvallation forms lobules which represent functional specialities. The cerebellum is divided in ten lobules: lobules I-V (which constitute the anterior lobe), lobules VI-IX (posterior lobe) and lobule X (flocculonodular lobe). The lobules VII and VIII are at the same time subdivided into VIIA and VIIB and VIIIA and VIIIB.

The exterior layer of the cerebellum is the cortex, which is formed mainly by gray matter. Going deeper into the structures, we can find an inner core (formed mainly by white matter) which encloses the deep cerebellar nuclei, the sole information output of the cerebellum. The cerebellar cortex is structured in three different cell layers: (1) **Molecular layer**: outer layer composed by two main cell types, basket, and stellate neurons. Both are inhibitory and regulate the Purkinje cells (PC). (2) **Purkinje cell layer**: located below the molecular layer. PCs are a highly important cell type in the cerebellum since represent the solo info output driving the info to the deep nuclei. (3) **Granular layer**: it is the deepest layer, and it is composed of excitatory granule cells whose axons form the parallel fibers. The *parallel fiber* (PF) reaches the PC dendritic arbor is located and making contact with the PC dendrites and is able to regulate the firing patter of PC. We also can find Golgi cells, which are inhibitory interneurons. The Golgi cells receive input from the parallel fibers and provide an inhibitory feedback to the cells of origin of the parallel fibers (the granule cells). Neurons in the deep cerebellar nuclei represent virtually all the output from the cerebellum. They receive inhibitory information from the PC and excitatory inputs from outside the cerebellum through the mossy and climbing fibers. The *climbing fibers (CF) are* originated in the brain stem (posterior part of the brain, continuous to the spinal cord, composed by the midbrain, the pons, and the medulla oblongata), particularly in the inferior olivary nucleus of the medulla oblongata. These axons make excitatory synapses with PC dendrites and with neurons in the deep cerebellar nuclei. The *mossy fibers (MF)* originate from several parts of the brain and spinal cord. These fibers form excitatory synapses with granule cells and with neurons of deep cerebellar nuclei. In turn, PCs receive two types of excitatory input from outside the cerebellum, one directly from the CF and the other indirectly via the PF of the

granule cells. Anatomical investigations in animals and post-mortem humans have established that cerebra-cerebellar connections are contralateral to each other and include an efferent cerebellar-cortical pathway from the cerebellar nuclei to the cerebral cortex through the thalamus and an afferent cortico-cerebellar pathway through the pons.

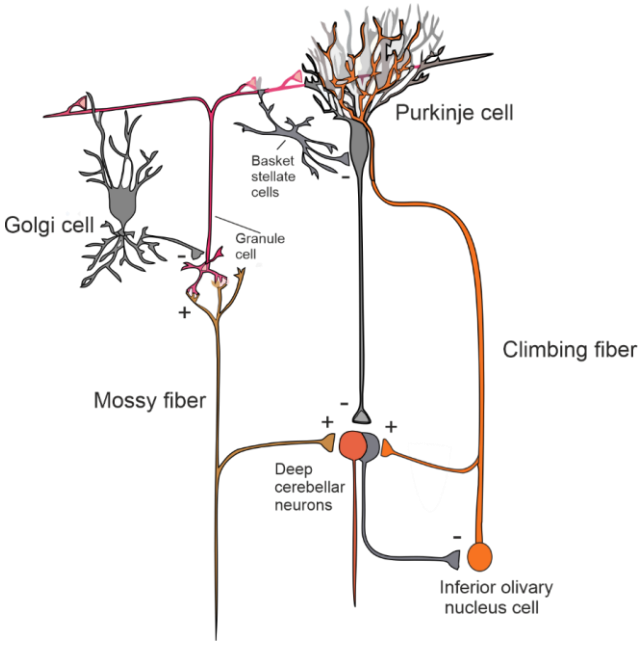


Figure 3 . Representation of main cerebellar circuits and its principle neurons. PC transmit the information to the deep cerebellar nuclei, which are the cerebellar output. The interactions between the cells are represented with (+) in case of excitatory connections and (–) when connections are inhibitory (from (Fernández et al., 2019)

Cerebellar implications in ASD

The cerebellum presents a topographic organization in which each region has a functionality based on their specific connectivity. In general, the anterior lobe and lobule VIII contain the representation of the sensorimotor cerebellum; lobules VI and VII. The VII is divided in two parts; VIIa, subdivided in Crus I/Crus II, and lobule VIIb. Those posterior lobes comprise the cognitive cerebellum; and the posterior vermis encompasses the limbic cerebellum (Fernández et al., 2019).

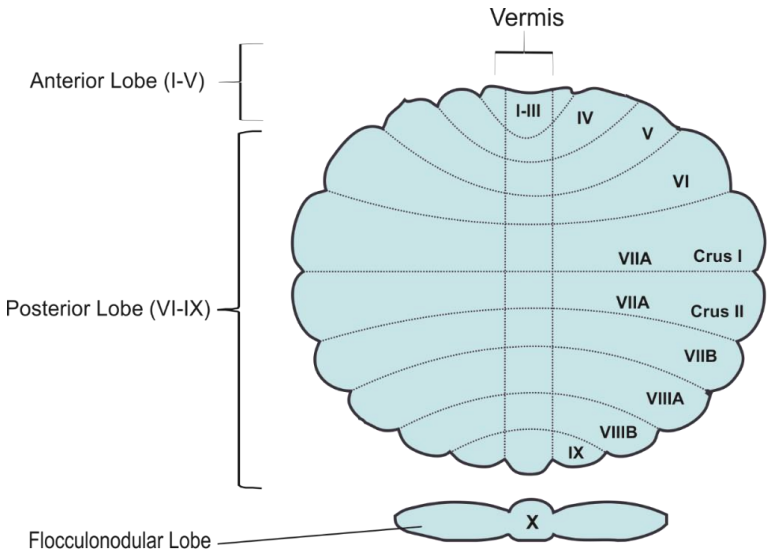


Figure 4. representation of the cerebellar lobules: anterior, posterior and flocculonodar and their respective subdivisions

Structural cerebellar abnormalities found in autism

The cerebellum has been reported as one of the most consistent sites of reported neuronal abnormalities in autism (Fatemi et al., 2012). Studies using human post-mortem brain tissue, found that the reduced size and number PCs is one of the most replicated findings in the brain of individuals with autism (Wegiel et al., 2014). This reduction in the number of neurons in autistic patients, is also reflected in a reduction in gray matter volume, smaller ratio of gray to white matter and smaller vermis (Bolduc et al., 2012; Courchesne et al., 2001). Besides structural and cellular alterations, molecular abnormalities have also been reported in the cerebellum of ASD individuals. Alterations in the distribution of the mRNA levels of glutamic acid decarboxylase 67 (GAD67), an enzyme involved in the synthesis of the inhibitory neurotransmitter GABA, have been found. Thus, decreased GAD67 mRNA has been reported in PC (Yip, Soghomonian, & Blatt, 2007), while increased GAD67 mRNA has been reported in cerebellar interneurons (Yip, Soghomonian, & Blatt, 2008). These cerebellar imbalances could account for the proposed E/I disequilibrium in ASD, as they could affect cerebro-cerebellar circuits (Hegarty, Weber, Cirstea, & Beversdorf, 2018).

The differences described in the cerebellum are not only present in humans, but different mouse model of ASD such Mecp2, SHANK3 or TSC1 replicate the human findings.

Rodent model	Cerebellar Structural Abnormalities	Functional Abnormalities	Reference
Fmr1 KO	• Decreased deep nuclei volume • Reduced number of neurons in deep nuclei • Increases astrocytes in deep nuclei • Reduced volume of cerebellar cortex • Reduced cerebellar volume during development • Elongated spines	• Deficits in eyeblink conditioning • Altered PF-PC synapses	(Ellegood, Jacob, Pacey, Hampson, Lerch, & Henkelman, 2010; Fatemi & Folsom, 2011; Gothelf et al., 2008)
Fmr1-PC KO	• Decreased cerebellar volume • Cellular loss in cerebellar nuclei • Elongated spines in PC	• Deficits in eyeblink conditioning learning • Altered synapses between PF and PC	(Koekkoek et al., 2005)
PC-*Tsc1*	• Abnormal spine density • Apoptosis of PC	• Reduced spontaneous PC firing rate • Impaired excitability • Deficits in eyeblink conditioning learning	(Kloth et al., 2015; Tsai et al., 2012)
Cntnap2 KO	• Larger cerebellar cortex • Larger inferior olivary cortex	• Deficits in eyeblink conditioning learning	(Ellegood, J. et al., 2015; Kloth et al., 2015)Ellegood 2015
Shank 3 Δc	• Decreased in spines density	• Deficits in eyeblink conditioning learning	(Kloth et al., 2015)
Pten-PC KO	• Reduced PC number • PC size abnormal • Thicker dendrites and axons • PC apoptosis	• Reduced spontaneous firing • Disrupted climbing and parallel fiber synapses	(Cupolillo et al., 2016)
patDp/+		• Learning • Eye blink conditioning	(Piochon et al., 2014)

Table 2. summarize of differents mouse model of ASD and their cerebellar alterations. (from (Fernández et al., 2019)

Crus I-II

In the present thesis, we focused on the Crus I-II characterization. These areas represent the cognitive and social cerebellum, due to their connections with different cortical regions implicated in language and social processing, such Brodmann´s area BA46 in the prefrontal cortex (Strick, Dum, & Fiez, 2009). Even more, the connection between the cerebellum and cortical areas in relation to social skills are supported by fMRI studies which show that the activation of Crus I-II is enhanced after socials paradigms (Jack, Englander, & Morris, 2011;

Jack & Pelphrey, 2015). A meta-analysis using over 350fMRI studies support the idea that the cerebellum plays a role in several social paradigms such as mirroring (i.e., observation of human motion) and mentalizing (i.e., interpreting other people's thoughts and intentions) (Van Overwalle, Baetens, Mariën, & Vandekerckhove, 2015a; Van Overwalle, Baetens, Mariën, & Vandekerckhove, 2015b). In addition, the reduction in PC density described above is more pronounced in the Crus I/II area in autism patients (Skefos et al., 2014). Further, in ASD patients the degree of reduction in gray matter of Crus I/II correlate with the severity of symptoms in social interaction and communication behavior (D'Mello, Crocetti, Mostofsky, & Stoodley, 2015; Riva et al., 2013). Not only structural abnormalities have been found in Crus I/II area in relation with ASD, but accordingly the functionality of these areas is also affected. In a task that requires perception and imitation of human actions, fMRI detected an engagement between the posterior superior temporal sulcus (pSTS) and the cerebellar region Crus I (Jack & Pelphrey, 2015). Interestingly, the degree of functional coactivation of pSTS and Crus I could predict social deficits in ASD in the "mentalizing skills" questionnaire, a parent report for specific social cognition skills based on imaginative mental activity that allows an understanding of the behavior of other people (intentions, needs, desires, or goals). Thus, stronger Crus I-pSTS interactions were associated with better mentalizing ability (Jack & Pelphrey, 2015). On a similar note, during a task that involves decoding the interactions between animated figures, aimed at examining the "theory of mind" network, that is, the ability to attribute mental states to others, a reduced cerebellar activation, particularly in Crus I, in participants with ASD was found (Kana et al., 2015).

The Cntnap2 Mouse model

Although it is clear that no animal model captures the full complexity of ASD in humans, animal models are powerful tools for studying the underlying cellular and molecular mechanism of specific ASD symptoms associated with ASD mutations. The mouse (*Mus musculus*), as a mammal, has been shown to present neurobiological similarities to humans and is considered to be a good model for neuropsychiatric disorder studies. A mouse knock out of Cntnap2 was generated in 2003 by Poliak and colleagues with the aim to study the role myelinisation (Poliak et al., 2003). A few years later, Strauss linked a nonsense mutation in the human gene CNTNAP2 as a causative of CDFE, a human syndrome with high penetrance of ASD (Strauss et al., 2006). This report put this model in the scope of ASD studies. It was in 2011 when Peñagarikano and colleagues validated the Cntnap2-KO mouse as an animal model of autism. After an exhaustive behavioral, physiological and neuronatomical evaluation, they concluded that the mouse fulfills construct, face and validity criteria to be considered a good model of ASD. Thus, the Cntnap2 model shoes the main core ASD symptoms in humans; communicative and social deficits and repetitive behaviors and restrictive behaviors (Peñagarikano et al., 2011). Moreover, the mouse presents other symptoms, which, even if they are not within the main criteria of autism diagnosis, are present in autism patients at a high prevalence and were reported in CDFE patients, such as

a hyperactivity and epilepsy (Peñagarikano et al., 2011). Further, treatment of the mice with risperidone, one of the few drugs approved to treat ASD associated symptoms, rescued hyperactivity and repetitive behavior and, similar to was described in humans, had no effect on sociability (Peñagarikano et al., 2011).

The neuropathology associated with the Cntnap2 KO mouse has been broadly studied by different groups in the last years, mainly focusing in the characterization of cortical areas. In the first characterization of the mouse it was reported that Cntnap2 mutants show defects in the migration of cortical projection neurons (Peñagarikano et al., 2011), similar to human patients with CDFE diagnosis (Strauss et al., 2006). The migration of the different cells into their final brain location during neurodevelopment is a key factor for normal functionality, in fact, defects in those processes have been reported as causative of epilepsy, intellectual disability and other neurodevelopmental disorders (Guerrini & Barba, 2010). In addition to the migration abnormalities, the Cntnap2 KO mouse also presents lower number of GABAergic interneurons (Peñagarikano 2011) and defects in spine stabilization in pyramidal cells (Gdalyahu et al., 2015). This alteration in the cortical neurons could be responsible for the defects in excitation/inhibition balance present in this area in the model. Interestingly, E/I imbalance has been linked to deficits in social behaviors and information processing (Yizhar et al., 2011) and the neuromodulation of this imbalance in Cntnap2 KO mice, either by increasing the excitability of interneurons or decreasing the excitability of pyramidal cells is enough to restore the social deficits of the model (Selimbeyoglu et al., 2017). In a more recent study, Antoine et al described alterations in the feedforward inhibition between cortical layers in the somatosensory cortex (SI) of the Cntnap2 mouse (Antoine, Langberg, Schnepel, & Feldman, 2019). This is an area where the integration of sensory information occurs. That maybe is not surprising, since alteration in the sensory processing has been described in this model (Peñagarikano et al., 2011) and is a symptom inside the spectrum (APA, 2013).

Cntnap2 knock-out mouse model and Cerebellum

Even the Cntnap2 mouse model of ASD is one of the most studied autism models and a lot of efforts and studies has been focusing in the characterization and description of the cortical areas, the cerebellum remain unstudied. The cerebellum seems to have a role in the ASD (Fernández et al., 2019) with different alterations present in both, patients with autism and animals models, but also the cerebellum, due to its connections with cortical areas associated with motor and non-motor behaviors, seems to have an important role in the modulation and control of these regions (Li & Mrsic-Flogel, 2020; Pisano et al., 2021). Pointing out the importance of cerebellum in the human behaviors understanding. Even though, a few other studies has been made using the Cntnap2 KO mouse model in order to characterize the cerebellum. In 2015, Kloth and colleagues, characterized the response in Cntnap2 KO mice after an eye-blink conditioning protocol, a cerebellar learning paradigm, revealing differences

in the % of correct response emitted by the mice after the learning process, suggesting a possible malfunctionality in the information processing in the cerebellum (Kloth et al., 2015). Besides a more recent study analyzed the cerebellar cytoarchitecture of Cntnap2 KO, reveling the presence of cellular heteropatia in the cerebellar molecular layer and alterations in the myelinated axons organization (Otazu et al., 2021)

Cerebellar information integrations approach

How the cerebellum integrates, the information is a good approach to try to understand how the circuit is working. Previous studies revealed that the Cntnap2 KO mouse present alterations in cerebellar information processing (Kloth et al., 2015) and the findings that connect the cerebellar alterations with ASD (Fernández et al., 2019) make the cerebellar characterization a good approach to study this model. For that the reason, we choose a well-known paradigm consistent in electrical whisker stimulation and simultaneously record the sensory evoked potential (SEP) in R-Crus I/II. The information of whisker conveys to the CrusI/II area from two pathways: from the trigeminal nuclei and from the somatosensory cortex (SI) (Mapelli & D'Angelo, 2007; Márquez-Ruiz & Cheron, 2012; Roggeri, Rivieccio, Rossi, & D'Angelo, 2008).

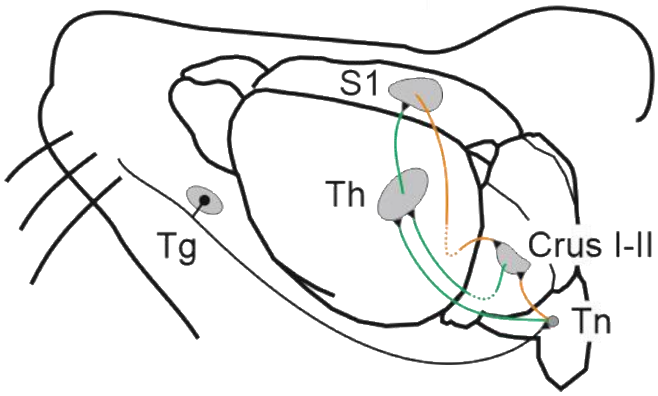


Figure 5. Schematic simplification of the mice whisker circuit connecting somatosensory and cerebellar cortices. Sensory information from whiskers is conveyed through Tn in the midbrain (black trace) to cerebellar CrusI-II by two different pathways (orange traces); a direct input comes from Tn and an indirect afference arrives from S1 (through pontine nucleus (dotted line), not showed in the figure). Also, CrusI-II projects to S1 through deep cerebellar nuclei (not showed in the figure, green dotted line) and Th. Abbreviations: Somatosensory cortex (S1), trigeminal ganglion (Tg), thalamus (Th) and trigeminal nucleus (Tn).

The well-characterized sensory-evoked potential is composed by two main components: the trigeminal component (T), appearing at ≈ 3ms, and the cortical component (C) at ≈12ms (Márquez 2012). Besides the SEP components are correlates with PC activity. The PC have two firing pattern : the simple spikes (SS), which occur spontaneously (Häusser & Clark, 1997; Raman & Bean, 1997) and can exceed 100 Hz in response to excitation from parallel fibres and complex spikes, which are triggered by climbing fibres excitation and consist of a characteristic high-frequency burst (Eccles, Ito, & Szentágothai, 1967; Thach, 1967). The PC simple spike firing is coincident with the latency in which we can observe the appearance of trigeminal component and, in the same way, the complex spike (CS) latency is synchronized with the cortical component (Márquez-Ruiz & Cheron, 2012; Tsutsumi et al., 2020).

2. Objectives

The autism in a neurodevelopmental disorder with a great incidence worldwide, becoming important health issue. There is not an effective treatment for the core symptoms of ASD, for that reasons, the study of the different brain domains altered in this disorders is a good approach to try to get a better understanding for get an effective treatment. Using different studies, in humans and in mouse model of autism, certain domains have been linked to ASD, especially those that classically have been linked to the control of social cognition (e.g. amygdala, striatum or prefrontal cortex). Nevertheless, the presence of abnormalities in cerebellar structures in ASD patients had open the field to study the function and the implication of this structure not only in ASD but also in the control of higher cognitive functions. For that reason, using s mouse model of autism previously validated, we performed an exploratory characterization of the cerebellum of the Cntnap2 KO mouse.

For our study, we performed the experiments with the aim to accomplish the following objectives:

1. Characterization of spontaneous Purkinje Cell in the cerebellar region R-Crus I/II in *in-vivo* Cntnap2 KO mice.
    1.1. Explore the possible modulation of PC spontaneous parameters using tDCS.
2. Morphological characterization of PC in a mouse model of autism Cntnap2 KO.
3. Characterization of cerebellar response and information processing after sensorial stimuli in Cntnap2 KO.
    3.1. Characterization of PC activity in response to sensorial stimuli.
4. Characterization of PC activity in response to sensorial stimuli.
5. Explore the role of Cntnap2 protein in the cerebellum and its implication in autistic behaviors.

3. Materials and methods

Animals

Two different mouse lines were used for the experimental procedures: 1) Adult male mutant mice lacking the Cntnap2 gene (Cntnap2-KO) and age matched wild-type counterparts (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). 2) Female and male Purkinje cell conditional Cntnap2 mice (cKO) were generating by crossing L7/Pcp2-Cre (Pcp2-Cre) transgenic mice stock number 010536 attained from The Jackson Laboratory (Bar Harbor, ME, USA) and mice with a loxP flanked Cntnap2 exon 3 (Cntnap2-loxP/loxP), donated by Dr. Daniel Geschwind (UCLA). Pcp2-Cre transgenic mice express Cre recombinase under the control of the mouse Purkinje cell protein (Pcp2). When crossed with a strain containing loxP site flanked sequence of interest, Cre-mediated recombination results in deletion of the targeted gene in the offspring (Figure X). Animals were housed 4-5 per cage on a 12-12 h light/dark cycle, at 21-23°C and 65-70% humidity. Food and water were provided ad libitum. Animal maintenance and experimental procedures were executed in accordance with the recommendations of the guidelines of animal care established by the European Communities Council Directive 2010/63/EU, as well as in agreement with the Spanish Legislation (Royal Decree 53/2013). Procedures were also approved by the Ethics Committee for Animal Welfare (CEBA) of the University of the Basque Country (UPV/EHU) and the Pablo de Olavide University (UPO).

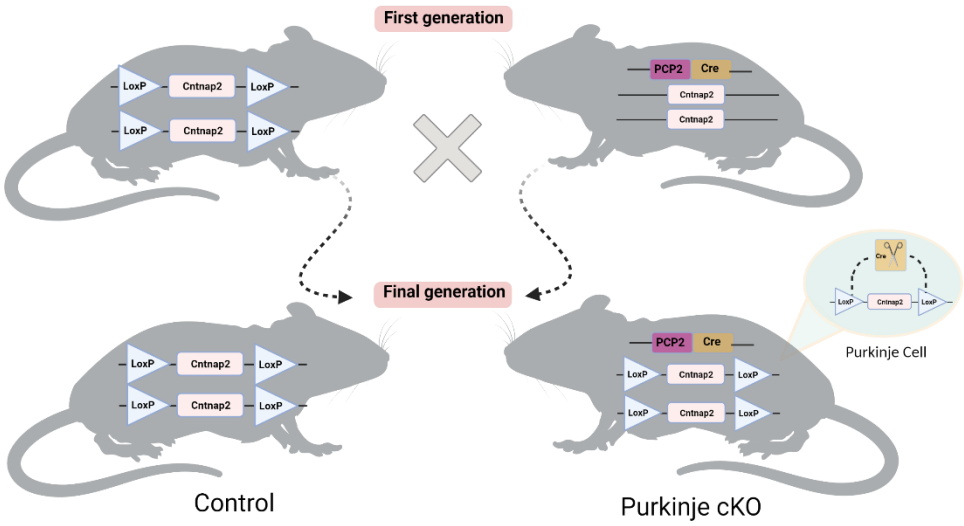


Figure 6. Schematic representation of the parental used to obtain the mice Purkinje cKO and their littermates control.

Genotype

Mouse genomic DNA was isolated from tissue biopsies following the protocol described in (Truett et al., 2000). Briefly, with this protocol we are able to obtain DNA from mouse tissue. The tissue, normally 2mm of tail is added to an Eppendorf contained 75µl of boiling buffer (25mM NaOH/ 0.2mM EDTA) and place in a thermocycler at 98º for 1 hour. One the boiling is end 75µl of neutralization buffer (40mM Tris HCl pH 5.5) is added to the Eppendorf were the DNA is present in the buffer.PCR was performed using Taq (DreamTaq™ Hot Start Green PCR Master Mix, Fisher) with the instructions provided by the manufactures. The sequence primer pairs (Integrated DNA Technologies) were the following:

- cKO: Cre F' 5'- GCGGTCTGGCAGTAAAAACTATC 3' R'5- GTGAAACAGCATTGCTGTCACTT 3'
- Control: F 5'- CTAGGCCACAGAATTGAAAGATCT3' R' 5- GTAGGTGGAAATTCTAGCATCATCC 3'
- Cntnap2 -/- : Wt F 5'- TGCTGCTGCCAGCCCAGGAACTGG 3' R 5'- TCAGAGTTGATACCCGAGCGCC 3'
- KO: 5'- AGCAGCCGATTGTCTGTTGT 3' R 5'- CTCACCCAATCTCACAAACAAG 3'

Mouse behavioral tests

Isolation-induced pup Ultrasonic Vocalizations

At postnatal day (P) 7 the pups were removed from the mother and individually gently placed in a small box. The recordings were measured inside a noise-protected box in order to avoid external noises. Ultrasonic Vocalizations (UsV) were monitored and recorded using Ultrasound Microphone (Avisoft UltraSoundGate CM16/CMPA, Avisoft Bioacoustics e.K., Germany), sensitive to frequencies 2-250 kHz connected to an Ultrasound recording Interface. Both devices were connected to a computer with a key that enables access to Avisoft Recorder (a Bioacoustics Recording Software that monitors real-time acoustic of sound signals. Avisoft Bioacoustics e.K., Germany). The microphone was located above the mouse pup, at a distance of 5 cm from the pups. Selected frequency ranged from 0 to 250 kHz. The number of vocalizations were measured semi automatically using Avisoft SASLab Pro.

Open-Field

The open-field test is used to measure the locomotor activity of the subjects. The test was performed at P 28-32. The mice were placed in an open arena for 20 minutes. The mice. They were placed in the middle of the field apparatus and let to move freely. The activity was

recorded by a camera fixed above the apparatus, and the results were individually analyzed using Avisoft Recorder software.

Three Chamber

The three-chamber is a classical test to quantify social behaviors in mice. The arena is a rectangular box divided into three chambers separated by methacrylate walls and with an opened door in the middle, allowing the mice to explore the three chambers freely. As habituation, the mice are first allowed to explore and be familiarized with the arena for 10 min, after that two cups are placed in the lateral chambers, remaining the center chamber empty. To test social preference, one of the cups has inside a mouse matched in sex and age with the experimental mouse and the other cup remains empty. The time spent by the experimental mouse exploring the cups was manually measured by an expert experimenter.

Self-grooming

Grooming is considered as a natural behavior for the mice but, when prolonged for an unusual period of time, it is considered as stereotyped or repetitive behaviors. Mice were placed individually in an empty cage and the spontaneous self-grooming was measured for 10 min.

Dark-light box

Dark-light box is based on the natural aversion of mice to a bright area. The arena is formed by two interconnected compartments, the smaller one is in darkness and it is considered as safe, the rest of the arena is highly illuminated. The mouse is placed in the safe box and allowed to explore the comportments for 5 min. The latency of the first transition and the accumulative time spent by the mice in the brightness area is a measure of anxiety.

T-maze

Spontaneous alternation in a T maze is a classical approach to test inflexibility in mice. The mice were placed on the base of the maze and were allowed to explore freely either the right or the left arm of the maze for ten consecutive trials. Once the mouse entered the arm (i.e. made a choice) it was allowed to explore it for 5 seconds. Usually, wild type mice tend to alternate arms every trial. The number of no-alternations is considered a measurement of inflexibility or ‘insistence of sameness’.

Hot Plate

To assess potential sensory deficits, we performed a Hot plate test, consisting of a plate warmed at 52,5 º. The mice were placed in the middle of the plate and the latency to the first

pain signal (paw licking or kicking) is measured. To avoid any harm to the animal, the maximum time is set at 15" at which the animal is removed from the plate.

In vivo electrophysiology

Surgery

For the surgical procedures, the animals were anesthetized with 4% isoflurane as induction and placed in a stereotaxic apparatus at a 2%-1.5% maintenance flow, which was kept along the procedures. Before starting surgery, the anesthetized state of the animals was confirmed by an absent reflex withdrawal of the hind paw to a strong pinch. Under aseptic conditions, an anteroposterior (AP) incision in the skin along the midline of the head, from the front leading edge to the lambdoid suture, was performed. The skull covering the cerebellum was exposed by retracting the muscles over the occipital bones. Subsequently, the periosteum of the exposed surface of the skull was removed and washed with saline. The animal's head was correctly positioned to mark the position of Bregma and Lambda as stereotaxic cero. Following the stereotaxic brain atlas (AP: -6,6 mm; and L: -2,6 mm, relative to Bregma) a craniotomy (2 mm ø) was drilled in the bone to expose the cerebellar cortex. The dura mater surface was sealed with wax bone (Ethicon, Johnson & Johnson, NJ, USA) until the recording of sessions.

Whisker stimulation

Whisker stimulation was performed by the implantation of a pair of flexible steel electrodes (Strand ø: 50.8 μm; Coated ø: 228.6 μm; Multi-Stranded PFA-Coated Stainless Steel Wire, A-M Systems, WA, USA) under the skin of the right whisker pad. The electrical stimulus consisted on a single square pulse (0.2 ms; 0.5-1 mA) delivered by an isolation unit (Cibertec ISU 210 BIP) connected to a stimulator device (CS420, Cibertec) and was applied every 10±2 s.

In vivo Recordings

After the surgery, the mice were allowed to recover for at least two days. For in vivo recordings, the animal's head was fixed to the recording table, using a screw previously implanted in the skull during surgery. The set up consisted in a treadmill with an infrared sensor to monitor the locomotor activity. All experiments were carried out with an amplifier (BVC-700A, Dagan corporation, MN, USA) connected to a dual extracellular-intracellular headstage (8024 Dual Intracellular & Extracellular Headstage, MN, USA). For recordings, the electrodes were manufactured following the protocol described in Sanchez-León 2021

(Sánchez-León et al., 2021) . Briefly, they consisted of glass micropipettes (Outside ø: 2.0 mm; Inner ø: 1.6 mm; length: 15 cm, with inner filament; A-M Systems, WA, USA) manufactured by pulling the glass capillary with a vertical pipette puller (Model PE-22, Narishige, Japan) and, subsequently, breaking the tip under an optical microscope to obtain tips with the appropriate diameter. For single-Purkinje cell activity, a micropipette with a tip diameter around 1-2 µm was used, subsequently the pipette was filled with 3 M NaCl and placed on a micromanipulator (Narishige MO-10, Japan). The pipette was inserted in the area of interest, at ~2 µm/s and spikes were detected based on visual (2002C and 2004C, Tektronix, OR., USA) and auditory (Audio monitor 3300, A-M Systems, WA., USA) cues. For SEP recordings a micropipette with a tip diameter between 8-10 µm was used, subsequently the pipette was filled with 3 M NaCl and placed on a micromanipulator (Narishige MO-10, Japan). To map the SEP, an electrical stimulus was delivered at the whisker pad every 2 s, the micropipette was lowered, and the current intensity adjusted until the maximum amplitude SEP was achieved. Then, the current intensity of whisker electrical pulses was reduced as to elicit a SEP with half of the maximum amplitude.

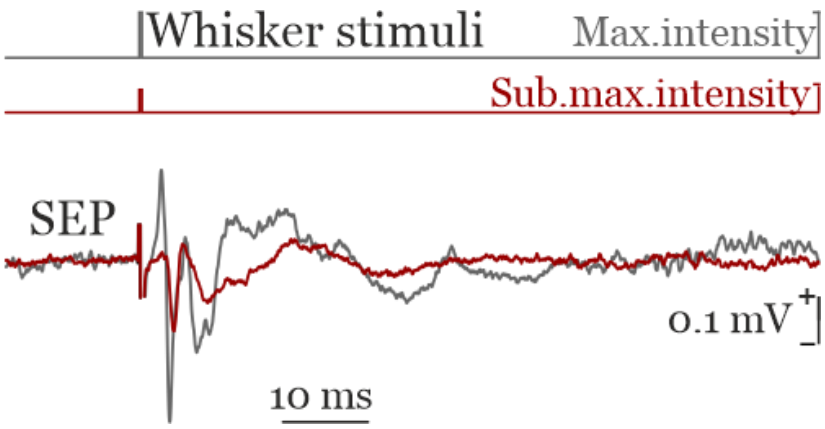


Figure 7: Comparison of Crus I/II-SEPs for two different intensities applied to whisker stimulation obtaining maximum N2-N3 amplitude (grey trace) and 50% of the maximum N2-N3 amplitude (red trace).

Transcranial direct current stimulation

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation technique (Nitsche & W. Paulus, 2000) that consists in the administration of low current through the scalp between a target electrode (located in the region that we want to modulate) and at least one reference electrode. Additionally, there are two stimulation modalities depending on the polarity of the target electrode: anodal stimulation, when the positive electrode is the target electrode, and cathodal stimulation, when the negative electrode is the target electrode. Transcranial currents were delivered by a battery-driven WPI A395 Linear Stimulus Isolator

(World Precision Instruments, FL, USA). They were applied between the target electrode (custom-made ring-electrode or polyethylene tubing) over the scalp (CrusI/II) and a reference electrode consisting on a rubber rectangle (6 cm2) attached to the back of the mice and moistened with electrogel (E10 ECI ELECTRO-GEL, Electro-Cap International).

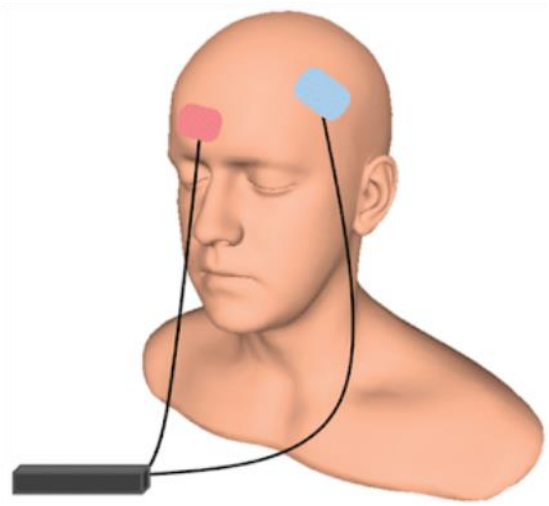


Figure 8. Schematic representation of tDCS technique. Red patch represents the active or target electrode, located in the area where we want to apply the stimulation. Blue patch is the reference electrode. The current flow between the electrodes is the responsible of the effect of tDCS from Dayan 2013.

tDCS immediate effects application

To characterize the direct effects of the electric current on the PC activity and to avoid plasticity changes, we applied alternating trials of 5s anodal or cathodal tDCS at 200 μA with an additional 5 s ramp-up and 5 s ramp-down, separated by 10 s of non-stimulation.

Data collection

Data were collected with a CED micro1401-3 and sampled at 25 kHz for single cell and LFP recordings, with an amplitude resolution of 16 bits. The remaining non-neuronal signals were sampled at 5 kHz.

Data analysis

Sensory Evoked Potential: Event-related potential (ERP) analysis was performed in EEGLAB rev.14.1.2 toolbox using Matlab 2015a software package. Recordings data were segmented in 70 ms windows using the electrical stimulation as trigger, baseline was corrected by subtracting the mean voltage level in the first 20 ms interval of the window (before whisker

stimulus). Data were averaged for each genotype to obtain the average SEP in EEGLAB toolbox and temporal periods were statistically compared.

Purkinje cell Electrophysiology: For single-cell recordings analysis, only well isolated neurons recorded during at least 100s were considered. A direct current (DC) remove process (time constant (s): 0,001-0,0004) was applied to reduce DC level drifts, and spikes were detected based on threshold-crossing algorithm of Spike2 software. All spikes were visually confirmed and Purkinje cells were identified by visualization of the Complex Spike (CS). Subsequently, the Simple and Complex Spikes of each neuron were analyzed using a Matlab custom-made script. The Predominant Firing Rate represents the mode of the firing rate, the Frequency of the firing rate that appears most often. The Coefficient of variation CV and CV2, as a measure of firing regularity, were calculated following the formulas described in Holt et al 1996: CV=σISI/(μISI), where ISI represents the inter-spike interval. CV2=(2|〖ISI〗_(n+1)- 〖ISI〗_n |)/((〖ISI〗_(n+1)+ 〖ISI〗_n)). The Complex Spikes were sorted manually offline for the duration of the recording.

Ex vivo experiments. Neuronal Morphology

Slice preparation

To analyze Purkinje cell morphology, neurons were filled in acute slices. Male from 4 to 8-week-old mice were anesthetized with isoflurane and decapitated. The brain was rapidly submerged in ice-cold cutting solution containing (in mM): 20 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 85 sucrose, 25 D-glucose and 60 $NaHCO_3$, saturated with 95% O_2/5% CO_2 (carbogen). The cerebellum was separated from the rest of the brain and the right part cut and glued to the cutting-dish. Parasagittal slices (250 μm thick) containing the Crus I area were prepared using a Campden Ci 7000smz-2 vibroslicer. Immediately upon cutting, slices were submerged in an artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 11.1 D-glucose, 21.4 $NaHCO_3$, 0.1 ascorbic acid and 0.4 kynurenic acid, bubbled with carbogen at room temperature (22-24 °C), and were left to recover for at least 1 h.

Purkinje cell morphology

PCs were filled with biocytin (Sigma) via passive diffusion and fixed in paraformaldehyde 4% for 24ºh at 4ºC. After fixation, the slices were treated with streptavidin Alexa 488 (Sigma) 1:500 in blocking solution for 48º at 4º. Slices were mounted using Prolong Antifade Gold mounting medium (Invitrogen).

Super resolution images were acquired using a confocal LSM880 Fast Airyscan microscope using a 25x/0.8 water (for dendrites analysis) and a 63x/0.4 oil (voxel size 49 x 49 x 211 nm, for spines analysis) Plan-Apochromat objectives. Sholl analysis was performed for each

neuron using Fiji ImageJ software (Figure X) and the number of intersections and the dendritic length were studied using 5um radio intervals from the soma. For spine quantification a dendrite area of 10um from each neuron was analyzed using Fiji imageJ software using a semi-automated process.

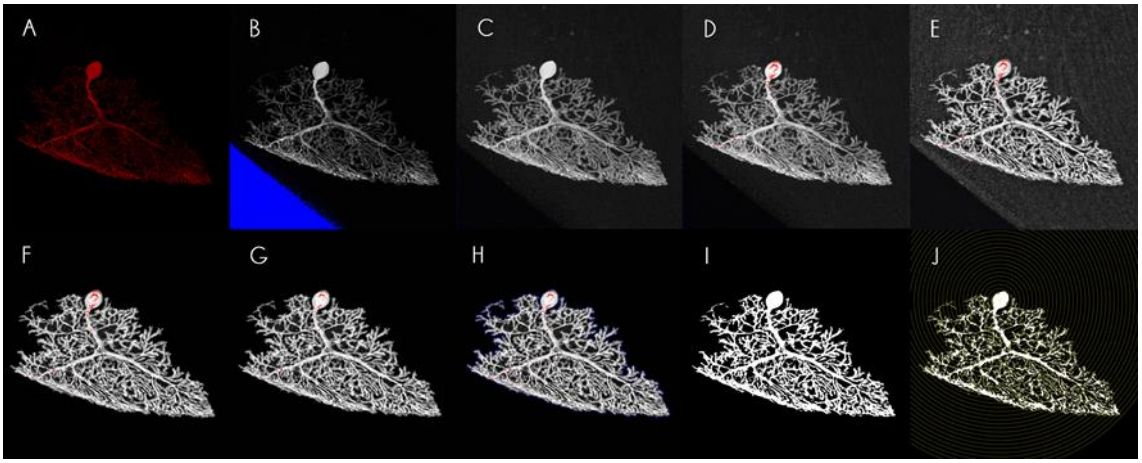


Figure 9. The transformation of the image of the Purkinje in each step of the Sholl analysis. (A) PC. (B) Hilo. (C) Gamma 0,5. (D) Brightness. (E) Local contrast. (F) Clear outside (G)Filter mean. (H)Sharpen. (I)Threshold. (J) Sholl.

Statistical analysis

Prism (GraphPad) and Matlab (MathWorks Inc.) were used for statistical analysis. The results display mean ± SEM. Statistical significance threshold was set at $p < 0.05$ (n.s, *$p<0.05$; **$p<0.01$;***$p<0.001$).

4. RESULTS

Lack of CNTNAP2 protein results in abnormalities in Purkinje cell spontaneous activity and morphology

Spontaneous in vivo PC activity is altered in Cntnap2$^{-/-}$ mice

Previous work shows an enriched expression of Cntnap2 gene in the cerebellum, being remarkable the expression founded in the PC layer (Gordon et al., 2016). As a first approach, we wanted to study if the lack of the CNTNAP2 protein would affect spontaneous PC activity. The area chosen to perform the experiments was Crus I/II because of its relationship with ASD (D'Mello et al., 2015; Skefos et al., 2014; Wang, Y., Xu, Zuo, Zhao, & Hao, 2020). Using a micropipette attached to a micromanipulator, we recorded unitary extracellular PC activity in awake mice (Figure A). PC are easily identified because of their two different spike waveforms: simple spikes (SS), which are generated by parallel fibers, and complex spikes (CS), which are generated by climbing fibers (Figure B). Both types of spikes have a synergic relation between them in terms of modulation; CS firing modulate the appearance of the SS and its activity (Tang et al., 2017). For that reason, our goal was the characterization of both spikes.

We found that the lack of Cntnap2 affects the number of CS fired by PC, which present lower CS firing frequency compared with WT controls (Figure 10C). Due to the low appearance rate shown by CS, we could not analyze more parameters, as was done for SS (see below). Regarding SS, we found that the SS firing rate remained unaltered, being the number of SS emitted by KO PC similar to WT, however the data dispersion seems to be higher in the KO suggesting a higher variability in the PC activity (Figure 10D). To further characterize SS, we explored the predominant firing rate, which represents the mode of the firing rate, in order to see whether PC tend to have a different predominant firing frequency depending on the genotype (Figure 10F). We did not find any difference between WT and KO mice. Our next step was to measure the heterogeneity or regularity in the SS firing pattern using the coefficients of variation 1 and 2 (Figure 10 G)(Holt, Softky, Koch, & Douglas, 1996). These coefficients are highly accepted as a measure of the intrinsic variability in firing pattern (CV1 and CV2). We found differences in CV1, which compares the interspike intervals in a spike train (Figure 1 H) but not in CV2 (Figure 10I), which compared the interspikes intervals only between adjacent intervals. This indicates that even if the overall spike train in KO PC presents higher variability, there is an intrinsic firing pattern between adjacent intervals.

Our results open a question about the role of the CNTNAP2 protein in the intrinsic properties of PC, revealing a possible role in the modulation of CS and the heterogeneity of the SS firing pattern.

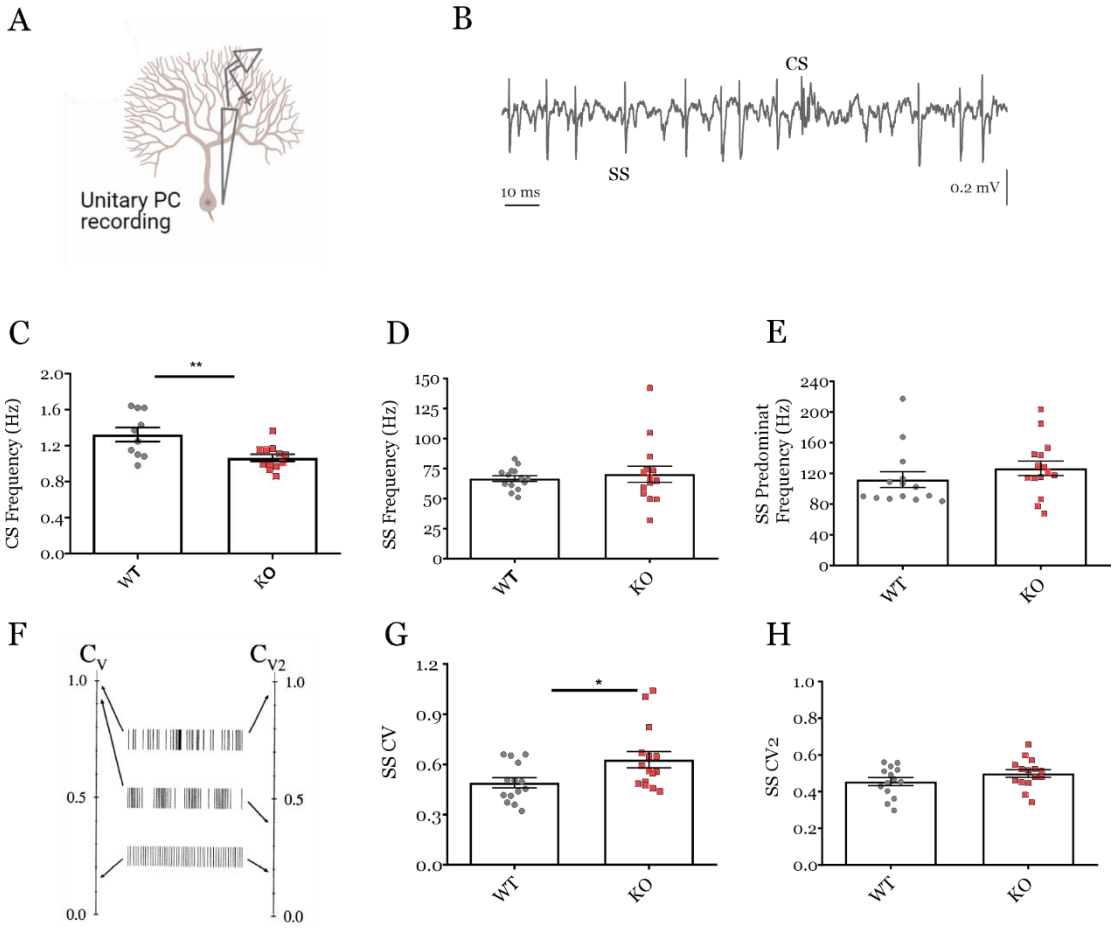


Figure 10. *In vivo* spontaneous activity of Purkinje cells. (A) Schematic illustration of an extracellular PC recording and a representative trace displaying simple spikes (SS) and complex spikes (CS). (B) CS firing rate representation, KO mice have a lower firing rate compared to WT mice. (C) The SS firing rate seems to be unaltered, but the data dispersion seems to be higher in KO mice (D) SS predominant firing rate. (E) Coefficient of variation (CV) of the inter-spike intervals for SS. (F) Coefficient of variation for adjacent inter-spike intervals (CV_2). Data are presented as mean ± S.E.M. n=14-15 neurons/genotype. Student's t-test (*p<0.05, **p<0.01).

tDCS modulates the firing rate and the coefficient of variation in Cntnp2KO PC

Transcranial direct current stimulation is a non-invasive neuromodulation technique consisting in the application of weak electric currents through the scalp (Nitsche & W. Paulus, 2000). This technique has demonstrated to have modulatory effects over neuronal populations (Nitsche & W. Paulus, 2000; Priori, A. Berardelli, S. Rona, N. Accornero, & M. Manfredi, 1998) and it is being used in a wide range of neurological and psychiatric conditions in humans (Grimaldi et al., 2016; Miterko et al., 2019; Reed & Cohen Kadosh,

2018; Stagg, Antal, & Nitsche, 2018). Previous experiments from the Marquez lab using WT mice have proven that after the application of this technique, the activity/firing of the neurons could be modified. Even though tDCS is not capable of inducing the neuronal firing, the current application is enough to modulate the firing rate, increasing or decreasing depending of the current used. We wondered if our KO PC were sensitive to this kind of modulation and if tDCS could be used as an approach to modify the altered parameters observed in the in-vivo spontaneous activity. For tDCS there are two kinds of current that can be applied, anodal, when the electrode located in the area that we want to stimuli is the anode (-) or cathodal, when the electrode located in the region of interest is the cathode (+) .We applied either anodal or cathodal 200µA currents following the protocol shown in figure A. The two main parameters that we used to study the SS are the FR and CV. We normalized the FR from data obtained 4s previous to the stimuli and this FR was considered a baseline of 100%. As expected, the application of tDCS had an effect on the FR, being this effect usually opposite between the opposite polarity currents (Figure B). Regarding the CV, we also observed a change depending on the current polarity applied (Figure C). It seems like the changes in the FR and CV have an inverted correlation with application of Anodal current (Figure D), nevertheless when the Cathodal current was applied we did not observe any changes (Figure E). These data indicate that tDCS could be a powerful tool to try to restore the deficits observed in the SS parameters of PC KO.

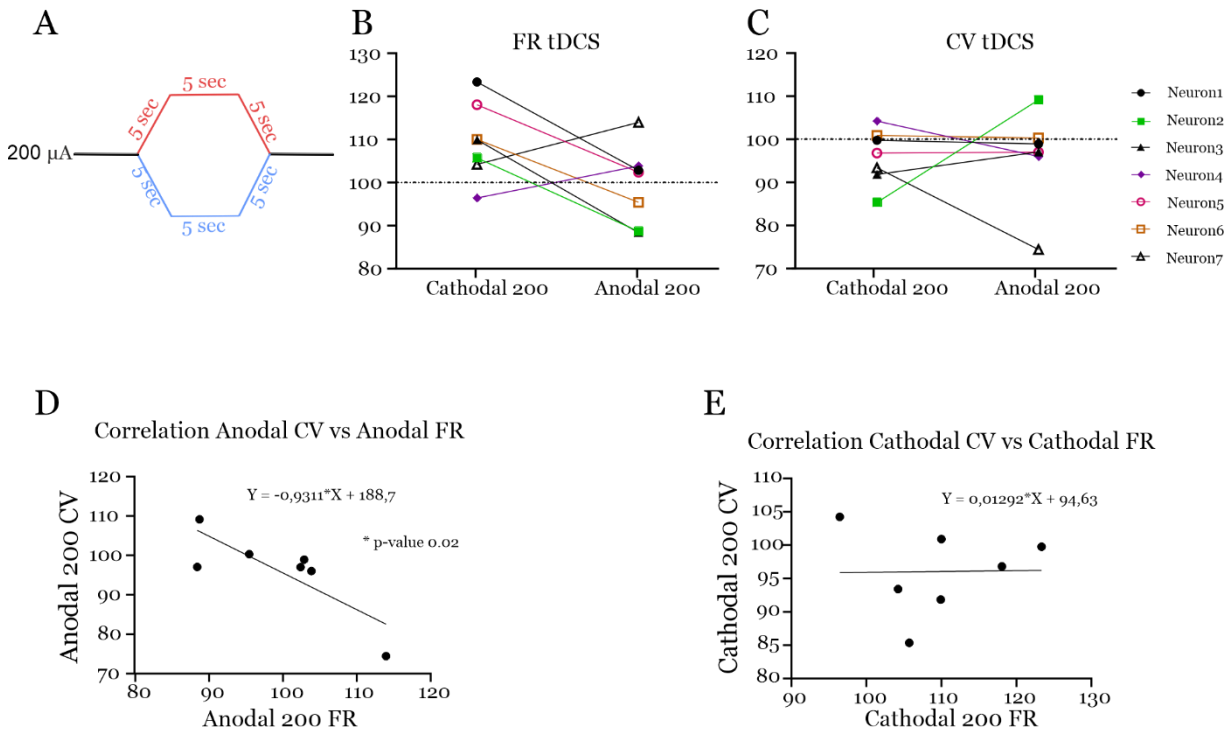


Figure 11. tDCS application in R-Crus I/II is able to modulate PC activity. (A) schematic representation of the protocol used for tDCS stimulation, red line represents anodal current and blue lines, cathodal. (B) firing rate is modulated in an increase or decrease

way depending the current used (C) effect of tDCS stimulation in Purkinje cell CV. (D) There is a correlation between the changes in the FR and CV during Anodal current application, (*p-value=0.02), (E) nevertheless, these changes are not present during the cathodal current. n= 7 neurons.

Mice lacking Cntnap2 present smaller PC, morphological atrophy and decreased arborization

Several postmortem studies using brains of patients with ASD, have revealed the presence of morphological abnormalities in PC, such as lower size, dendritic abnormal arborizations and PC atrophy, especially in the Crus I/II area (Skefos et al., 2014; Wegiel et al., 2014). Besides, several mouse models of autism recapitulate those morphological alterations in PC and cerebellum (Fernández et al., 2019). Also, Cntnap2 seems to have a role in the development, arborizations and spine stabilization of several neuronal types, including PC (Argent et al., 2020). To determine whether the observed deficits in the PC spontaneous activity could be related with alterations in their morphology, we characterized the morphology by biocytin staining and confocal microscopy. We measured, using ImageJ software, the total length of PCs from soma to the most apical point in the dendritic arbor (Figure B) and the soma diameter (Figure C). In both cases, our results showed differences between genotypes, being the PCs from KO mice smaller than WT. We also studied the complexity of the dendritic arbor using Sholl analysis, in this analysis we took the center of the neuronal soma as initial point and from this point, the software creates concentric circles separated by 5µm. The analysis considers the number of intersections between the circles and the dendritic arbor, as a measure of complexity (Figure D). We also measured the point where the maximum arbor complexity is reached. KO PC show a maximum value of dendritic arborization or complexity in a shorter distance from the soma compared to WT PC (Figure E). Also, we found that absence of *Cntnap2* decreases the total number of intersections (figure F). Even if our results show a clear difference in the arborization complexity, the size of the neurons in KOs are smaller. To differentiate whether the observed reduced complexity is simply due to a reduced neuronal size, we used the regression coefficient, which takes into account the size of the neuron and represent the rate of the decay of branching. We found (Figure G) that this reduced complexity is not due to the smaller size, but to a decrease in the complexity arborization, as the distribution of the total intervention along the distance from the soma (Figure H) reveals. Finally, we characterized if the lack of Cntnap2 has an effect in the spine density of PC (Figure I) and we did not find any difference between WT and KO mice (Figure J).

Our studies are in agreement with previous works indicating a role for the Cntnap2 protein in the development and arborization of the PC (Argent et al., 2020).

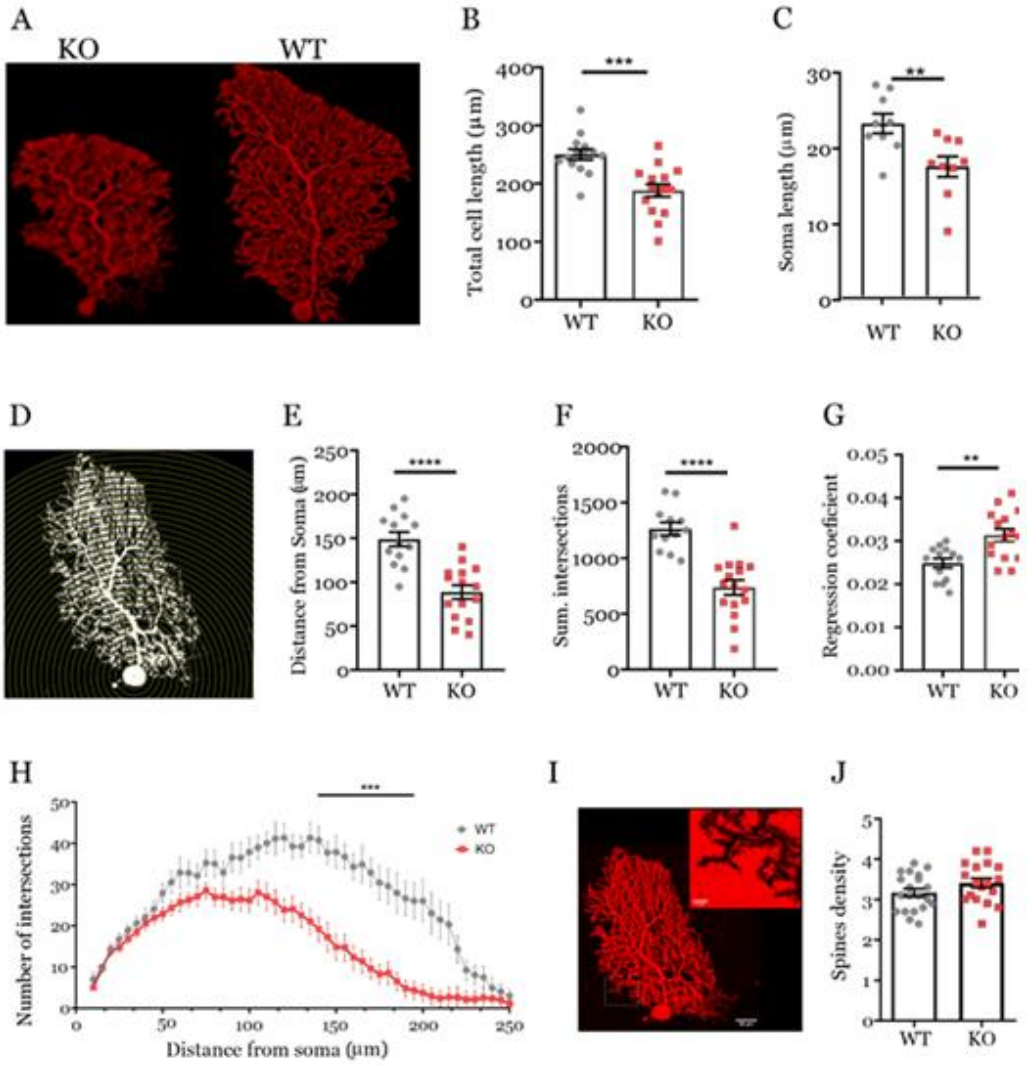


Figure 12. Neuroanatomical characterization of Purkinje cells. (A) representative fluorescence image of PC KO and WT injected with biocytin and labeled with Streptividine. (B) whole length of PC in WT and KO mice and (C) represent the measurement of the soma. (D) representative illustration of Sholl analysis and its radius. (E) indicate the distance from the soma to the dendritic arbor which present more complexity. (F) representation of the total number of intersections counted after Sholl analysis. (G) regression coefficient, representing the number of intersections in function of the size of the cells. (H) sholl analysis showing the number of intersections as a function of the distance from the soma. Two-Way ANOVA with repeated measures ***p-value<0.005. (I) Representative images from a PC its spines. (J) Spine density expressed as number of spines/microns. Data are presented as mean and ± S.E.M. ***p-value total cell length=0.002; **p-value soma length= 0.007; ****p-value maximum radius distrance from soma< 0.0001; ****p-value sum.intersec. =< 0.0001; **p-value regression coefficient<0.019; ***p<0.001. n= 13-16 per genotype

Cerebellar response to sensory-evoked stimuli is altered in Cntnap2-/- mice

Atypical sensory processing is one of the characteristics included within the diagnosis of autism (APA, 2013) including hearing, touching, or smelling alterations. Cntnap2 KO mice have been shown to present abnormalities in sensorial processing (Dawes et al., 2018; Scott et al., 2018) and an altered cerebellar function in a cerebellar related task (Argent et al., 2020; Kloth et al., 2015). Due to the observed alterations in PC activity and morphology, we wondered if circuits requiring cerebellar involvement could be affected, since the PC represent the sole cerebellar information output.

Sensory-evoked potential characterization in R-Crus I/II

To characterize the Sensory-evoked potential (SEP) activity in response to whisker stimuli, we used a well-known paradigm, and following the protocol described in Marquez-Ruiz and Cheron 2012, we applied a subcutaneous electrical stimulation to the ipsilateral whisker pad in alert mice (Figure A). The neuronal response after the stimulus is well characterized and is formed by two main components (Figure A left): the trigeminal (peaked at 3.79±0.69ms from whisker stimuli) (Figure) (T) and the cortical (peaked at 12.57±1.12 ms) (C) components (Mapelli & D'Angelo, 2007; Márquez-Ruiz & Cheron, 2012; Roggeri et al., 2008) . Although the trigeminal component is composed by 3 subcomponents (N1, N2, N3), in the present study we studied the trigeminal component as a whole, since the variability of the subcomponents made distinguishing them hard. The cortical component amplitude decreases when the animal is moving, therefore only the events recorded when the animal was immobile were taken into account for the analysis. First, to study the SEP, we performed an intensity profile, in order to know how the different components of the SEP changes behaved depending on the intensity of whisker stimuli delivered. The final amplitude of the different components linearly depended on the intensity of the electrical stimuli applied to the whiskers (Figure B, C, D), therefore, the current intensity of whisker electrical pulses was adjusted as to elicit a N2-N3 component with half the maximum amplitude. This permitted to abolish saturation in the signal, possible damage in the animal and to stablish a comparative point between the animals (Figure E).

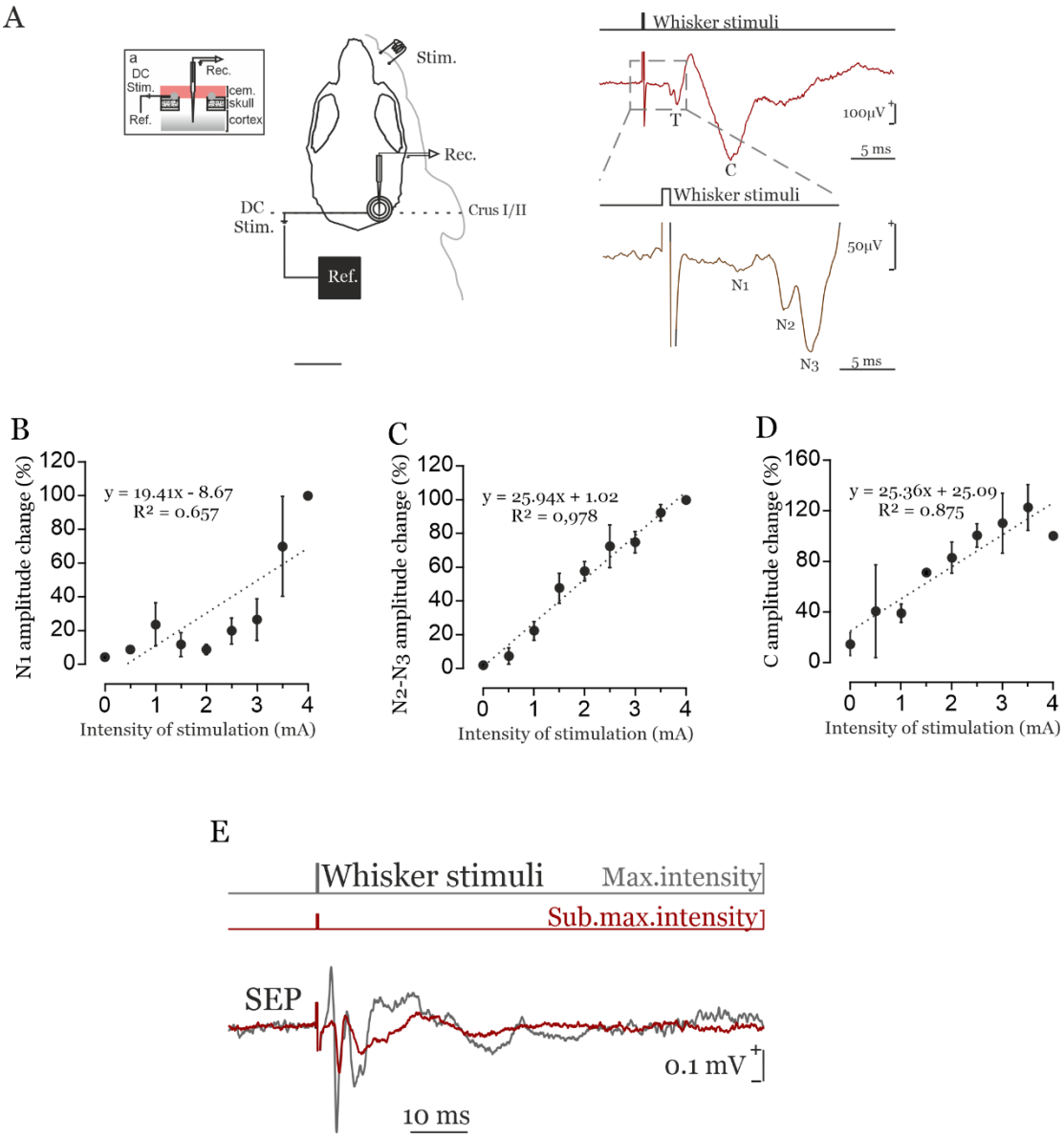
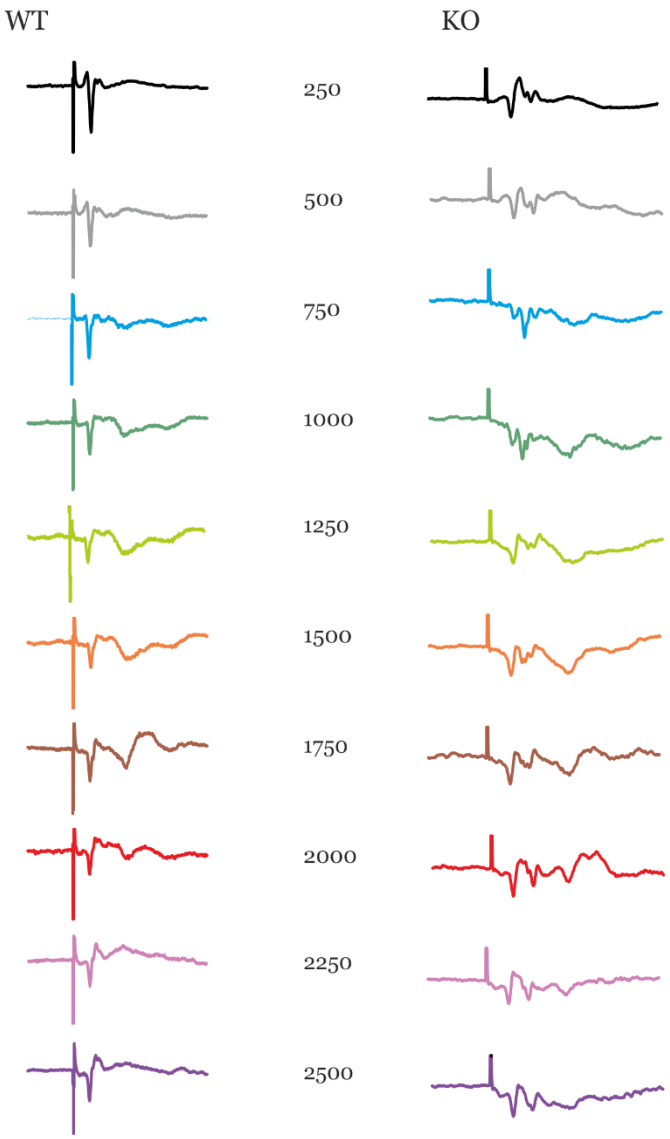


Figure 13. Characterization intensity profile of SEPs in CrusI-II. (A) left experimental design for Set 2 experiments with concurrent Cb-tDCS and recordings on Crus I/II, right, profile of sensory evoked potential by whisker electrical stimulation showing the different components (trigeminal component (T); N1, N2 and N3; cortical component. (B-D) quantification of the amplitude change in N1 (B), N2-N3 (C) and C (D) components regarding intensity applied to whisker electrical stimulation. Data normalized with respect maximum amplitude recorded at 4 mA. (E) comparison of CrusI-II-SEPs for different intensities applied to whisker stimulation obtaining maximum N2-N3 amplitude (black trace, n= 10), and 50% of the maximum N2-N3 amplitude (green trace, n= 10).

Once we had a primary approximation of SEP, we performed a depth profile in WT and KO animals, in order to see how the different components changed with depth and to delimitate a range of depth to use in future experiments. Lowering the pipette from 0 µm to 2500 µm in both WT (Figure B left) and KO (Figure B right) and delivering an intensity to evoke the submaximal amplitude we obtained profiles that differed between genotypes, as it is shown in figure X . In order to have a standardized measure to all the mice, we recorded the SEPs

layer at 1500 µ approximately, avoiding the PC layer. Since for the characterization only a few animals were used, it remains to be seen if these differences could be replicated using a bigger n and whether the differences between the genotypes are significate.

A



B

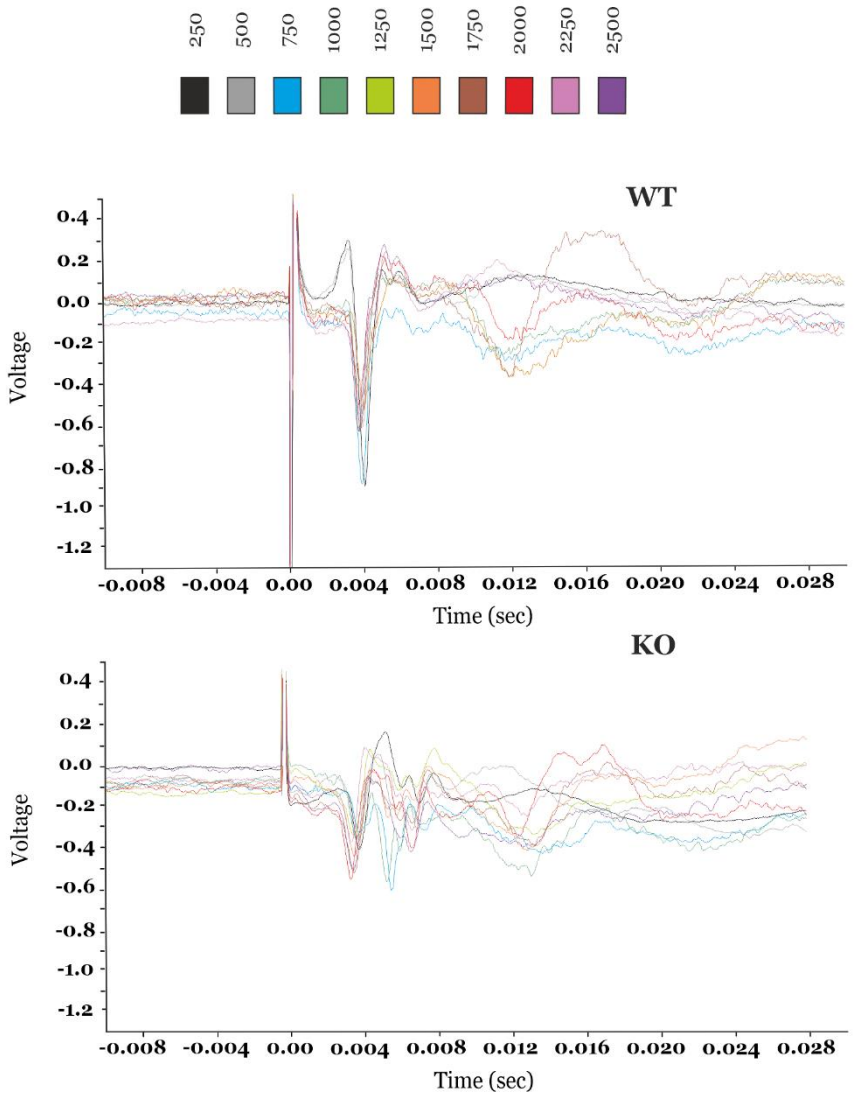


Figura 14. Characterization depth profile of SEP. (A) to charazterized the depth profile we lowered the micropippete 250µm every 2s using 1.5mA whisker stimulus. Left row represent que SEPs in a WT animal and right row represent KO mouse. (B) Merge of SEPs obtained at different depth in WT mouse (up) and KO mouse (down).

Sensory evoked potential is altered in Cntnap2$^{-/-}$ mice

Due to the alterations that we observed during the profile characterization we performed a deeper analysis using event-related potential (ERP) analysis. Using the same approach (figure A) we stimulated the ipsilateral whisker pad in alert mice and recorded the SEP formed in Crus I/II. Before start the experiment in each mouse, we stablished the depth and the intensity of the stimuli until the submaximal response were found below the PC layer.

Results from ERP analysis are shown in Figure XX. The ERP represents the average response of all mice used for the experiments, and vertical bars represent the latencies where we found

statistical differences between the genotypes. Although the trigeminal components seem unaltered, we found at 6.46 ms an aberrant component in the KO SEP which does not appear in the WT. In the same way, later, at 12.87 ms where the cortical component appears in WT mice, we couldn't find a similar one in the KO SEP.

Our results indicate that cerebellar sensory processing is altered in Cntnap2 KO mice, denoted by the appearance of aberrant components with unknown origin and the absence of the cortical components, which is driven from the somatosensory cortex.

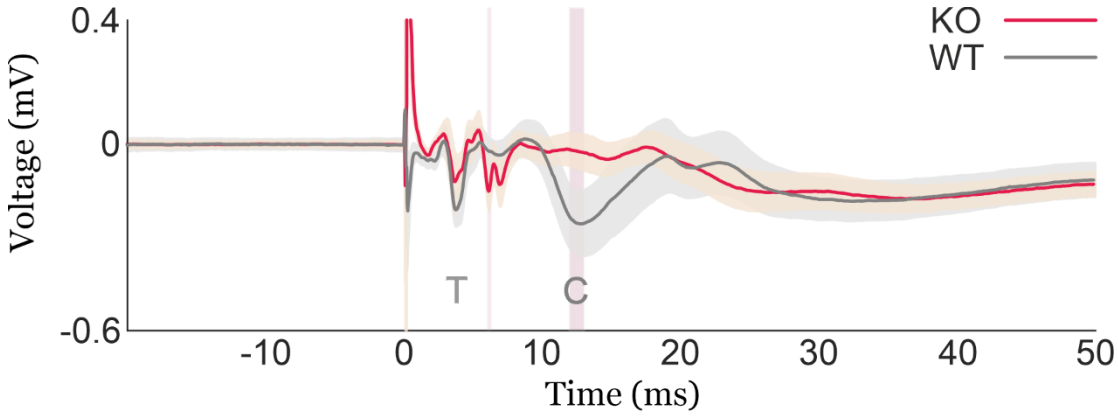


Figure 15. Event-related potential (ERP) analysis comparing the average SEP traces for 11 KO (red trace) and 9 WT mice (black trace). Trigeminal component is indicating as T and cortical component as C. Vertical bars indicate statistically different latencies, corresponding to the cortical peak (C) in WT at 12.87±0.40 ms, which is absent in KOs, who show a novel negative peak, presumably an anticipated cortical response, at 6.46±0.14 ms. Student's t-test ($p<0.05$)

R-Crus I/II Purkinje cell activity is altered in response to whisker stimuli

Previous studies using a similar stimulation approach have demonstrated the role of certain PC activity in the SEP pattern in WT mice, specifically the appearance of the trigeminal component correlates with SS burst and the cortical component is coincident with CS firing (Márquez-Ruiz & Cheron, 2012; Tsutsumi et al., 2020). To characterize if the alterations present in the SEP components of KO mice could be related to an abnormal PC response after whisker-stimuli, we recorded unitary extracellular PC activity in alert mice while we applied an electrical shock in the ipsilateral whisker pad. Figure A and B represent the histogram of accumulative spikes from all the PC neurons recorded after the stimuli application in WT and KO mice, respectively. The accumulative SS firing pattern seems to be unaltered along the time after the whisker stimuli, representing a similar pattern of emission at 3 ms latency after whisker stimulation, which in our ERP analysis remain unaffected. We also studied the complex spikes, represented in Figure C (WT) and D (KO). In this case, we found differences in the firing pattern. WT PC peaks at 12ms, coincident with the cortical component. On the other hand, KO PC peaks at 6 ms, coincident with the aberrant component observed in the

ERP analysis that is not present in WT mice. We did not observe a peak around 12ms, which is coincident with the cortical component; on the contrary, we found a dispersion in the CS firing along the time after whisker stimuli. That observation led us to think that maybe the stimulus induced a disorganized CS firing, so we counted the number of CS appearing after the whisker stimuli (Figure G) and we found that KO PC tend to fire more CS after whisker stimuli than WT. In short, we found a correlation between the activity of PC and the aberrant component observed in the SEP, specifically it seems that it could be caused by an altered CS pattern. Further, not only the CS firing pattern but also CS firing frequency upon whisker stimulation seems to be altered.

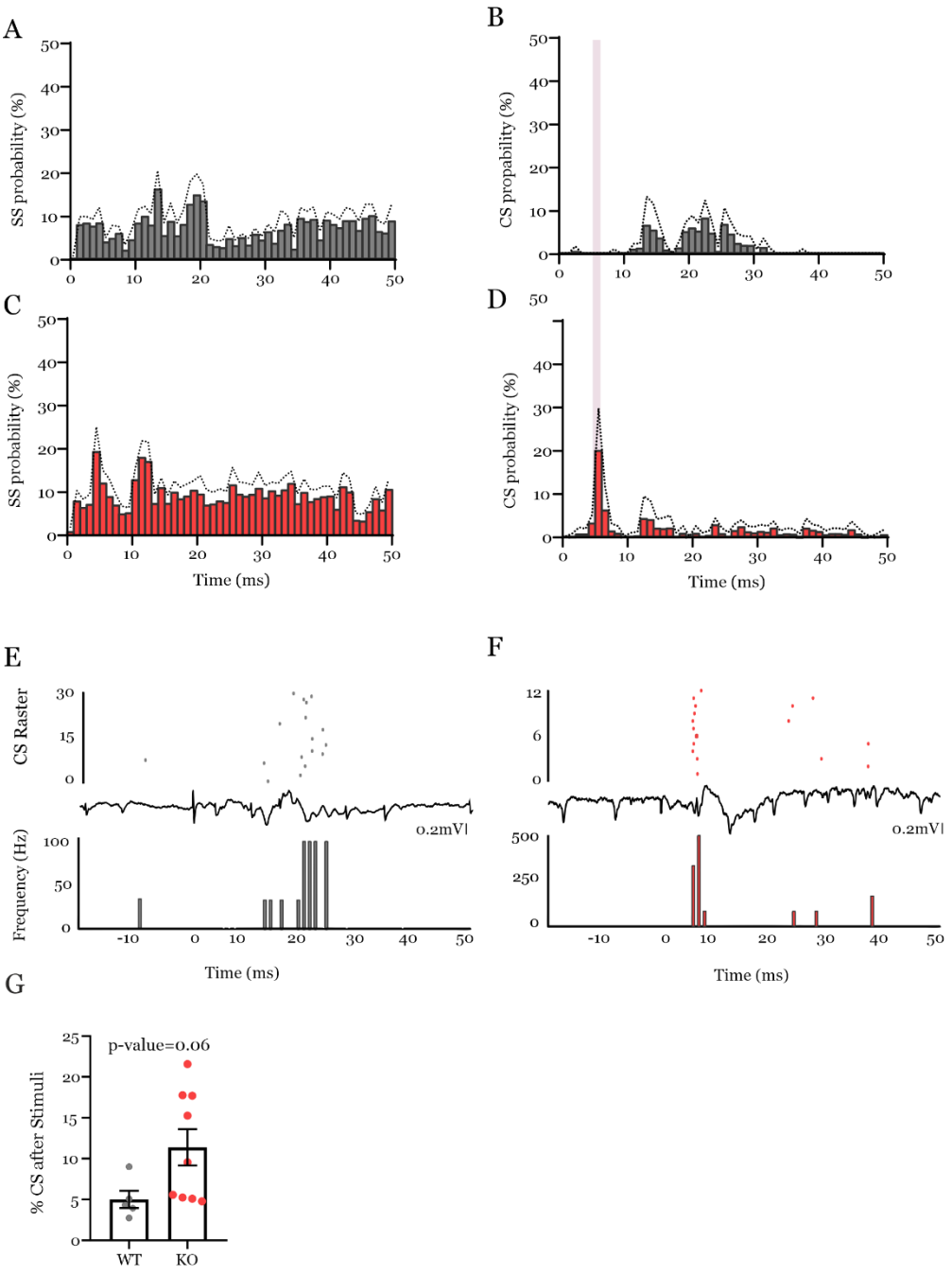


Figure 16. Graphical representation of histogram of accumulative firing of PC activity after whisker stimuli. (A) Temporal firing pattern for WT SS and (B) for KO. (C) histograms represent the CS firing patter in WT and (D) in KO PC. Note that CS appear at post-stimulation latencies concordant with the C component in WT, while they appear earlier in KO mice, likely indicating an anticipated C response in the animals. The graphs represent the mean ± S.E.M (discontinuous line). n=7-10 PC per genotype. E and F represent an example of a CS activity in WT and KO respectively, with itself SEP, the accumulative firing pattern are coincident with the components of the SEP. Figure G represent the percentage of CS appearing after the arriving of whisker stimuli. Student's t-test (p-value=0.06) Mean and SEM are show for the measurement

Behavioral characterization of the conditional deletion of Cntnap2 in PC

Our results have revealed an alteration in PC and cerebellar sensory information processing in a mouse model of autism. Nevertheless, the role of the cerebellar expression of the Cntnap2 protein regarding the core behavioral symptoms of ASD is still unknown. To characterize the role of CNTNAP2 specifically in PC with regards to the global behavioral phenotype of ASD we used LoxP/Cre technology, to generate a mouse lacking Cntnap2 specifically in PC. For that purpose, we used Cntnap2 floxed mice and the promoter Pcp2 driving Cre expression, which is specific of Purkinje cells. Once we generated the experimental (Figure) controls and cKO mice, we performed a complete battery of behavioral tests as it relates to autism as it was performed for the Cntnap2 full KO in Peñagarikano et al.,2011 (Peñagarikano et al., 2011).

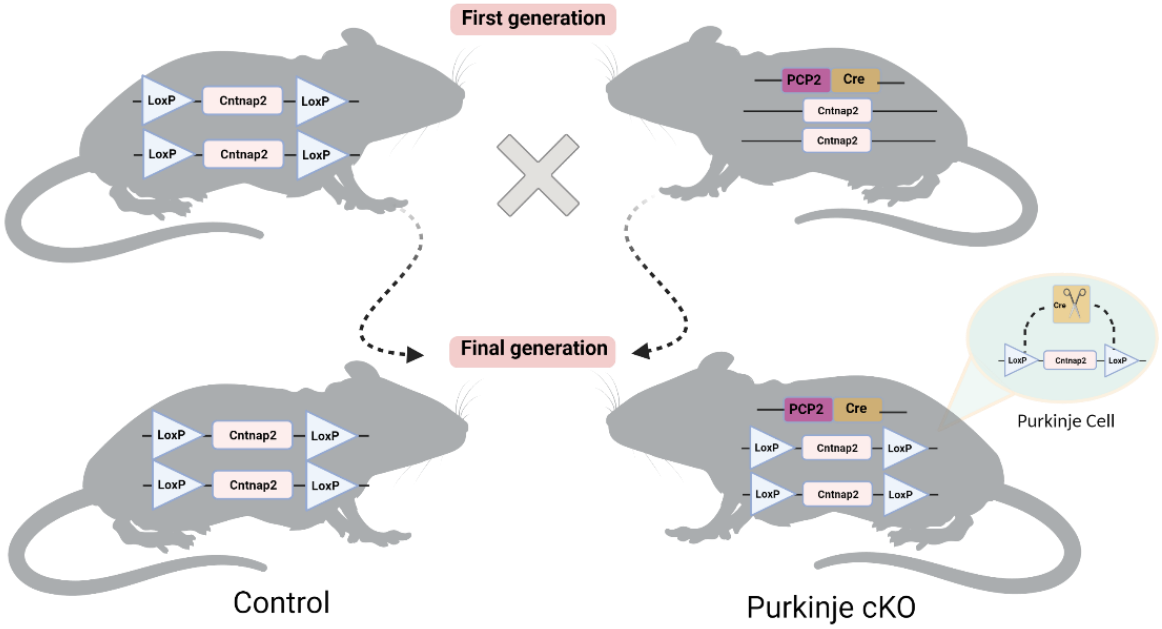


Figure 17. Schematic representation of the parental used to obtain the mice Purkinje cKO and their littermates control.

	Cntnap2 Purkinje cKO	Cntnap2 KO-/-	Test
Alterations in communicative behaviors	✓	✓	Ultrasonic Vocalizations at P7
Hyperactivity	✓	✗	Open field
Repetitive behaviors	✓	✗	Grooming
Social behaviors deficits	✓	✗	Three-chamber
Behavioral inflexibility	✓	✓	T-maze
Anxiety behaviors	✗	✗	Dark-light Box
Sensorial alterations	✓	✗	Hot plate

Table 5. Comparative table showing the results previously obtained in the Cntnap2 full KO (Peñagarikano et al., 2011)with the recent results obtained after the behavioral characterization of Conditional Purkinje Cntnap2 KO.

Cntnap2 PC cKO mice present an aberrant number of ultrasonic vocalizations

Mice are social animals which use ultrasonic vocalizations (UsV) to communicate between them. Those vocalizations are emitted in a certain frequency range (≈30-110kHz), above the human hearing range (Branchi, Santucci, & Alleva, 2006). These UsV are composed by a sequence of syllables, which can be formed by different elements or notes that are separated between them by a period of silence (Arriaga & Jarvis, 2013; Holy & Guo, 2005). The frequency and duration of UsV can change depending on the context (Arriaga & Jarvis, 2013), and such parameters are believed to be important for the transmission of information. For example, pups emit UsV when they are separated from the mother for a short period of time (Scattoni, Crawley, & Ricceri, 2009), and the response of the mother can change depending on the type of UsV emitted by pups, in terms of frequency and duration. In this set of experiments, we individually separated P7 pups from their mother and gently placed them over a specialized box for 5 mins to record the UsV emitted. Figure A shows an example of the different syllables emitted and their frequency. We found that Cntnap2 cKO pups emitted more UsV than WT. Previous studies from our lab demonstrated that not only the number

but also the type of UsV emitted are different between the full knock-out and its copartner WT at this age, so we wondered if those parameters were also altered in the cerebellar conditional model. We analyzed the duration of the calls (B) and the frequencies at the start (D), end (E) and the maximum frequency raised (F), and we did not find differences between genotypes in any of these parameters. Even though our results point out a possible alteration in the communicative system, our results, showing an increase in the number of USVs emitted, are opposite to what was previously described in the full-knock out, which present a reduction in the number of UsV emitted in a similar test at the same age.

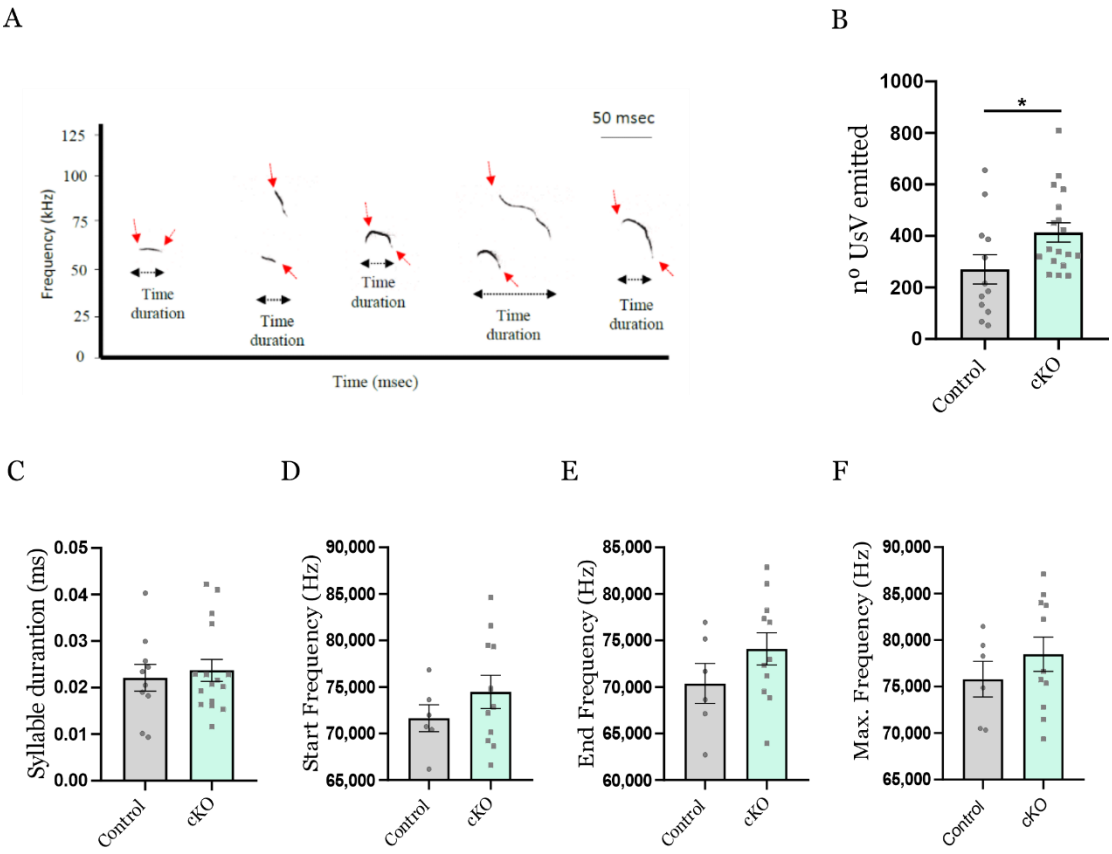


Figure 18. cKO mouse presents alterations in communicative system. (A) squematic representation of UsV. Red arrows represent the star and end peaks of each syllables. The UsV are compose by different syllables, which are represent in a relation between frequency and time. (B) number of UsV emitted by pups at P7 during 5 mins after being separated from their mother, cKO presents higher number of UsV compared to Control. (C) analyses of duration of the syllables which compose the UsV. (D-E) analysis of syllables frequency at the start (D), end (E) and the maximum frequency reach by the syllables. No differences were found between the genotypes in these parameters. The graphs represent the mean ± S.E.M. Student's t test (* p-value= 0.03). n= 11-16 animals per genotype.

Cntnap2 cKO mice present normal locomotor activity

Next, we measured the locomotor activity of Cntnap2 cKO mice, as hyperactivity was described in the full mutant (Peñagarikano et al., 2011), patients harboring CNTNAP2 mutations (Strauss et al., 2006) and is a comorbid condition frequently seen in autistic patients (APA, 2013). For that purpose, we assessed their locomotor activity in an open field test, consistent of an open arena in which the animal can move freely. Our results show no differences between genotypes, neither in the distance traveled (Figure B) nor in the mean speed (Figure C). In addition, we determined that both genotypes explore the arena in the same way and do no spent more time in a specific area. Our results demonstrated that conditionally deleting Cntnap2 in PC is not enough to recreate the hyperactivity phenotype present in the full KO.

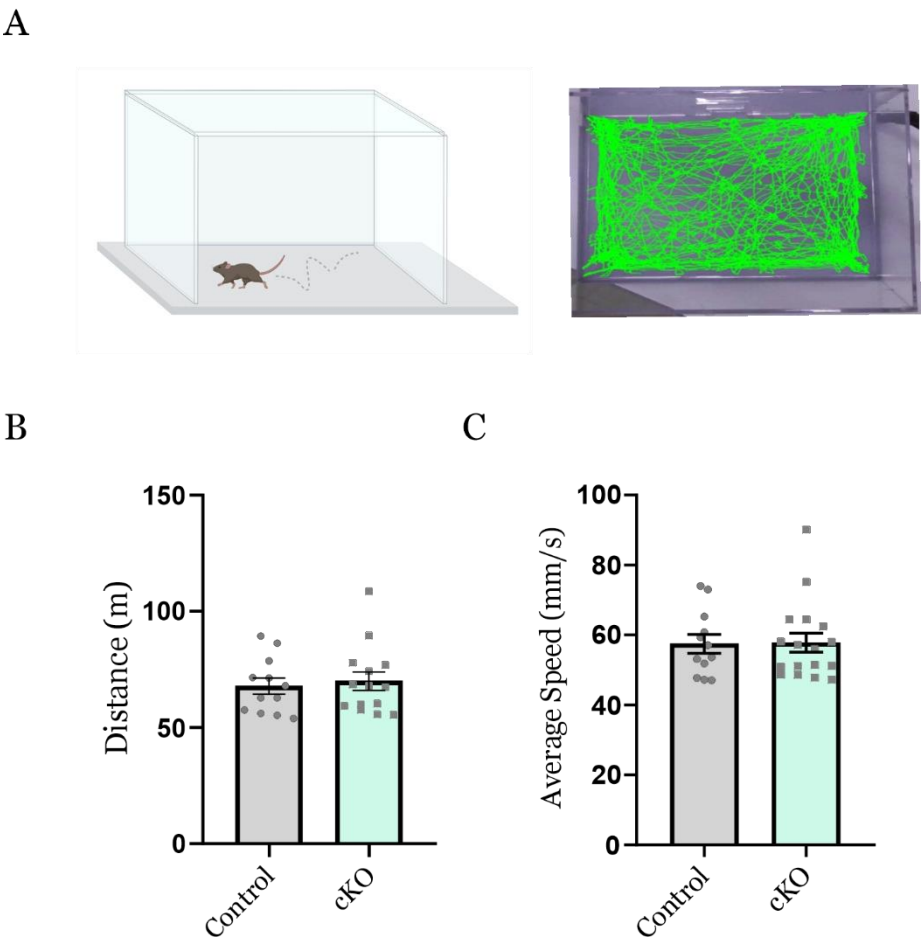


Figure 19. Locomotor activity comparison between Controls and cKO mice. (A) left figure, illustration of the open field apparatus used to measure the locomotor activity, right figure, representation of the software tracking following the animal. (B) traveled

distance by the mice during 20 minutes. (C) average speed of the traveled distance. No differences were found neither in the distance nor in the average speed. The graphs represent the mean ± S.E.M. Student's t test were used to determine the differences between the genotypes. n= 12-17 per genotype.

Cntnap2 Purkinje cKO mouse presents normal social interaction in the 3 chamber test

Mice, as a social species, display social preference behavior, that is, a preference to interact with a conspecific over an inanimate object if given the chance, as measured in the three-chamber test, being this a powerful tool to study social behaviors in animal models. Mice lacking the Cntnap2 gene display an impairment in social behavior in the three-chamber test (Peñagarikano et al., 2011). In this test, the animal is free to choose to explore either an inanimate object or an unfamiliar conspecific mouse matched in age and sex. The time spent interacting with either of them is compared as a measure of social preference. In this way, WT mice tend to spend significantly more time inspecting the unfamiliar mouse than the object. Our results show that both genotypes, controls and cKO, spend significantly more time exploring the conspecific, and therefore show no social deficits (figure). Besides the exploration index, measured as the time exploring the social cup minus the time exploring the object divided by the total exploration time $\frac{(T.exploring\ social - T.exploring\ object)}{T.exploring\ social + T.exploring\ object)}$ in which we take into account the exploratory capacity of the mouse, does not show differences between genotypes. Although lacking Cntnap2 evoked social deficits in mice, it seems like the mutation restricted to the PC is not enough to reproduce these behaviors, indicating that other circuits are implicated.

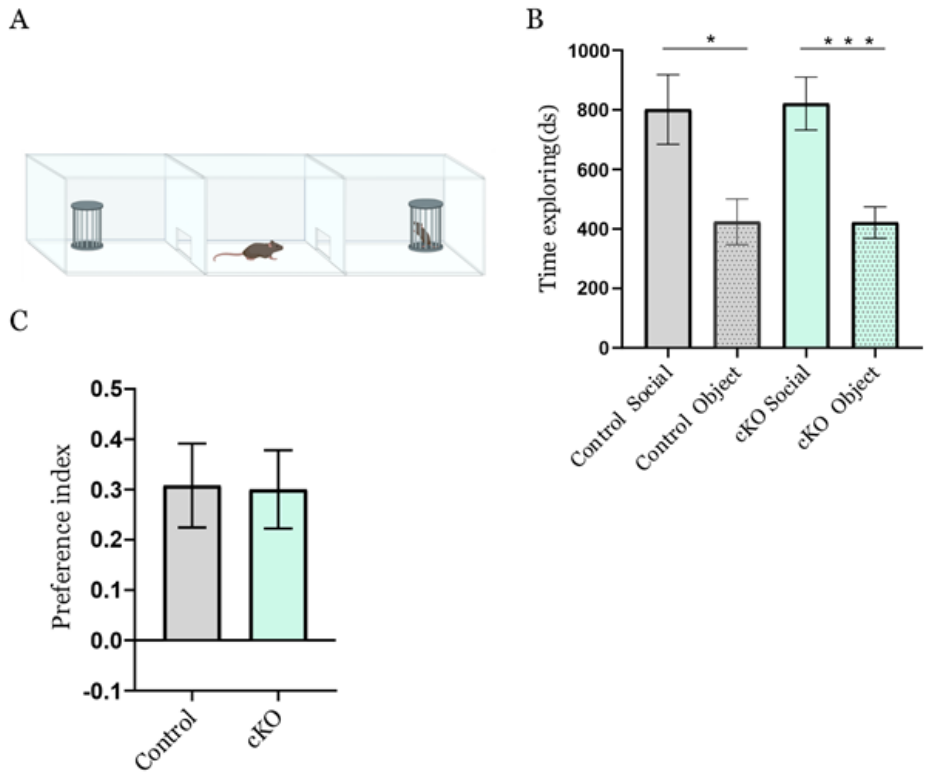


Figure 20. cKO mice does not present social deficits after performing three-chamber test. (A) representative illustration of three-chamber test. Experimental mouse has to choose between the object. (B) time spent by the mouse exploring the object. Both genotypes tend to expend more time with the social object. (C) preference index does not show any difference between Controls and cKOs. The graphs represent the mean ± S.E.M. Student's t test were used to determine the differences. (*p-value= 0.022; ***p-value= 0.001) n= 12-17 per genotype.

Cntnap2 Purkinje cKO mouse do not present anxiety-like behaviors

Our next step was trying to identify possible anxiety behaviors in the conditional Cntnap2 Purkinje KO. The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light. The test apparatus consists of a small dark safe compartment (one third) and a large illuminated aversive compartment (two thirds)(Crawley, Jacqueline & Goodwin, 1980). The time spent in the bright area and the latency to transit from the dark box to the bright area are indicative of anxiety-like behaviors. Our results show that the Cntnap2 cKO tend to spend the same time as the control in the bright area. In the same way, not significantly differences were found in the latency of transition between genotypes. Similar to the full KO, lacking Cntnap2 in PC does not induce anxiety-like behaviors in the mice.

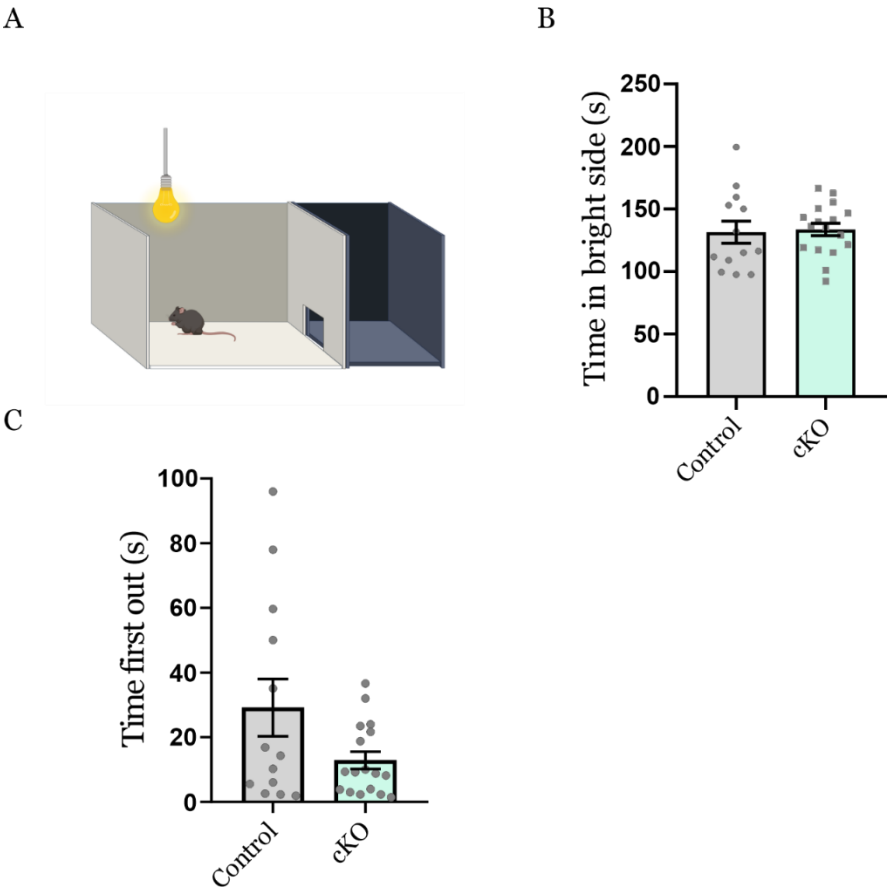


Figure 21. cKO mouse does not present anxiety-like behaviors in the light-dark box. (A) representative ilustration of the box, with the two compartiment, light and dark compartiement. (B) time spent by the mice in the bright side. This is a marker of anxiety levels. (C) latency in perform the first out from the dark compartiment to the light one. No differences were found in neither of the measuremnts. The graphs represent the mean ± S.E.M. Student's t test were used to determine the differences. n= 12-17 per genotype.

Lacking Cntnap2 in PC is enough to replicate inflexibility behaviors but not repetitive behaviors

Previous studies have been stablished a relation between the lack of Cntnap2 and the presence of repetitive behaviors and behavioral inflexibility (Peñagarikano et al., 2011). We wondered whether the lacking Cntnap2 in PC is enough to create these deficits. First, in order to assed the behavioral inflexibility we use the T-maze test. The test is consisted in a maze with three arms. The arm located in the middle is the largest and is the starting point for the experiment. The mouse is placed in the start point and is free to choose which arm (left or right) wants to explore, this process is repeated for 10 times. Due to exploratory nature of mice, they tend to alternate between the arms in every trial but, when inflexibility behaviors are present, they tend to go to the same arm repeatedly. Our results, after 10 trials, show how the Purkinje cKO have tendency to choose the same arm, representing inflexibility behaviors. We also measured the self-grooming as a measure of repetitive behavior. The self-grooming

is a complex stereotyped behavior involving rapid movement of forepaws over facial area and along the body (Moretti, Bouwknecht, Teague, Paylor, & Zoghbi, 2005). To study the role of the Cntnap2 protein in this behavior, we measured the time that the mice spent doing self-grooming for 10 minutes. No differences between controls and cKOs were found in our experiments (Figure), suggesting that the role of the protein in the cerebellum is not enough to replicate the deficits observed in the full KO regarding the self-grooming.

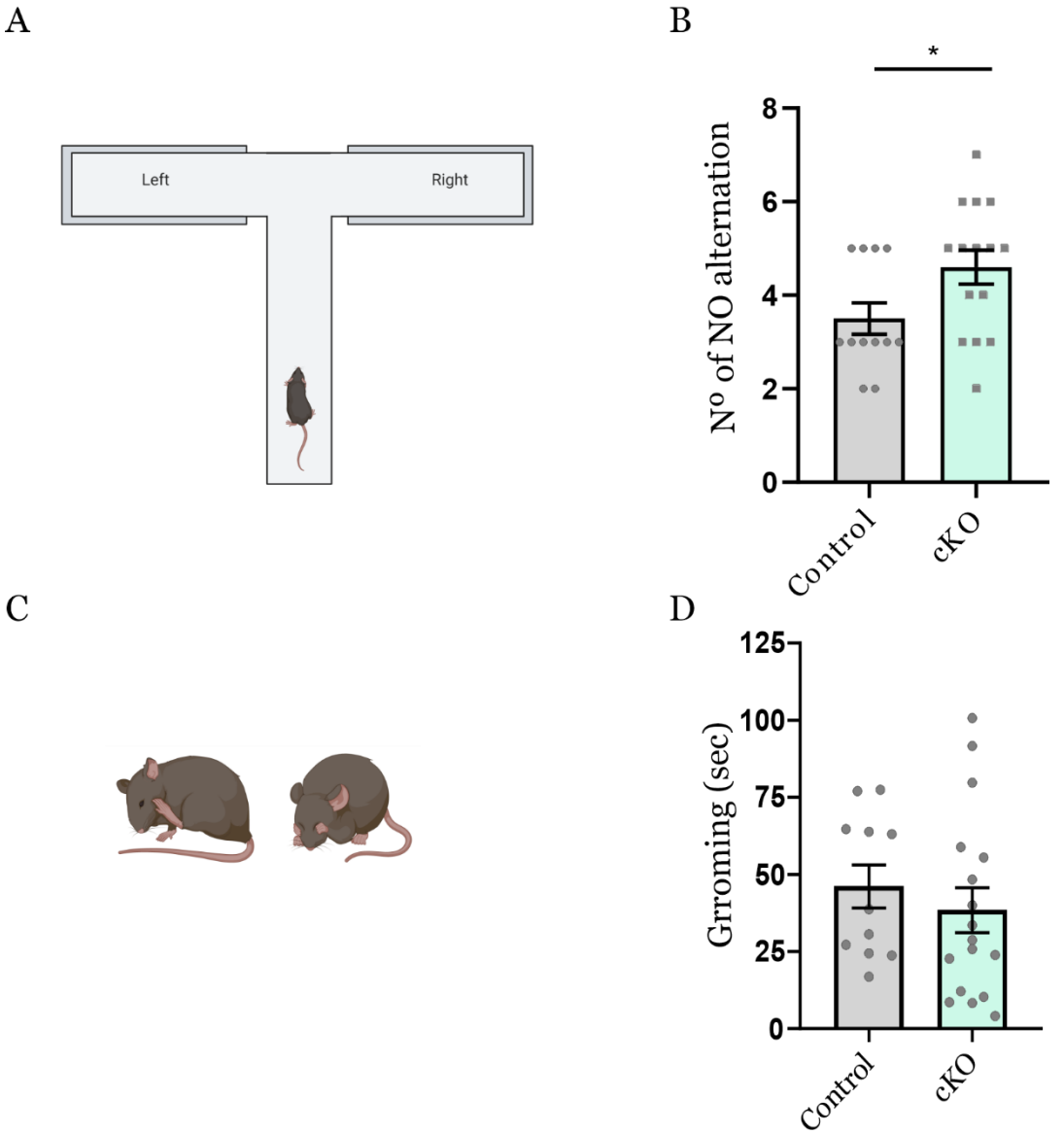


Figure 22. cKO mice present alterations in T-maze test but not repetitive behaviors. (A) representative illustration of T-maze test, the mouse has to choose between the two arms. (B) number of no alternations performed by the mouse in ten trials. cKO animals tends to turn to the same arm more times than Controls. (C) illustration of grooming movement. (D) time expend by the mouse performing grooming movements during 10 mins. The graphs represent the mean ± S.E.M. Student's t test were used to determine the differences (*p-value=0.03).) n= 12-17 per genotype.

Purkinje cKO mouse does not present alterations in Hot plate

Since 2013 the American Psychiatric Association (APA) added sensory sensitivities to the symptoms that help diagnose autism (APA, 2013). Autism's sensory issues can involve both, hyper-sensitivities (over-responsiveness) and hypo-sensitivities (under-responsiveness). Previous studies using the full-knock out had shown alterations in the hot plate test, indicating that the mice lacking Cntnap2, present sensorial alterations (Peñagarikano et al., 2011). The hot plate test consists in introducing the animal into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated up to 52.5º. We placed the mice inside the cylinder, which remains there up to a maximum of 15 seconds. We measured the latency of the first signal of pain in the mouse (usually shown as jumping, paw licking or kicking) and we did not find any difference in the latency of response between Control and cKOs mice.

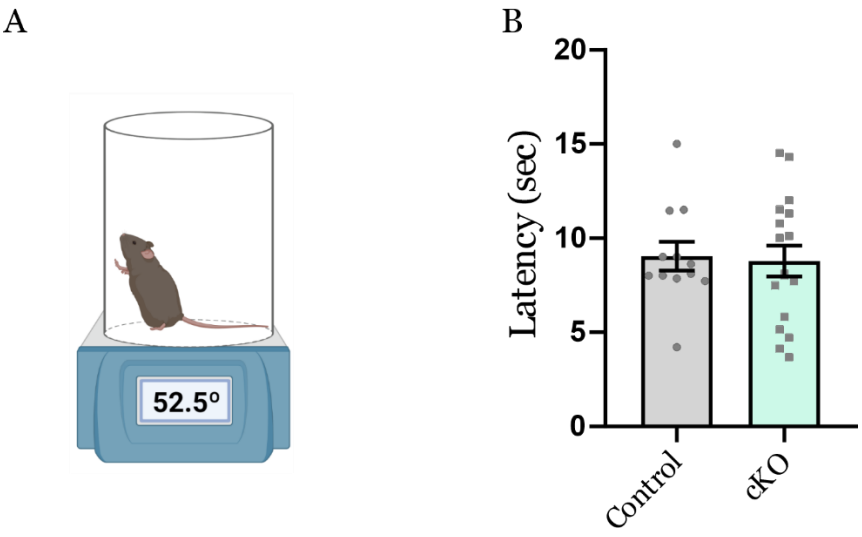


Figure 23. cKO mice present a normal sensorial reactivity in hot plate test. (A) representative illustration of the hot plate apparatus. (B) latency to perform the first pain signal up to a maximum of 15 seconds. No differences were found between the genotypes. The graphs represent the mean ± S.E.M. Student's t test were used to determine the differences. n= 12-17 per genotype

5. DISCUSSION

Classically, the cerebellum has been set aside in the biology of emotions and superior cognitive tasks. Nevertheless, in the recent years a huge number of studies stablished the cerebellum as an important structure in the modulation of emotional and cognitive functions and, consequently, has been implicated in a diverse group of neuropsychiatric disorders, including ASD and schizophrenia (Andreasen & Pierson, 2008; Fernández et al., 2019; Marsden, 2018). In fact, the cerebellum has been reported as one of the most consistent sites of abnormalities in patients with ASD (Fatemi et al., 2012). Also, different mouse models of autism replicate the observations made in humans, presenting alterations in cerebellar morphology and functionality (Fernández et al., 2019). Despite its relatively homogeneous structure, in terms of cellular composition, the cerebellum shows functional compartmentalization. Specifically, the Crus I-II area of lobule VII, has been shown to be especially relevant in the regulation of cognitive and social behavior due to its connection with cortical areas related with emotional processing and language (D'Mello et al., 2015; Fernández et al., 2019).

There is growing evidence of the involvement of the cerebellum in the integration of sensory information. Alterations in sensorial processing have been described as part of the symptomatology of ASD (APA 2013). Different mouse models of autism, including the Cntnap2 knock-out mouse, present alteration in cerebellar processing of sensory information (Kloth et al., 2015). However, the functional alterations of Purkinje cells in Cntnap2 mice, the only output from the cerebellar circuit, has not been studied. The results presented in this Doctoral Thesis reveal the presence of anatomical and functional abnormalities in the cerebellum of KO mice.

CNTNAP2 modulates spontaneous Purkinje cell activity

Our results reveal the implication of Cntnap2 in the spontaneous activity of PC in cerebellar R Crus I/II, an area widely linked to ASD. The action potential output of Purkinje cells has been classified into two distinct types: simple spikes, which occur spontaneously (Häusser & Clark, 1997; Raman & Bean, 1997) and can exceed 100 Hz in response to excitation from parallel fibres and complex spikes, which are triggered by climbing fibre excitation and consist of a characteristic high-frequency burst (Eccles et al., 1967; Thach, 1967).

The lack of Cntnap2 protein produces alterations in the activity of the PC in several parameters of both kinds of spikes. These findings are also replicate in other genetic mouse models with autistic phenotypes and mouse with perinatal injuries, such hypoxia, linked to ASD hypoxia (Wang, S. -., Kloth, & Badura, 2014) (see table below).

We found that the SS firing rate seems to be unaltered while the CS firing rate was decreased in KOs. Also, the data dispersion was found to be higher in KO compared to WT, which could suggest a potential alteration in the rhythmicity of the firing, for that reason, we analyzed the parameters CV and CV2 to find patterns of emission. The coefficient of variation (CV) tells us about the heterogeneity of the firing pattern which compares the interspike intervals in a spike train, with the aim to find synchronic between the neurons firing. When we are recording in vivo neurons, as in our case, is also useful to use the CV2, a CV modification which compared the interspikes intervals only between adjacent intervals with the aim to identify patrons of emission (De Schutter & Steuber, 2009; Holt et al., 1996). Our results, based on CV but not CV2, demonstrated that the emission pattern is more heterogeneous in KO compared to WT. This indicates that even if the overall spike train in KO PC presents higher variability, there is an intrinsic firing pattern between adjacent intervals which CV2 is able to identify. Similar results were found in a mouse model of cerebellar damage (Hx) where they found alterations in CV but not in CV2 (Sathyanesan, Kundu, Abbah, & Gallo, 2018).

The same PC parameters (SS, CS, Cv and Cv2) have been used to explore and study different models with mutations or cerebellar damages linked to ASD. Table XX shows a recapitulation of ASD animal models and the results of PC spontaneous activity characterization. As it can be seen in the table below, alterations in firing frequency in SS are present in the majority of the models studied, which present a lower firing rate compared to WT. Although our model does not present differences in this parameter, this finding is similar than the model Pc-MECP2 present. Both models show a higher data dispersion in the SS firing rate. Probably, with a higher number of neurons analyzed we could observed this deficits in the SS and, therefore we hypothesize that this data variability could be linked to the alterations observed in the CV. This is a really useful parameter, since tell us about how the PC population in emitting its firing pattern. Is normal to find that models who present alteration in the SS firing rate, present at the same time, alterations in the CV, but no necessarily in the CV2. Together with the SS parameters, the CS even less studied, probably due to the difficult to identify it, is also altered in our model and in other model linked to ASD (see table below). Cntnap2 have been linked to small-conductance potassium channels (Poliak et al., 1999), those channels have been demonstrated to be necessary to develop a normal complex spike in PC(Zagha, Lang, & Rudy, 2008). Based on previous works we hypothesize that the Cntnap2 functionality clustering the Kv channels could be responsible of the alterations that we observed in our model in terms of deficits in the CS firing rate.

Taken together our results with the previous one, it seems like the spontaneous activity of PC is altered in ASD related models, with a remarkable alteration in the CV, similar results has been found in our Cntnap2 model in cortex, where a lack of synchrony or deficient communication between different neurons have been described (Antoine et al., 2019; Peñagarikano et al., 2011). How this deficits in the spontaneous activity in Cntnap2 KO could affected to the entire

cerebellar circuit is unknown and how phenotypically this alterations could be manifesting in information processing.

		Simple spike (SS)			Complex spike (CS)	
	Model	**FF**	**CV**	**CV_2**	**FF**	**Refs**
Affections linked to ASD	CACNA1A-P601L	↓	↑	↑	↓	Hoebeek 2005
	CAMK2B KO	↓	↑	↑	↓	Van Woerden 2009
	CACNA1A-S218L	↓	↑	↑	=	Gao 2012
	PC-$BK^{-/-}$	↓	n	n	↓	Sausbier 2004 Chen 2010
	Chronic sub-lethal hypoxia (Hx)	↓	↑	=	=	Sathyanesan 2018
	PC-$MECP2^{-/-}$	=	↑	↑	=	Achilly 2020
	CNTNAP2 KO	=	↑	=	↓	Fernández 2021
	PC-Tsc1	↓	n	n	n	Tsai 2012
Cerebelar damage model	$SCA6^{84Q/84Q}$	↑	=	↓	n	Jayabal 2017
	$SCA6^{84Q/+}$	=	↑	n	n	Jayabal 2016

Table 6. Table summarizing finding in PC spontaneous activity in difference mouse model of ASD or ASD related model.

tDCS modulation of CV and FR

Transcranial direct current stimulation is a non-invasive neuromodulation technique consisting in the application of weak electric currents through the scalp (Nitsche et al., 2008) and has proved to be effective in humans in the modulation of the connectivity of different brains areas, and is currently considered a promising therapy in ASD (Amatachaya et al., 2014; Esse Wilson, Quinn, Wilson, Garcia, & Tesche, 2018). Previous studies demonstrated that the application of transcranial electrical stimulation (tES) can modulate neuronal activity. Specifically, Voroslako and collages in 2018 showed, using rats, that transcranial stimulation over the neocortex modulate the FR of cortical neuros, increasing or decreasing the firing rate compared to control condition, depending on the currents applied. (Asan, Lang, & Sahin, 2020; Vöröslakos et al., 2018) Specifically, PC neurons has shown to be mouldable with transcranial stimulation. In this case, the authors use other technique called transcranial alternating current stimulation (tACS). In many ways tACS is similar to tDCS as a neuromodulatory technique, but instead of applying a direct electrical current, tACS oscillates a sinusoidal current at a chosen frequency to interact with the brain's natural cortical oscillations. In the paper published by Asan and collegues the CV was shown to increase with higher transcranial stimulation intensity and the stimulation

entrained firing activity in a frequency-dependent manner, with stronger phase-locking to the stimulus cycle at higher frequencies (Asan et al., 2020).

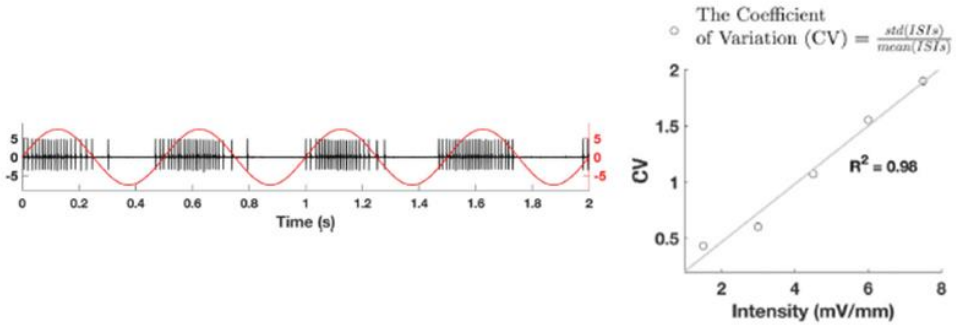


Figure 24: From (Asan et al., 2020). Typical PC response varying between the phases of transcranial alternating current stimulation. On the left, CV vs. stimulation current intensity correlation in PC activity.

As we observed alterations in the FR of SS as well as in the CV of PC in Cntnap2 mice, as a proof of principle of whether tDCS could be used to modulate PC activity in the model, we performed tDCS stimulation over R-CrusI/II and in-vivo PC unitary recordings in alert KO mice. Our results show a bidirectional effect in both the SS firing rate and the CV in KO mice, depending on the currents applied. Currents can be applied in two different polarities, denominated as anodal and cathodal depending if the active electrode positioned over the stimulated region acts as an anode or as a cathode, respectively. When we applied cathodal current the FR increased compared to control conditions in 5 of 7 neurons analyzed and, conversely, anodal current application induced a decrease in the FR. Although the CV is also modulated, this modulation is not that clear as the observed in FR. Only 2 of 7 neurons seems to show a strong modulation, whereas the rest of neurons show a subtle modulation.

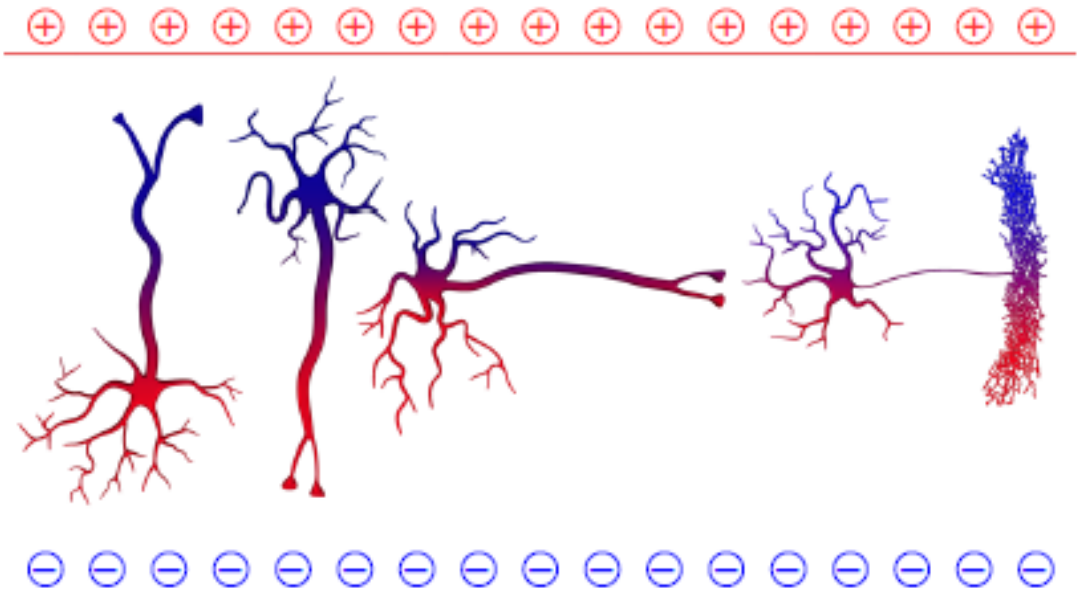


Figure 25. Idealized neurons representing the relationship between different neuronal features and tES impact on membrane polarization. The orientation and morphology of the different neuronal compartments (soma, dendrites and axon) with respect to the electric field determine whether the neuron will be depolarized or hyperpolarized. (From (Liu et al., 2018)).

Since the orientation in the neurons with respect the electric field determine the possible effect of the stimulation in the neuron response (Chan & Nicholson, 1986; Rahman et al., 2013), we hypothesize that the mixed effect observed in our result is due to the orientation of the neurons. We studied the relation between the parameters in each condition in terms of figuring out whether there is a relation between the FR and CV. During anodal current conditions it seems like there is an invert relation between the FR and CV, nevertheless during cathodal current this relation does not appear.

It is worth mentioning that our results differ from the reported data obtained in similar experiments. For example, Voroslako et al using rats described a decreased FR with cathodal and increased FR with anodal and our modulation in the CV in PC is not as clear as Asan and colleagues described. These differences could be related to: 1) the chosen area of study, as Voroslako used cerebral cortex and Asan cerebellar vermis. The Crus I/II area, due to its specific anatomical characteristics (i.e. circumvolutions) represents a more heterogeneous area in this sense because the orientation of the neurons is not maintained through the area. 2) due to the transcranial stimulation used, since we used tDCS and Asan and colleagues used Transcranial Alternating Current Stimulation. Since there is not an effective treatment for the core symptoms of ASD, we hypothesize that transcranial stimulation could be a powerful tool for the treatment. Has been prove that the application over the scalp is enough to modulate the neuronal activity. Here, we described how PC neurons are sensitive to this technique, Besides, different studies postulated the transcranial stimulation as an approach to treat different psychiatric disorders, nevertheless the basic science underlying to this

changes remains unclear (Godfrey, Muthukumaraswamy, Stinear, & Hoeh, 2021; Oberman et al., 2016; Sánchez-León et al., 2018).

Regulation of Purkinje neuron morphology by CNTNAP2

Deficits in dendritic arborization and dendritic spines abnormalities has been described in animals model of ASD and patients with autism diagnosis (Martínez-Cerdeño, 2017; Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011). Besides Cntnap2 gen has been reckon as a protein with an important role in the arborisation processes and spine stabilization. Knockout and knock-down studies showed that it is required for the normal development of neuron dendritic arborization and spines in different neurons types as interneurons or cortical neurons (Canali et al., 2018; Gao et al., 2018; Gdalyahu et al., 2015; Lazaro et al., 2018; Truong, Rendall, Castelluccio, Eigsti, & Fitch, 2015). More specifically, a recent paper using cultures, observed a deficits in dendritic arborization in PC lacking Cntnap2 (Argent et al., 2020).

Our results show that lacking Cntnap2 results in neuron reduced size in general, smaller dendritic arbor and reduced soma size. Besides, this reduction in the size is accompanied with a reduction in dendritic arbor complexity, that means that PC KO neurons present lower number of branches, and those branches are less complex. The results present in this doctoral thesis are consistent with similar works published recently in where studying the cerebellum maturation, suggests that Cntnap2 protein is necessary for correct PC dendrites maturation (Argent et al., 2020). Besides, neuroimagen studies using humans have been demonstrated that CNTNAP2 loss of function mutations are associated with cerebellar abnormalities, including hypoplasia of the cerebellar vermis and hemispheres and reduced cerebellar gray matter (Rodenas-Cuadrado et al., 2016; Tan, Doke, Ashburner, Wood, & Frackowiak, 2010).

Cntnap2 protein also has reported to have a role in the spine stabilization in neurons and interneurons in cortex, where a mouse KO of this protein present a deficit in the spine stabilization and density(Anderson et al., 2012; Gao et al., 2018; Gdalyahu et al., 2015), nevertheless our results in PC indicate that the protein is not necessary to maintain the spine density in this area. The observed deficits in arborization could potentially be responsible for the alterations in the PC spontaneous activity. The less complex arborization likely leads to a decreased number of synapses, specifically there is a smaller dendritic arbor to synapse with climbing fibers, which could lead to the decreased frequency of CS seen in our model. Nevertheless, even the SS is induced by synapse between PC dendritic arbor and PF, we did not find differences in between genotypes but, since the SS is due to a massive connections between PC and PF, a smaller dendritic arbor induce more variability in the firing rate reflexing in the CV.

Purkinje cells present an altered response after sensory evoked potential in Cntnap2 knock-out mouse

Based on previous evidence and our results demonstrating an alteration in PC spontaneous activity and morphology, we next wanted to test whether the functionality of the circuit could be altered. We used a well-characterized paradigm consisting in electrical whisker stimulation and recorded sensory evoked potentials in cerebellar area Crus I. In R Crus I/II this same paradigm evoked a well-characterized sensory evoked potential (Brown & Bower, 2002; Roggeri et al., 2008) and a specific activation of the PC (Cheron, Dan, & Marquez-Ruiz, 2013). Sensory information reaches R Crus I/II area from two main pathways; the trigeminal nucleus and the somatosensory cortex (S1), creating two different components in the SEP characterized accordingly by a trigeminal (T) (Morissette & Bower, 1996; Roggeri et al., 2008) and a cortical (C) (Brown & Bower, 2002; Diwakar, Lombardo, Solinas, Naldi, & D'Angelo, 2011; Mostofi, Holtzman, Grout, Yeo, & Edgley, 2010; Parasuram, Nair, Naldi, D'Angelo, & Diwakar, 2018) component.

Our results identified remarkable differences between the obtained SEP between WT and KO mice. The trigeminal component, appearing 3.94±0.13 ms which is supposed to be the result of the parallel fiber and PC interactions (Bengtsson & Henrik Jörntell, 2007; Márquez-Ruiz & Cheron, 2012), seems to remain unaltered. However, the cortical component, resulting from the interaction between climbing fiber and PC (Márquez-Ruiz & Cheron, 2012) that appears at 12ms in WT is not present in KO mice, and an aberrant component is found at 6.46±0.14 ms. These results seem to indicate that the cortical component is anticipated in KO mice, although other potential explanations remain possible. Whisker stimulation had been previously applied in our model to study. Antoine and colleagues applied deflecting movements to mouse whiskers and record the response in the corresponding section of somatosensory cortex in L2/3 layer demonstrating that the neuronal response in SI is affected in our mouse model of autism. Besides, using in vitro approach, studied the inhibition circuit between the layer 4 and 2 of somatosensory cortex, describing differences between WT and KO mice in terms of mutants showed reduced feedforward inhibition in layer 2/3 of S1 (Antoine et al., 2019). The S1 cortex of Cntnap2 KO mouse have been studied, finding alterations in the synaptic transmission, fewer interneurons $PV^{+,}$ delayed in the myelination process and axonal excitability deficits, we hypothesize that the alterations present in our model in S1 are affecting the cortical component. Those alterations in the somatosensory cortex, could affect the integration of sensory stimuli and the transmission to cerebellar cortex. Since alterations in the SEP are present in our model after whisker stimulation and we reported alterations in the spontaneous PC activity besides, we wanted to decipher how PC alterations could modify the SEP. For that reason, we analyzed the PC activity after whisker-stimulation, using the same protocol as used in the SEP study.

As mentioned above, whisker information induces a response in the PC activity and reaches the cerebellum via two routes; the trigeminal nucleus-parallel fiber pathway and the inferior olive-climbing fiber pathway (Kleinfeld, Berg, & O'connor, 1999),creating the two characteristic PC spikes, SS and CS respectively. These two firing types are linked to the creation of the different components of SEP. The T component is the result from the interaction between PC and PF, which induces SS firing, and C component is the result from the interaction between PC and CF, inducing CS firing. Recording of individual PC upon whisker stimulation revealed alterations in PC activity. Individual analysis of each type of spike revealed that the timing of appearance of the stimulus-evoked CS is anticipated in Cntnap2 KO mice, being the latency of appearance similar to the latency of the aberrant components around 6ms that we observed in SEP. Besides, upon stimulation, CS in KO mice seem to remain altered longer, we found that 50 ms post-stimuli the frequency of CS tends to be superior in KO compared to WT. Those results seem to indicate an altered integration of climbing fiber inputs and are in agreement with previous works where, using an eye-blink conditioning task, a task dependent on the cerebellum which involves the pairing of a conditioned stimulus (led light) to an unconditioned stimulus (air puff) and a correct cerebellar processing is required for this form of classical conditioning. They described a decreased response probability was related to a dysfunction in olivocerebellar circuit in Cntnap2-KO mouse (Kloth et al., 2015). The appearance of the stimulus-evoked CS has been shown to be modulated by activity from the somatosensory cortex, since the suppression of S1 activity abolishes CS appearance upon tactile stimulation (Shimuta, Sugihara, & Ishikawa, 2020) . Further, diminishing S1 activity lengthens and enhancing it shortens CS appearance latency (Brown & Bower, 2002). These data would suggest that increased activity of S1 could be responsible for the observed CS anticipation in the Cntnap2 model. In fact, decreased inhibitory markers and increased excitation/inhibition ratio leading to higher cortical sensory gain have been described in Cntnap2 mice (Antoine et al., 2019; Peñagarikano et al., 2011), which could account for the observed deficits. Even though, regarding the SS which is responsible for the trigeminal component formation (Márquez-Ruiz & Cheron, 2012), we could not find any differences between the two genotypes. Being consistent those results whit the SEP, in which we did not identify differences in the T component. We hypothesize that the alterations observed in SEP are due to a malfunction in the Climbing fiber-PC input.

Role of cerebellar Cntnap2 protein in behavioral core symptoms of autism

Finally, in order to determine the role of the cerebellar Cntnap2 protein in ASD we selectively removed the Cntnap2 protein from PC, using Cre-Lox technology. Since Autism diagnosis is based purely on behavioral criteria, as biological markers have not been identified yet (Silverman et al., 2010), behavioral approaches using conditional models of genes whose full

knockout recapitulate the autism symptomatology are a powerful tool to understand the circuits underlying the core symptoms of ASD. The Cntnap2 full knockout presents the main core deficits of autism, and it is one of the most relevant mouse models nowadays (Peñagarikano et al., 2011). However, the contribution of the cerebellar Cntnap2 protein to the core symptoms is still unknown.

To address this point, we tested behavior relevant to ASD core symptoms, including analysis of communicative system, repetitive/stereotyped behavior, behavioral inflexibility and social behavior. In addition, we also assayed other behaviors which are commonly comorbid with ASD: anxiety, hypersensitivity to sensory stimuli, and hyperactivity (APA, 2013).

	Cntnap2 Purkinje cKO	Cntnap2 KO-/-	Test
Alterations in communicative behaviors	✓	✓	Ultrasonic Vocalizations at P7
Hyperactivity	✓	✗	Open field
Repetitive behaviors	✓	✗	Grooming
Social behaviors deficits	✓	✗	Three-chamber
Behavioral inflexibility	✓	✓	T-maze
Anxiety behaviors	✗	✗	Dark-light Box
Sensorial alterations	✓	✗	Hot plate

Table 7. table with the behavioral differences between full knock-out Cntnap2 mouse and the Cntnap2 Purkinje conditional.

Communication

Mice use a sophisticated system to communicate between them composed by USVs emitted in a frequency above the human hearing limit (Branchi et al., 2006). They emit USVs in different environmental situations; one of them is when pups are separated from the mother and littermates (Scattoni et al., 2009). USVs are composed by one o more syllables which can be classified based on the duration and the frequency peaks of the syllables (Scattoni, Gandhy, Ricceri, & Crawley, 2008). Our results show differences in the number of USVs

between cKO and Control mice. cKO tends to emit a higher number of syllables in 5 minutes, this result differs from the results previously publish with the full KO, where the number of USV emitted under similar conditions is lower (Peñagarikano et al., 2011). Nevertheless, an increased number of USVs emitted by pups is not a rare finding in mouse models with autism-like phenotypes as; Nfr1$^{+/-}$ (Maloney et al., 2018) TSC2$^{+/-}$ (Young, Schenk, Yang, Jan, & Jan, 2010), BTBR (Scattoni et al., 2008), Mecp2 (Picker, Yang, Ricceri, & Berger-Sweeney, 2006), PatDp1 (Nakatani et al., 2009), PC-Tsc1(Tsai et al., 2012) and NFKB1 (Premoli et al., 2019). There are different theories that could explain the increase in the number of USVs. Since the number of UVS tends to decreased with the age could be that the cKO present deficits in the maturation and its increased USVs is due to a differencial maturity stage compared to WT. Other theory widely used to explain the increased number of USVs in models as Nfr1+, TSC2 or PatDp1 linked the higher number of USV with a increased anxiety state in the pups in response to the stress to be separated from the mother (Crawley, J. N., 2007; Krömer et al., 2005) this altered state does not to be present in adult lige, as in Nfr1 which in adulthood does not present anxiety like behaviors (Molosh 2004). Finally, another theory linked the USVs to the humans babies crying, where the babies with autism tend to performed larger crying compared to neurotypical babies (Johnson, 2008). Some of these genes, as Cntnap2, are associated with cerebellar damage: TSC2$^{+/-}$ (Boronat, Thiele, & Caruso, 2017), Mecp2 (Ellegood, J. et al., 2015) and TSC1 (Weisenfeld et al., 2013). Foxp2, a transcription factor highly express in the cerebellum has been associated with language impairment in humans and mices (Fujita & Sugihara, 2012). Besides, Foxp2 interact with Cntnap2 downregulating its expression (Vernes et al., 2008). Therefore an overexpression of Foxp2 in the cerebellum have been linked to a high number of USVs emitter by mice (Fujita-Jimbo & Momoi, 2014). We hypothesize that this could be one of the reason for we observed differences in the number of USV. We did not find any differences in other parameters such as syllable duration or syllable frequency contrary to findings in the full KO (Burkett, Day, Peñagarikano, Geschwind, & White, 2015) nevertheless, this change has been reported in others model such NFKB1 mouse which presents autistic phenotypes present alternations in number of USV emitted but not in frequency or duration parameters.

Cntnap2 Purkinje conditional mouse present behavioral inflexibility but no repetitive behaviors

Self-grooming in mouse is an innate behavior that is involved in hygiene maintenance and other physiologically important processes, including thermoregulation and social communication and an aberrant rodent self-grooming can be related to human disorders in which repetitive behaviors are a symptoms (Kalueff et al., 2016). Regarding the circuit, involve in this behavior, the basal ganglia, especially the striatum and its dopaminergic inputs control rodent self-grooming motor behavior. Other secondary areas are also implicated as the amygdala which is involver in context-specific modulation of self-grooming or the cerebellum via its multiple projections to the basal ganglia or cortex, is implicated in the

control and coordination, and in fine-tuning of self-grooming movements (Kalueff et al., 2016). Our results, after measuring the time doing grooming by the mice, did not show any difference between the genotype, in the opposite directions of what was observed in the full-KO (Peñagarikano et al., 2011). We hypothesize that this results are expected, since the circuit implicated in the self-grooming is mostly drive by the basal ganglia and our mutant only present the lack of the protein in the PC, being the lack of Cntnap2 protein in PC not enough to replicate the results of KO mouse. Behavioral flexibility is the ability to adapt behavior in response to changing environmental demands (Ragozzino, 2007). People with autism have considerable difficulty performing tasks requiring behavioral flexibility both in their everyday lives and on neuropsychological tests (Hill, 2004) and it is inside the main symptoms of ASD (APA 2013). Is a complex behavior regulated by different areas and its projections between them such prefrontal cortex, orbitofrontal cortex and dorsomedial striatum (Uddin, 2021). The cerebellum also seems to have a role in the control of this behavior, probably due to its connection with the PFC. Experimental studies in rodents support the conclusion that the cerebellum exerts a modulatory influence on functions linked to the PFC (De Bartolo et al., 2009). In rodents, loss of PC postnatal induce inflexibility behaviors in the animals (Dickson et al., 2010). Our result in agreement with the characterization previously publish in the full KO (Peñagarikano et al., 2011), where the mice were test using T-maze as a measurement of behavioral inflexibility, show that cKO present behavioral inflexibility in this test, tending to visit the same arm in the different trials. We hypothesize that the Cntnap2 loss in the PC induce to the appearing of this behavior due to its connections with areas direct linked to this behavior as PFC. Besides the presence of behavioral inflexibility in a mouse with loss in PC reinforces our theory, signaling the cerebellum as important area for the control of behavioral inflexibility.

Lacking Cntnap2 is not enough to replicate some autistic phenotypes observed in full knock-out

While some behaviors related to ASD have been replicated in the cKO, others behaviors linked to lacking Cntnap2 protein in mouse could not replicate in our conditional mouse. Hyperactivity is a symptom inside the spectrum and it is estimate that the co-ocurrance between ASD and attention deficit hyperactivity disorder in children is 40-80% (Leitner, 2014), besides, the full knock-out present hyperactivity compared to WT after the open field test (Peñagarikano et al., 2011). In animals attention deficit hyperactivity disorder is caused by the dysregulation of areas such PFC, striatum and cerebellum (Castellanos & Proal, 2012; Durston, van Belle, & de Zeeuw, 2011). In our results, none differences where find between cKO and controls in either distance travelled or velocity. This results is consistent with other conditional ASD mouse where the locomotor activity was not altered (Tsai et al., 2012).Even the cerebellum seems to be implicated in hyperactivity behaviors, surprisingly, we did not

find any differences between our conditional and control mice, suggesting maybe the implication of more areas, which are doing a compensatory effect over the phenotype.

Finally, in order to characterise the role of the protein in the cerebellum we performed a social test. The social cognition have been widely studied and different areas such mPFC, amygdala, Lateral Septum or Nucleus Accumbes linked to a correct social behavior develop (Fernandez, Mollinedo-Gajate, & Penagarikano, 2018), nevertheless, the cerebellum is also implicated, especially due its connections with cortical areas such mPFC and basal ganglia (Fernández et al., 2019). For that purpose we use the three chamber test, which is a well-know test used to determine whether the mouse present alterations in social behaviors. Mice, as social animals, tens to expend time interacting with other courtpartnes rather than inanimate object. Our results did not show any difference between the genotype, being these results surprising for us. Previous works using conditional cerebellar mouse such Tsc1, a protein linked to ASD in humans, showed deficits in social behaviors (Tsai et al., 2012)and the chemomodulation of PC in wild-type animals is enough to create social deficits (Stoodley et al., 2017). Therefore, we hypothesize that there is some compensatory system involve in the regulation of this behavior which is enough to rescue the possible defect creating by lack of Cntnap2.

6. Conclusions

1. Lacking of Cntnap2 protein in a mouse model of autism, induces alterations in the PC spontaneous activity. Reflexed in abnormalities in the two patterns of PC emission: single and complex spike.

2. tDCS technique is able to modulate the PC activity in the Cntnap2 KO mouse.

3. PC morphology in Cntnap2 KO is altered, demonstrated that the lack of this protein induce a smaller and less complex dendritic arborization compared to WT.

4. The Cntnap2 KO mouse present cerebellar integration processing, after the application of sensorial stimuli. The component of the cerebellar SEP in KO mice are significantly differences of those present in WT animals.

5. The aberrant component present in the SEP in KO mouse are due to Purkinje cell complex spike firing.

6. The lack of Cntnap2 protein in the PC is not enough to replicate some of the behavioral autistic phenotypes seen in the full knock-out. Nevertheless, there is a presence of alterations in communicative system and behavioral inflexibility.

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