IDENTIFICATION OF MODIFIER GENES DRAVET SYNDROME IN A *Drosophila melanogaster* MODEL

Bachelor's thesis

Degree in Biotechnology

Escola Tècnica Superior d'Enginyeria Agronòmica i del Medi Natural

Universitat Politècnica de València

Author: María del Carmen Martín Carrascosa

Academic tutor: Dr. Máximo Ibo Galindo Orozco
External co-tutor: Andrea Tapia González

València, July 2021
Academic year 2020-2021

TITLE: Identification of modifier genes in Dravet Syndrome in a *Drosophila melanogaster* model.

TÍTULO: Identificación de genes modificadores en el Síndrome de Dravet en el modelo *Drosophila melanogaster*.

TÍTOL: Identificació de gens modificadors a la Síndrome de Dravet en el model de *Drosophila melanogaster.*

SUMMARY

Epilepsy is a common chronic neurological disorder that represents a serious threat to the health of the approximately 50 million people who suffer from it worldwide, since the available treatments are not fully adequate. In our case, we will focus on Dravet Syndrome (DS), a severe type of child-hood onset epilepsy and is characterized by mostly myoclonic seizures accompanied by prolonged tonic-clonic seizures. DS usually manifests during the first year of life, following a febrile illness. Subsequently, there will be a delay in psychomotor development and behavioral disorders will appear. In addition, the risk of sudden unexpected death in epilepsy (SUDEP) associated with the syndrome is 15 times higher than in any other epilepsy beginning in infancy.

As for the genetic cause, this condition is caused in more than 80% of cases by mutations, usually de novo heterozygous, in the *SCN1A* gene that codes for the alpha subunit of the $Na_v1.1$ channel. Furthermore, it has been shown that, although there are patients with similar mutations in the sodium channel gene that result in a similar loss of function, there may be large differences both phenotypically and in terms of clinical outcome, thus complicating an effective clinical prognosis.

Because of the large phenotypic differences mentioned above, we have focused on the hypothesis that there are modifier genes that influence *SCN1A* expression although they segregate independently of *SCN1A*. This hypothesis is supported by other experimental studies carried out with other genetic pathologies, as well as by two works carried out in this same research group, and on which I am going to base my project by choosing six possible modifier genes: *CLCN1*, *CACNA1A*, *CHRNB2*, *KCNQ3*, *CHRNA4* and *PAX6.*

In this work, we have used as a model *Drosophila melanogaster* presenting the $para^{bss1}$ variation of the *para* gene, being this the homologue of *SCN1A* in fruit flies. The epileptic phenotypes given by the *bss1* mutation cannot be completely repressed by pharmacological treatment, a fact very similar to what happens in patients with DS. Our project has used gene silencing via RNA interference and the *GAL4*/UAS system to obtain phenotypes with repressed expression of fly homologues of the human genes of interest.

The experiments developed comprise a RT-qPCR to check the expression level of the genes involved -and thus validate the functioning of the *GAL4*/UAS system-, a flight test, a negative geotaxis test and *a posteriori* locomotion assay of one of the phenotypes following interesting results.

Thus, we have concluded that the human genes *CLCN1*, *KCNQ3* and *PAX6* could be used in the future in the diagnosis and treatment of Dravet syndrome.

RESUMEN

La epilepsia es un trastorno neurológico crónico común que representa una seria amenaza para la salud de los aproximadamente 50 millones de personas que la sufren, dado que los tratamientos disponibles no son completamente adecuados. En el que caso de este trabajo nos vamos a centrar en el Síndrome de Dravet (SD), una enfermedad rara que resulta en un tipo severo de epilepsia que comienza en la infancia y que está caracterizado por convulsiones mayoritariamente mioclónicas acompañadas de prolongadas crisis tónico-clónicas. El SD normalmente se manifiesta a lo largo del primer año de vida, a raíz de un cuadro febril. Posteriormente, se producirá un retraso en el desarrollo psicomotor y aparecerán trastornos de conducta. Además, el riesgo de muerte súbita inesperada en la epilepsia (SUDEP) asociado al síndrome es 15 veces mayor que en cualquier otra epilepsia que comience en la infancia.

Genéticamente, SD es provocado en más del 80% de los casos por mutaciones, normalmente *de novo* heterocigóticas, en el gen *SCN1A* que codifica para la subunidad alfa del canal $Na_v1.1$. No obstante, aunque haya pacientes con mutaciones similares en *SCN1A*, pueden existir grandes diferencias tanto fenotípicas como en lo relacionado al resultado clínico, complicando así un efectivo pronóstico clínico.

Debido a las grandes diferencias fenotípicas mencionadas, nos hemos centrado en la hipótesis de la existencia de genes modificadores que influyen en la expresión de *SCN1A* aunque se segregan de manera independiente de este. Esta hipótesis es respaldada por otros estudios experimentales realizados con otras patologías genéticas, además de por dos trabajos realizados en este mismo grupo de investigación, y en los cuales voy a basar mi proyecto mediante la elección de seis posibles genes modificadores: *CLCN1*, *CACNA1A*, *CHRNB2*, *KCNQ3*, *CHRNA4* y *PAX6.*

En este trabajo, hemos utilizado como modelo *Drosophila melanogaster* presentando la variación $para^{bss1}$ del gen *para*, siendo este el homólogo del *SCN1A* en las moscas de la fruta. Los fenotipos epilépticos dados por la mutación *bss1* no pueden ser reprimidos por completo mediante tratamiento farmacológico, un hecho muy similar al que sucede en los pacientes con DS. Nuestro proyecto se ha valido del silenciamiento génico vía RNA de interferencia y del sistema *GAL4*/ UAS para la obtención de fenotipos con la expresión de los genes de interés reprimida.

Los experimentos desarrollados comprenden una RT-qPCR para comprobar el nivel de expresión de los genes implicados -y así validar el funcionamiento del sistema *GAL4*/UAS-, un ensayo de vuelo, un ensayo de geotaxis negativo y un ensayo de locomoción *a posteriori* de uno de los fenotipos tras unos resultados interesantes.

De esta forma, hemos llegado a la conclusión de que los genes humanos *CLCN1*, *KCNQ3* y *PAX6* podrían utilizarse en el futuro como en el diagnóstico y tratamiento del síndrome de Dravet.

KEYWORDS: Dravet Syndrome; seizure; modifier gene; locomotion; negative geotaxis; flight.

PALABRAS CLAVE: Síndrome de Dravet; crisis epiléptica; gen modificador; locomoción; geotaxis negativa; vuelo.

AUTHOR: María del Carmen Martín Carrascosa.

ACADEMIC TUTOR: Dr. Máximo Ibo Galindo Orozco.

EXTERNAL CO-TUTOR: Andrea Tapia González.

València, July 2021

ACKNOWLEDGEMENTS/ AGRADECIMIENTOS

El mayor de los agradecimientos va para mi madre. Gracias por vivir la carrera con el mismo entusiasmo e ilusión con la que yo la he vivido. Gracias por leerme temas y temas de todas las asignaturas, y aprenderte los exámenes conmigo. Juntas no había asignatura que se nos resistiese, por muy aburrida y pesada que fuese. Gracias a mi padre por todos los kilómetros recorridos para llevarme a la UPV y para devolverme a casa, y por todas las horas de espera paciente en el coche. A Andrés, por hacerme más entretenida la *televida* y el *telecole*.

Muchas gracias a Ibo por acogerme en su laboratorio a pesar de las complicaciones derivadas de la pandemia. Gracias por tu carácter cercano y afable, por salir del despacho y ayudarme siempre que lo he necesitado. Andrea, supongo que no te gustan los agradecimientos cursis ni sentimentales, así que soy directa: gracias por todo, absolutamente todo. Por hacerme de guía en el laboratorio, por escuchar mis dudas y problemas del trabajo, por conseguirme un cuaderno súper chulo, por los regalos de cumple y mil cosas más. Lamento que tan solo te den 10 horas por tutelarme, sin duda te mereces más. Del centro de investigación Príncipe Felipe, también me gustaría agradecer a Isabel del Pino y a Álvaro su ayuda. Y a Xavi, por los fantásticos almuerzos que me prepara.

A continuación, me gustaría dar las gracias a las personas que me llevo de la carrera, sin vosotros los cuatro años habrían sido más largos y difíciles. Mención especial para Mar, la amistad más bonita que me llevo de la UPV. Sin olvidar a Javi, innumerables almuerzos en el CIPF, días en la universidad y en el telecole, siempre unidos a mil trabajos. A Migue, por los almuerzos y las risas en clase. Y a todos los demás, ya sabéis quiénes sois.

Con este trabajo concluyo cuatro años maravillosos del que puedo decir que ha sido el grado universitario de mi vida.

A mi yo de niña, por haber aprendido a
quererse y a confiar ciegamente en sí misma.

Y a quien lo hizo posible: infinitas gracias, mamá.

INDEX

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS AND ABBREVIATIONS

BDSC	Bloomington Drosophila Stock Center
BS	Bang-sensitive
cac	*cacophony*
CACNA1A	Calcium voltage-gated channel subunit alpha 1 A
CACNA1G	Calcium voltage-gated channel subunit alpha 1 G
CACNB4	Calcium voltage-gated channel auxiliary subunit beta 4
CFE	Cryptogenic focal epilepsy
C. elegans	Caenorhabditis elegans.
CGE	Cryptogenic generalized epilepsy
CHRNA4	Cholinergic receptor nicotinic alpha 4 subunit
CHRNB2	Cholinergic receptor nicotinic beta 2 subunit
CLCN1	Chloride voltage-gated channel 1
Clc-α	Chloride channel alpha
CNS	Central nervous system
CyO	Curly of Oster
D. melanogaster	*Drosophila melanogaster*
DNase	Desoxirribonuclease
DS	Dravet syndrome
dsRNA	Double-stranded RNA
elav	Embryonic lethal abnormal visual system
GABA	γ–aminobutyric acid
GAL4	Galactose-responsive transcription factor GAL4
GEFS+	Genetic epilepsy with febrile seizure plus
HLF	Hepatic Leukemia factor
ICEGTC	Intractable childhood epilepsy with GTCs

ILAE	International League Against Epilepsy
IS	Infantile spasms
KCNQ	Potassium voltage-gated channel subfamily Q
KCNQ2	Potassium voltage-gated channel subfamily Q member 2
KCNQ3	Potassium voltage-gated channel subfamily Q member 3
MAE	Myoclonic Atonic Epilepsy
Mb	Mega-bases
mRNA	Messenger RNA
nAChRα1	Nicotinic acetylcholine receptor alpha 1
nAChRα4	Nicotinic acetylcholine receptor alpha 4
Na_v1.1	Sodium channel protein type 1 subunit alpha isoform 1
Na_v1.9	Sodium channel protein type 1 subunit alpha isoform 9
NIG-Fly	Fly stocks of National Institute of Genetics
Nipagin	Methyl 4-hydroxybenzoate
Ø	Diameter
para	*paralytic*
para^{bss1}	*paralytic* gene with bang-sensitive 1 allele
PAX6	Paired box 6
POLG	DNA polymerase gamma, catalytic subunit.
rcf	Relative centrifuge force
RNAi	RNA interference
RNase	Ribonuclease
rp49	Ribosomal protein L32
RT-qPCR	Retro transcriptase-quantitative polymerase chain reaction
SCN1A	Na^+ voltage-gated channel alpha subunit 1
SCN2A	Na^+ voltage-gated channel alpha subunit 2
SCN8A	Na^+ voltage-gated channel alpha subunit 8

SCN9A	Na^+ voltage-gated channel alpha subunit 9
shRNA	Short hairpin RNA
SIGEI	Severe idiopathic generalized epilepsy of infancy
SMEI	Severe myoclonic epilepsy of infants.
SMEIb	Borderline severe myoclonic epilepsy of infants
SUDEP	Sudden unexpected death in epilepsy
TE	Transposon element
toy	Twin of eyeless
TRiP	Transgenic RNAi Project
UAS	Upstream activating sequence
VDRC	Vienna Drosophila Resource Center
WT	Wild type

1. INTRODUCTION

1.1 SEIZURE DISORDERS AND EPILEPSY

Human seizure disorders represent a serious health concern due to the large number of affected people and due to the inadequacy of available treatments. Seizures are symptoms of an acute illness, hence can be 'provoked seizures' or they can also occur during epilepsy, 'unprovoked seizures'. Around 10% of the population will suffer one or more seizures – provoked or unprovoked- during their lifetime, but only 1% to 3% of the population will suffer from epilepsy which is a common chronic neurologic disorder that affects around 50 million people worldwide (Dare *et al*., 2021; Shneker & Fountain, 2003). There have been several definitions and classifications for epilepsy and seizures over the years. The first modern classification was proposed in 1964 (Gastaut *et al*., 1964) but its international use began in 1970 (Gastaut, 1970). The distinction of epilepsy and seizures, which started in 1970, is important as a large percentage of patients who suffer from seizures are unclassifiable under any type of epilepsy (Falco-Walter *et al*., 2018). However, the clearest cases of epilepsies are channelopathies which involve mutations and trafficking defects of ion channels, producing expression and function abnormalities (Hirose, 2006; Parker *et al*., 2011).

Throughout the years, the classifications were updated several times based on the accumulated clinical experience by the International League Against Epilepsy (ILAE). Nowadays, an epileptic seizure is defined as: "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain." (Fisher *et al*., 2005). And epilepsy is given when someone suffers an epileptic seizure and their brain "demonstrates a pathologic and enduring tendency to have recurrent seizures" (Fisher *et al*., 2014).

In 2017, the ILAE updated its classification of the different types of epilepsy -thus modifying the 1981 classification- based above all on the 2010 classification. It grouped under the name of generalized seizures those that arose and involved networks that were distributed between both hemispheres. On the other hand, it assigned the term focal to those that only originated in connections of a specific hemisphere. Finally, those for which there was not enough evidence to classify them in any of the previous categories were grouped under the term unknown onset. The name "unclassified" was reserved for those in which the information was really scarce and little or nothing was known about them (Fig.1) (Fisher *et al*., 2017; Moshé *et al*., 2015).

In 2010, as a result of a modification of the causes of epilepsy by the ILAE, the following categories were established: genetic, structural or metabolic epilepsies and unknown origin. An epilepsy is considered genetic if genetic factors play an important role, either because the genes responsible have been inherited or due to *de novo* mutations that may or may not be inherited. In contrast, in structural and metabolic epilepsies there is a genetic or non-genetic cause that results in a structural or metabolic alteration (Moshé *et al*., 2015).

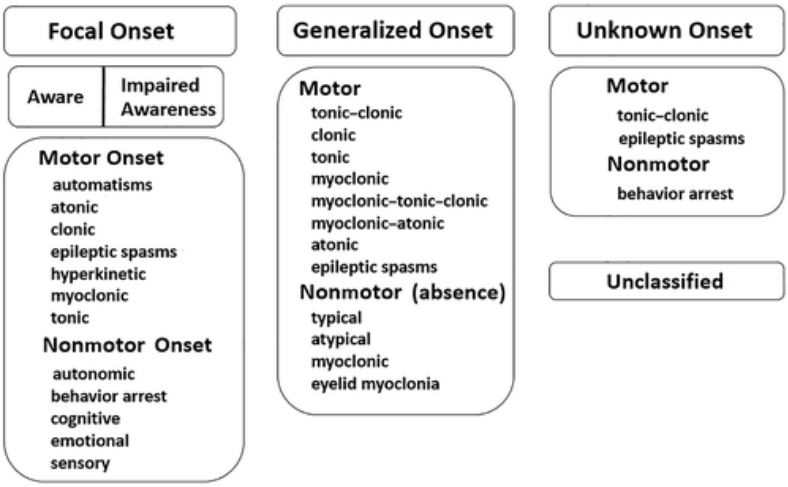

Figure 1. Current classification of seizure types according to the ILAE. *The onset nature defines how epilepsy types are according to the 2010 modification by the ILAE. Adapted from Fisher et al., 2017.*

Mortality is an important topic when considering epilepsy. Although not all forms of epilepsy are associated with a reduction in life expectancy, in the case of pediatric epilepsies there is a high mortality due to various comorbidities and low mortality of patients due to other causes. In developed countries, sudden unexpected death in epilepsy (SUDEP) is the most common cause of mortality associated with epilepsy. This is a category of death for epileptic patients that occurs in the absence of a true structural cause and appears to be heterogeneous with respect to the mechanisms and circumstances involved. In childhood-onset epilepsy, SUDEP usually occurs in patients suffering an intractable epilepsy, who are not in remission and in those with a known epilepsy, and it rarely occurs before adulthood (Kalume *et al*., 2013; Kearney, 2013; Moshé *et al*., 2014; Nashef *et al*., 2012).

In the case of this project, we will focus on Dravet syndrome a pediatric genetic epilepsy consequence of *de novo* mutations and associated to a 15-fold greater risk of SUDEP than other childhood-onset epilepsies (Kearney, 2013).

1.2 DRAVET SYNDROME

Dravet syndrome (DS) is a severe type of epilepsy characterized by the onset of prolonged febrile and afebrile seizures in infancy -the majority being myoclonic- that usually begin throughout the first year of life and that will evolve into drug-resistant epilepsy (Steel *et al*., 2017). In DS, as it happens in similar epileptic syndromes, seizures give as result damage of the neural tissue by means of oxidative stress, inflammation and metabolic imbalance (Pearson-Smith & Patel, 2017; Rana & Musto, 2018). There will also be a delay in cognitive and motor development, and conduct disorders may appear. It is classified as a rare disease, affecting only one person in every 20,000 or 40,000 (Kano *et al*., 2015).

First of all, is crucial to remark on the predominant types of seizures that come with DS: myoclonic seizures, tonic seizures, clonic seizures and tonic-clonic seizures (Fig. 1). They can have a focal or a generalized onset. Firstly, myoclonic seizures normally are sudden, short-lasting mild or forceful jerks that affect some or the whole body; they are normally too short to

affect consciousness although some people can have them in clusters of several seizures over a period of time. Second, in the case of tonic seizures if the onset is generalized all body muscles will become stiff, while if the onset is focal just some of the muscles will tighten. Thirdly, clonic seizures last for a few minutes and might affect consciousness, they cause the body to shake and jerk. Finally, the tonic-clonic seizures, which last for a few minutes, always have a generalized onset and have two stages: an initial tonic-like stage which is shortly followed by a clonic-like stage. Prolongued tonic-clonic seizures are characteristic of DS, often precipitated by fever (EPILEPSY ACTION, 2021; Kearney, 2013).

Apart from cognitive delay, motor difficulties and other effects, we must consider that DS has associated a greater SUDEP risk than other pediatric epilepsies, therefore being a major concern to families and caregivers. Although little is known in human patients, according to the findings of Kalume *et al*., (2013) who studied the mechanism of premature death in Scn1a heterozygous KO mice, SUDEP could be caused by apparent parasympathetic hyperactivity immediately following tonic-clonic seizures, leading to lethal bradycardia and electrical dysfunction of the ventricle in mouse models (Kalume *et al*., 2013; Kearney, 2013).

The syndrome was described for the first time in 1978 (Dravet, 1978), giving it the name of severe myoclonic epilepsy of childhood or SMEI. Afterwards, the name was changed to Dravet syndrome (DS) as the myoclonic component of this epilepsy is not always present and the symptomatology presents some variability. Seizures usually occur in conjunction with fever or illness, so they are usually first classified as febrile seizures; thus delaying the correct diagnosis of the syndrome (Dravet, 2011).

Originally, two forms of Dravet could be differentiated depending on the symptoms, the typical or nuclear form; and the borderline form in which the myoclonic component was absent or very subtle. However, nowadays it is preferable to include the “borderline” cases under the DS definition. Consequently, a wider range of phenotypes with heterogeneous causes are categorized as DS (Steel *et al*., 2017).

Although, as we mentioned above, the syndrome was clinically described in 1978, it was not until 2001 when DS was firstly described from a genetic point of view when *de novo* variants of the *SCN1A* gene, which encodes the voltage-gated sodium channel $Na_v1.1$, were discovered to cause more than 80% of DS cases. Nevertheless, although the aforementioned gene is now considered a model for the study of epilepsy genetics, no alterations of *SCNA1* are found in 30-20% of tested cases, therefore, several genes associated with a DS-like phenotype have recently been described. Furthermore, even DS patients with similar loss of function variants can present important phenotypic differences (i.e. from severely disable to mildly disable), thus prediction of clinical outcomes becomes difficult and inaccurate, causing major uncertainty and concern to parents and families (Claes *et al*., 2001; de Lange *et al*., 2020; Kearney, 2013; Steel *et al*., 2017).

Consequently, the genetic intricacies of this syndrome that produces childhood-onset drug-resistant genetic epilepsy have to be studied. Therefore, it is important to know the implications of the *SCN1A* gene encoding the altered $Na_v1.1$ sodium voltage-dependent channel in the majority of diagnosed cases. However, we must consider that currently it is known that even two patients having the same *SCN1A* mutation can have very different clinical outcomes,

therefore it is logical to conclude that genetic and non-genetic conditions may exist that modify the classical DS nuclear form.

1.2.1 *SCN1A* GENE

The *SCN1A* gene is localized in the 2q24 chromosomal region and encodes the α subunit of the $Na_v1.1$ channel, a voltage-gated sodium channel (Fig. 2A). Voltage-gated sodium channels play an essential role in the generation of the action potential rising phase in excitable cells (i.e. neurons and myocytes). They are large internal membrane proteins with selective sodium-ion permeation. In mammals, these channels are encoded by at least ten genes and are located in the brain and in muscle cells like myocytes. The different sodium channels have remarkably similar functional properties, but small changes in sodium-channel function are biologically relevant (Fig. 2B) (Yu & Catterall, 2003).

Sodium voltage-gated channels exist in two principal sets of conformations, conducting and nonconducting and they are formed by an α subunit ($Na_v1.1$-$Na_v1.9$) associated with auxiliary β subunits (β1–β4). First of all, the 260kDa α subunit has the voltage sensors and ion-conducting aqueous pore. The ion-conducting pore is contained entirely within the α subunit in four internally repeated domains (I–IV) and each domain consists of six α-helical transmembrane segments (S1–S6) and a pore loop connecting S5 and S6 (Catterall *et al*., 2010). Secondly, the presence of the 33–36 kDa β subunits is required for the correct functioning of the pore as they modify the kinetics and voltage-dependence of gating and serve as cell adhesion molecules interacting with other components (i.e. the extracellular matrix and the cytoskeleton). There are different combinations possible of α and but the channels $Na_v1.1$ from the mammalian brain are a complex of 260 kDa α subunit -codified by *SCN1A*-, β1 (36 kDa), and β2 (33 kDa) subunits (Catterall, 2000).

Although, as we have mentioned, voltage-gated sodium channels are present both in muscle cells and in the brain, we are going to focus on the location of $Na_v1.1$ in the latter. This sodium channel is expressed in GABAergic interneurons which develop an important role in pathophysiology and acquired epilepsies due to the release or impaired release of the inhibitory neurotransmitter γ–aminobutyric acid (GABA) (Fig. 2C). Mutations in *SCN1A* cause loss of function of the voltage-gated sodium channel, impairing the generation of action potentials at high frequency by the neurons affected and therefore causing the release of GABA to be reduced. Therefore, the lack of activation of these sodium channels results in excess neuronal activity since GABAergic inhibitory neurons control neuronal excitability by the release of their inhibitory neurotransmitter (Hedrich *et al*., 2014; Yu *et al*., 2006).

Although the final effect on inhibitory interneurons remains the same, there is a wide range of phenotypes related to alterations in $Na_v1.1$. Due to this, Ragsdale (2008) and Catterall *et al*., (2010) proposed the hypothesis that the spectrum of severity of the forms of epilepsies associated with variations in the sodium channel is linked to the severity of the loss-of-function mutations and how much they impede the action potential of GABAergic neurons. Mild impairment of $Na_v1.1$ function provokes febrile seizures; moderate to severe impairment of the channel function by nonsense mutations together with altered mRNA processing causes the variety of phenotypes seen in GEFS+ epilepsy; and very severe to complete loss of function causes Dravet syndrome due to the haploinsufficiency of the gene. Nevertheless, the

presence of comorbidities can alter the resulting phenotype even between individuals with the same $Na_v1.1$ alteration (Catterall *et al*., 2010; de Lange *et al*., 2020).

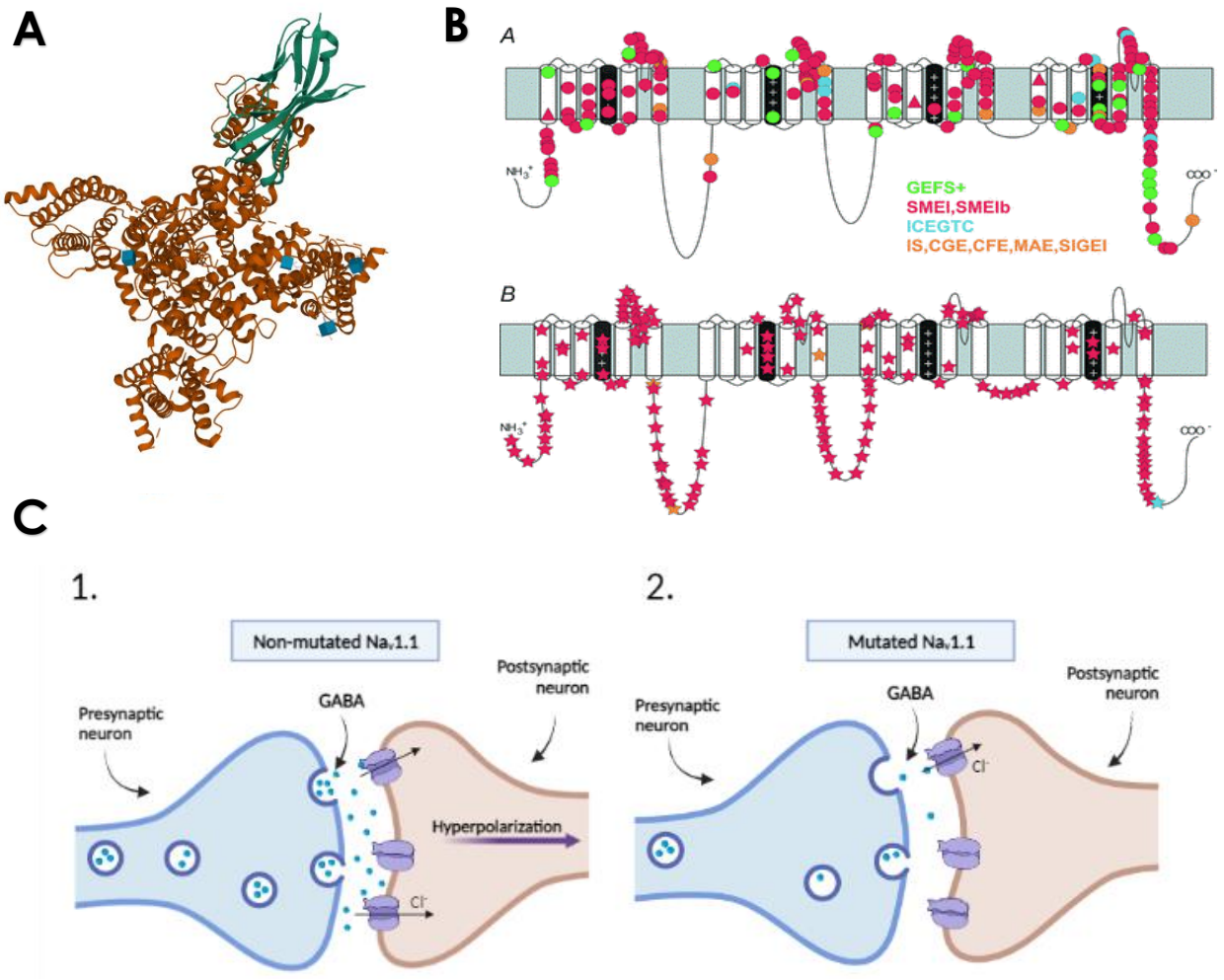

Figure 2. $Na_v1.1$ structure, its mutations in epilepsy patients and further physiological effects.
(A) Biological assembly: Cryo-EM structure of the full-length human $Na_v1.1$-β4 complex at 3.3 Å resolution. Retrieved Pan et al., 2021. (B) Mutations in $Na_v1.1$ channel in patients with epilepsy. Above: missense mutations (circles) and in-frame deletions (triangles). Below: truncation mutations (stars). Clinical type of epilepsy indicated by color: GEFS+, generalized epilepsy with febrile seizures plus; SMEI, severe myoclonic epilepsy of infancy (nowadays called Dravet syndrome); SMEIb, borderline SMEI (nowadays included inside Dravet phenotype) ; ICEGTC, idiopathic childhood epilepsy with generalized tonic–clonic seizures; IS, infantile spasms; CGE, cryptogenic generalized epilepsy; CFE, cryptogenic focal epilepsy; MAE, myoclonic astatic epilepsy; SIGEI, severe idiopathic generalized epilepsy of infancy. Retrieved from Kearney & Meisler, 2009. (C) View of the presynaptic neuron, synaptic cleft and postsynaptic neuron in two different scenarios of $Na_v1.1$. 1, GABA secretion by the presynaptic neuron occurs after the functional sodium channels have transmitted the action potential and will cause hyperpolarization and consequent loss of sensitivity to stimuli in the postsynaptic neuron. 2, GABA secretion is reduced given the impairment of high-frequency action potential generation by the mutated Nav1.1 channels: hyperpolarization does not occur. Created with Biorender.

As previously commented, more than 80% of DS patients have pathogenic variants or mutations in *SCN1A* in spite of the different phenotypes produced. The loss of function of the gene can be given by several types of mutations being the most pathogenic ones *de novo* mutations; however, in less than 10% of cases, they are inherited from mosaic affected or

unaffected parents. About the type of mutations, approximately half of the patients have truncation variants and the other half present missense variants (Scheffer & Nabbout, 2019).

However, several factors have been proposed as possible modifiers of the final phenotype of Dravet syndrome and, in general, of all epilepsies which are produced by the lack of function of the *SCN1A* gene. Among these factors, apart from mosaicism of a pathogenic variant of the gene and variations in regulatory regions of *SCN1A*, we find variations in modifier genes that could influence the phenotype (de Lange *et al*., 2020).

1.3 MODIFIER GENES

Modifier genes are those genes which are able to alter or influence the expression or function of another gene, although they segregate independently from the main mutation. As it has been established for several other genetic disorders, modifier genes can interfere in different pathological aspects such the onset, progression, treatment or severity (de Lange *et al*., 2020; Kearney, 2011; Guo *et al*., 2015; Vélez *et al*., 2016) and according to several investigations there are strong indications that genetic background can modify the phenotype and clinical outcome of pathogenic *SCN1A* variants DS, both in human patients and Scn1a knock-out mice (Catterall *et al*., 2010; de Lange *et al*., 2020; Hawkins & Kearney, 2016; Yu *et al*., 2006).

Due to the high phenotypic variance, knowledge of modifier genes has proven to be key to better understand the pathophysiological mechanisms, and therefore try to find new therapeutic targets for drug-resistant epilepsy and the other clinical disabling manifestations associated with the syndrome. There are several potential modifier genes which have already been identified: variants in *SCN9A, SCN8A, SCN2A, HLF, POLG, KCNQ2, CACNB4, CACNA1G*, and *CACNA1A* might influence clinical outcomes (de Lange *et al*., 2020).

Potential loci identified in mouse models contain genes encoding for GABA receptors, ion channel genes and genes associated with epilepsy and neuronal hyperexcitability. What is more, overexpression of rare variants of neuronal hyperexcitability controlling genes has been identified in severely affected DS patients (de Lange *et al*., 2020; Hammer *et al*., 2017; Miller *et al*., 2014).

However, each modifier gene itself only contributes to a small portion of the variability of the syndrome and each patient may be affected by different modifiers or by several modifiers simultaneously. In addition, no clinically relevant modifiers have been found at present, so there are no tests to detect them, hence it is impossible to use them clinically. It is therefore crucial to dedicate further research both to identify relevant modifier genes to understand both the clinical variability of the syndrome as to find a therapeutic target for this drug-resistant disease (de Lange *et al*., 2020).

So, in previous projects in my current lab, they were engaged in trying to identify and narrow down a list of possible modifier genes. First, a selection of candidate modifier genes of *SCN1A* which are conserved in *D*. *melanogaster* was performed by Ñungo, (2018). And one year later, Hernandez (2019) performed a theoretical and experimental review of the work done by Ñungo (2018). Therefore, for my current project a series of promising human genes were selected considering both aforementioned works (Hernández, 2019; Ñungo, 2018).

But, even though modifier genes play an important role in neurological diseases, isolating them in human patients is challenging, thus genetic screens in model organisms have to be performed (Kearney, 2011). Therefore, for this project the *Drosophila* model was selected as it was also chosen in the case of Ñungo (2018) and Hernández (2019).

1.4 THE *Drosophila* MODEL

Drosophila or fruit fly is a model organism widely used in scientific and medical research. This is due to several advantages. It is easy to maintain and culture in the laboratory with a short generation time and a small budget investment. In addition, they are easy to manipulate genetically, they also have a compact genome and many orthologous genes associated with human disease (Hales *et al*., 2015).

First of all, with regard to the life cycle, the process of developing from fertilized egg to adult requires an average of 9-10 days at 25ºC; however, the modification of the temperature enables control of the development speed. Upon fertilization, embryogenesis is completed and followed by the larval development which is completed five days after fertilization. Afterwards, larvae metamorphose within a hard, protective chitin-based pupal case constituted from the outer larval cuticle. Meanwhile the pupae stage lasts, the adult structures get formed from a collection of tissue-specific progenitor cells named imaginal discs and which are present in the larvae. Finally, adult flies emerge from the pupal or puparium in which is called eclosion and after 8 - 12 hours they become sexually active (Hales *et al*., 2015).

To understand why *D. melanogaster* is a good model organism for screens, it is necessary to know its genomic constitution. The vast majority of protein-coding genes are found in the euchromatic region while the heterochromatic region is mainly composed of simple sequence repeats (Celniker & Rubin, 2003). However, due to investigations developed over the last two decades, it is believed that nearly 65% of human disease-causing genes have a functional homolog in flies and most of these homologs are expressed in *Drosophila* tissues that perform the function of the equivalent human tissue. What is more, *Drosophila* presents little gene redundancy, hence offering a good model to study human mutated genes (Dare *et al*., 2021; Ugur *et al*., 2016).

The nervous system in *Drosophila* and the human nervous system work in a similar manner. Both are required to process information related to vision, hearing, olfaction, proprioception and taste, and in both the aforementioned information is conveyed to the CNS, where it is analyzed to provide the most suitable motor output. Furthermore, numerous properties such as genetic, cellular and electrophysiological, remained conserved between both organisms, albeit the gross anatomy of their brains differs (Ugur *et al*., 2016).

Even though many different types of neurons are required to process information in *Drosophila*, fruit flies have probably a million-fold fewer neurons overall compared to vertebrates. Therefore, the reduced complexity of the *Drosophila* nervous system allows an ease to assess in depth the function of genes and neuronal networks (Ugur *et al*., 2016).

In order to study human diseases using a fly model, there are three main strategies which have been developed: reverse genetics, forward genetics and the recently established,

diagnostic strategy. In the present project we are going to make use of the reverse genetics approach in order to identify possible modifier genes in DS. Broadly speaking, the approach consists of creating mutations of human genes in their fly homologs to study their phenotypes in vivo. There are mainly three methods to diminish or abolish expression of a gene in flies: targeted gene disruption, transposon-mediated mutagenesis and excision of existing transposable elements (TE), and gene silencing (Ugur *et al*., 2016). This work will take advantage of the ease to perform gene silencing via RNA interference (RNAi) and the *GAL4*/UAS system, as it will be detailed later.

1.4.1 SEIZURE DISORDERS IN *Drosophila*

As previously described before, there exist some relevant similarities between fly nervous system and human nervous system. For example, there is a high evolutionary conservation of voltage-gated, ligand-gated channels and transmitter receptors of several molecules such as acetylcholine, glutamate and γ–aminobutyric acid (GABA). However, an electrical shock applied to a fly is sufficient to induce neuronal spiking activity resembling human seizures (Dare *et al*., 2021; Parker *et al*., 2011).

Humans and *Drosophila* have in common several features in seizure phenotype: they have a seizure threshold, electroconvulsive shock treatment increases ulterior seizure activity threshold, genetic mutations modulate seizure susceptibility, seizure activity spreads through the central nervous system presenting spatial segregation and *Drosophila* seizure phenotype can be modified and diminished by anti-epileptic drugs (AED) used in humans (Dare *et al*., 2021).

In order to study seizure disorders in *Drosophila*, mutant collections are created. Although seizures can be provoked to wild type flies (WT) using electrical stimulus, the aforementioned collections have lower sensitivity threshold and seizures can occur due to thermal or mechanical stimulus. One of those collections is the bang-sensitive (BS) *paralytic* class in which there are 14 mutant alleles representing 12 genes, which produce different gene products. The *paralytic* gene, which is the fly orthologue of *SCN1A* human gene, codes for a voltage-gated Na^+ (Na_V) channel and it has an allele of great importance named bang senseless ($para^{bss1}$). This allele is a severe BS mutation resulting in the most sensitive phenotype from an electrophysiological and behavioral point of view. Furthermore, it expresses a prominent tonic-clonic-like phenotype and is the most difficult type of BS mutant to suppress both genetically and pharmacologically, thus it has been presented to model human intractable epilepsy (Dare *et al*., 2021; Howlett *et al*., 2013; Parker *et al*., 2011).

The importance of the *paralytic* gene began to be considered in 1989, when it was identified as a sodium channel coding gene in *Drosophila* by Loughney *et al*., (1989). Furthermore, the same study confirms that mutations in this locus lead to an alteration in the structure of the channel. But not only that, it is already proposed the hypothesis that in the case of *para* mutants the action potentials of neurons are blocked at high temperatures due to the possible scarce presence of these sodium channels.

But focusing now on the BS mutants, the majority of *para*^{bss1} flies show perfectly normal behavior under standard circumstances. Abnormal behavior is induced by means of mechanical shock, a tap of the culture vial or brief vortex mixing (a “bang”), choosing the latest due to higher reproducibility. The resulting seizure pattern is complex (Fig. 3) and six phases are distinguished (Parker *et al*., 2011):

1. Initial seizure. It lasts several seconds and is characterized by leg shaking, abdominal muscle contraction and beating of wings, among others.
2. Initial paralytic period. Flies remain immobile and unresponsive under mechanical stimulus.
3. Tonic-clonic-like activity period. The fly stays in a quiescent state which is interrupted by episodes of clonus-like activity.
4. Recovery seizure. It resembles both the initial seizure and clonus-like activity.
5. Refractory period. Further seizures cannot be provoked yet.
6. Complete recovery. Flies have recovered bang sensitivity.

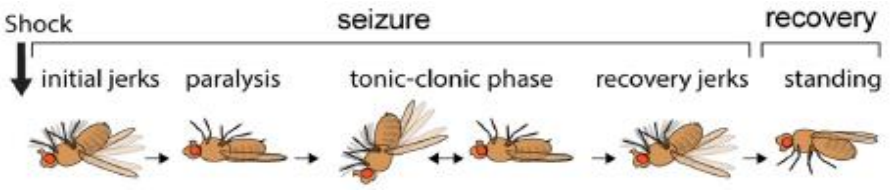

Figure 3. Drosophila para^{bss1} phenotype complex seizure pattern. *The process is composed by a general seizure phase and a recovery phase. General seizure phase is formed by initial seizure or jerks, initial paralysis, tonic-clonic-like activity and recovery seizures or jerks. Recovery phase is formed by an initial refractory period and by the complete recovery of bang sensitivity. Retrieved and adapted from Dare et al., 2020.*

To conclude, thanks to the extensive literature and experimental procedures developed previously, *para*^{bss1} *D. melanogaster* are a suitable model organism to carry out the identification of potential modifier genes through the silencing of human orthologous genes.

1.5 INTERFERENCE RNA (RNAi)

As we all know, RNA interference (RNAi) constitutes a powerful research tool to reduce the expression or induce a “knock down” of a certain gene or set of genes. Research has taken advantage of the originally-endogenous system, modifying it to its purpose and creating libraries to silence the majority of genes of several organisms like *C.elegans* and *Drosophila* in order to perform genome-wide screening (Boutros & Ahringer, 2008; Mohr, 2014).

As every technique, RNAi presents advantages and disadvantages. On the one hand, it allows the immediate knowledge of all the identified genes and it makes it easier to identify lethal mutations. On the other hand, not every gene is susceptible to the technique providing incomplete and variable silencing, and the knockdowns cannot be inherited by the progeny unless that construct is expressed as a transgene, among other disadvantages (Mohr, 2014). In the case of *Drosophila,* RNAi technique is quite developed, having protocols, software tools, databases and wide libraries around the globe. The most renowned collections are the Transgenic RNAi Project (TRiP) (http://www.flyrnai.org/trip), Bloomington Drosophila Stock Center (BDSC) (http://flystocks.bio.indiana.edu/), National Institute of Genetics (NIG)-Fly

(http://www.shigen.nig.ac.jp/fly/nigfly/) and Vienna Drosophila Resource Center (VDRC) (http://stockcenter.vdrc.at/control/main).

To carry out RNAi in *Drosophila*, it is possible to opt either for an injection of 200 to 2000 base pairs into the blastoderm or for the insertion of the construct as a transgene. We selected the last option since it allows gene silencing to be inherited by the offspring (Perrimon *et al*., 2010). Firstly, transgenes introduced in flies encoded for long dsRNA hairpins but the field evolved towards the use of optimized vectors with constructs encoding short hairpins (shRNA) introduced in the flies by means of site-directed approaches. Therefore, the expression and subsequent knockdown were improved, even in the germline (Mohr, 2014).

The RNAi technique is usually combined with the *GAL4*/UAS system in order to silence the desired target genes in adult flies in a heritable manner. The regulatory and structural *GAL* genes are required for the growth of yeast on galactose but the induction of them is dependent on the transcriptional activator Gal4 which operates through an upstream activating sequence present in their promoters called UAS_{GAL} (Traven *et al*., 2006). Nevertheless, it has been demonstrated that the *GAL4*/UAS system can operate not only in yeast but also in various animal cells or organisms such as *Drosophila* where it has been widely used for the regulation of the expression of target genes (Fig. 4) (Asakawa & Kawakami, 2008).

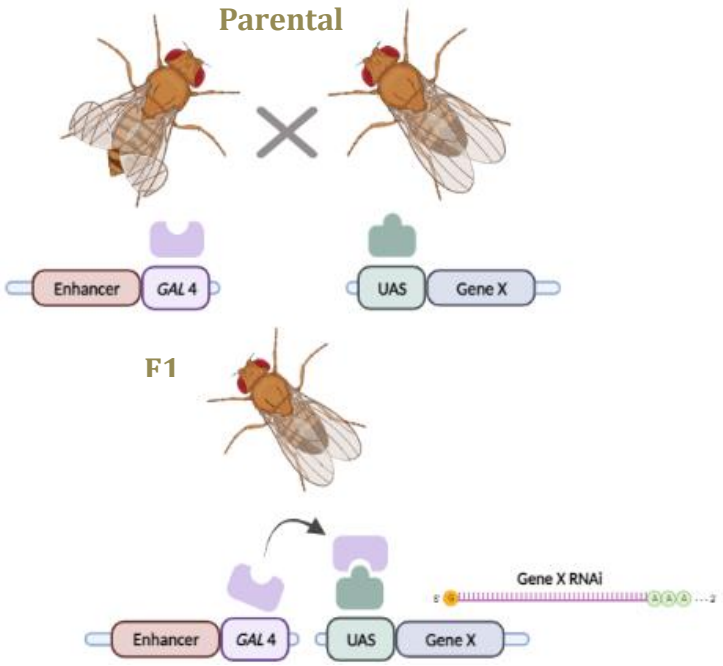

Figure 4. Illustrative representation of the specimens used and functioning of the GAL4/ UAS system. *Drosophila virgin para^{bss1} females express balancer CyO (curvy wings), GAL4 and specific promoter (left). Drosophila males express the transgene downstream of UAS (right). Flies necessary to make the crossing and obtain the GAL4/ UAS system and the silencing of the corresponding gene (Gene X).* Created with Biorender.

In conclusion, for the present project we will take advantage of the RNAi method combined with the *GAL4*/UAS system with the purpose of identifying modifier genes in DS using a *D. melanogaster* model.

2. OBJECTIVES

Due to the great variability associated to the DS phenotype even between individuals with very similar loss-of-function mutations and to the need of finding clinically relevant modifier genes to be used in the diagnosis and as therapeutical targets of the disease, the main objectives we have established for this project are the following:

- Validation of the *GAL4*/UAS system in *para*^{bss1} *Drosophila melanogaster* gene expression.
- Identification of candidate genetic modifiers of the *SCN1A* gene utilizing *para*^{bss1} *Drosophila melanogaster* homologous model through:
    - Negative geotaxis tests.
    - Flight test.

3. MATERIALS AND METHODS

3.1 *D. melanogaster* COLLECTION

The flies are grown in polystyrene transparent tubes (Dominique Dutscher, *Drosophila* tubes narrow 28.5 x 95 mm) with cellulose acetate plugs (Genesee Scientific Cat No. 49-101) and standard corn mill medium (see annex). *Drosophila* stocks are kept at 18ºC to slow down their life cycle but the experimental strains (Table 1) are kept at 25ºC.

Table 1. Specification of the D. melanogaster gene, the individuals genotype and how they are mentioned along the project.

D. melanogaster gene	BDSC stock code	Genotype	Mentioned in the text as
cac	27244	y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02572}attP2	cac^{RNAi}
ClC-α	53337	y^1 sc* v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMC03566}attP40/ CyO	$ClC\text{-}\alpha^{RNAi}$
KCNQ	27252	y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF02562}attP2	$KCNQ^{RNAi}$
nAChRα1	28688	y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03103}attP2	$nAChR\alpha1^{RNAi}$
nAChRα4	31985	y^1 v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.JF03419}attP2	$nAChR\alpha4^{RNAi}$
toy	33679	y^1 sc* v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS00544}attP2	toy^{RNAi}
para	Made in the lab	$\frac{para^{bss1}}{para^{bss1}}; \frac{elav}{CyO}; \frac{+}{+}$	$para^{bss1}$
None	25709	y^1 v^1; P{$y^{+t7.7}$=nos-phiC31\int.NLS}X; P{$y^{+t7.7}$=CaryP}attP40	Control

The crosses are specified in the annex. As the balancer CyO, which confers curvy wings, has seemed to interfere in the results obtained in previous projects (Hernández, 2019), flies with an empty RNAi have been chosen to be the control group.

3.2 ASSAYS

For every assay male control flies ($para^{bss1}$ + empty modifier gene RNAi*)* and male RNAi-engineered flies ($para^{bss1}$ + modifier gene RNAi) were used unless otherwise specified. Males are used because they present a stronger phenotype than females due to the fact that they are hemizygous.

3.2.1 NEGATIVE GEOTAXIS

There were 3 biological replicates and 3 repetitions of the experiment. Every fly group consisted of 10 to 15 individuals depending on the progeny availability from each cross (n = 10-15).

In this behavioral assay we are going to take advantage of the *Drosophila*'s reflex of climbing after being knock down to the bottom of the vial. Negative geotaxis is a widely used method for the screening of locomotion modifiers and for the assay of diverse *Drosophila* models of neurodegeneration: Alzheimer's disease, Parkinson's, ageing and motor disorders (Ali *et al*., 2011; Cao *et al*., 2017; Liu *et al*., 2015).

First of all, flies are introduced in an empty tube -without food- for 3 minutes. Afterwards, another test tube is attached to the top of the first tube using adhesive tape. The bottom test tube was previously marked at 8 cm from its ground level. A video camera was set to record 10-second videos of the flies' movement. To begin the experiment, the conjoined tubes are tapped against the bench in order to set all flies at ground level. Immediately, the camera starts recording for 10 seconds. Finally, the flies which go beyond the 8 cm threshold in those 10 seconds are counted.

3.2.2 FLIGHT ASSAY

For this assay there were 3 biological replicates. On average each fly group was made up of 34 individuals (n = 34).

First of all, the flies are introduced in an empty tube for 10 minutes, so they get used to fasting. The flight test consists in dropping the *Drosophila* tubes in the 90 cm flight tester, which is built as specified in 'An Improved Method for Accurate and Rapid Measurement of Flight Performance in *Drosophila*' (Babcock & Ganetzky, 2014), in order to determine flight performance. The flight tester interior plastic sheet is coated with adhesive glue (Bricofam) hence flies are glued to the cylinder wall at variable height dependent on the time necessary for the fly to produce enough thrust to make contact with the cylinder adhesive surface. A 'drop tube' is added to ensure that flies enter the flight tester at the same velocity, thus reducing variability associated with manipulation (Fig. 5A).

Afterwards, the plastic sheet is removed and placed on a flat white surface, so we are able to photograph it (Fig. 5B). The images collected are analyzed using the free software ImageJ (Wayne Rasband, NIH) following the protocol specified in Babcock & Ganetzky (2014).

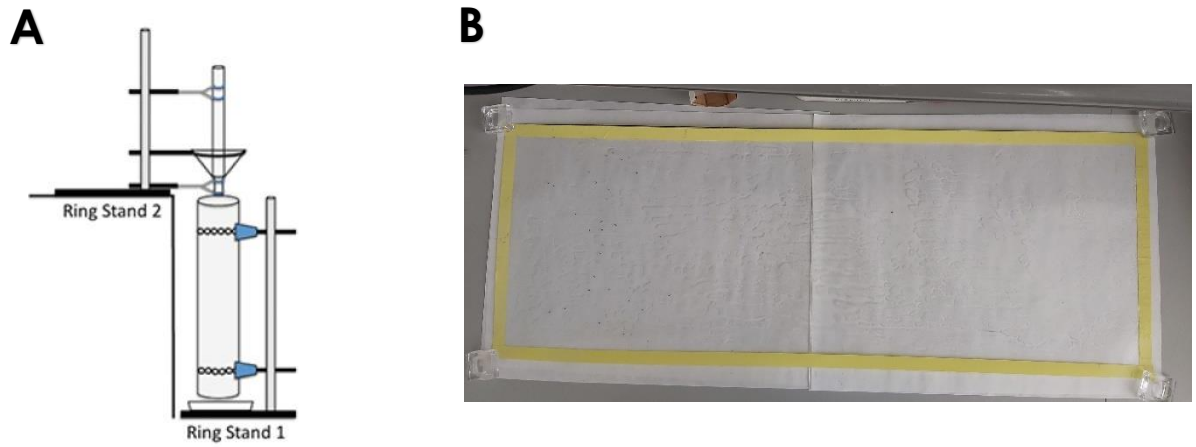

Figure 5. Representation of the two main steps of the flight assay. *(A) Representation of the flight tester tube; retrieved form Babcock & Ganetzky (2014). (B) Photograph of the plastic sheet which covers the interior of the flight tester.*

3.2.3 LOCOMOTION ASSAY

For the locomotion assay 3 biological replicates were used. The genotypes utilized were 18 control *para*^{bss1} (n = 18) and the 15 experimental fly progeny *para*^{bss1} + *toy* ^{RNAi} (n = 15).

First, the flies are introduced in an empty tube -without food- for 15 minutes, so they get used to fasting. Afterwards, they are individually introduced into a 90 mm Petri dish (Thermo Fisher Scientific Cat. No. 101/IRR) to delimit a closed circular area. However, as the bottom lid is used, the actual area is 85 mm approximately. The Petri dish is located on the top of a white surface with a light source below. A 10-minute fly track video is recorded from the top by a camera connected to the program VirtualDub (VirtualDub.org; free software) which previously has had its parameters correctly adjusted as it is detailed in the paper 'A Low-cost Method for Analyzing Seizure-like Activity and Movement in *Drosophila*' (Stone *et al*., 2014).

The analysis of the videos recorded is done with the EthoVision XT 15 (Noldus, Wageningen, the Netherlands). The model organism of the program is set to *Drosophila* (larvae) and the program is manually adjusted. Tracked feature is set at center-point detection. Units of distance, time and rotation are determined to be centimeters (cm), seconds (s) and degrees (deg), respectively. For the arena the whole Petri dish is selected (ø 8.5 cm) and a concentric circular zone (ø 4.5 cm) is marked. The track duration for the analysis is set at 10 min after a delay of 2 seconds.

The dependent variables of interest are time spent in movement, time spent in zone (ø 4.5 cm) and distance moved of the center-point. The time spent in movement of the center-point is averaged according to the total fly number in each sample, control (n = 18) and *toy*^{RNAi} (n = 15). The movement thresholds were set at 0.20 cm/s and 0.05 cm/s. The statistics calculated for the different dependent variables involved the cumulative duration, frequency and latency to first, but what is interesting for us is the cumulative duration of the movement.

3.2.4 MOLECULAR BIOLOGY STUDIES

To observe the differential expression of the genes involved and to validate the *GAL4*/UAS system in *para*^{bss1} *Drosophila*, a RT-qPCR is performed. Regarding the sample size, there were three replicates of each well and the RNA was extracted from 20 fly heads of each phenotype.

As mentioned, for the protocol fly heads were used exclusively. This is because the progeny obtained from the respective crosses will express the *elav* gene (embryonic lethal abnormal visual system) which is required for the post determinative development of the nervous system, with lethal alleles causing loss of function. Therefore, due to the function of *elav* as a neuron-specific marker, our six genes will only be repressed in neuronal tissue (Koushika *et al*., 1996; Yao and White, 1994).

The protocol used for the extraction of RNA from 20 fly heads was performed as follows. First 600 µL of Trizol (Thermo Fisher Scientific; Waltham, Massachusetts, USA) were added to the fly heads and the mix was homogenized with a mortar. After letting the mix incubate on ice for 5 min, 120 µL of chloroform (Scharlab SL; Sentmenat, Barcelona, España) were added, shaken vigorously for 15 s and incubated on ice. Then, a centrifugation at 12,000 rcf for 15 min at 4ºC was performed. The aqueous phase was transferred to a new micro centrifuge tube and 300 µL of isopropanol (Scharlab; Sentmenat, Barcelona, España) were added. It is important to shake well to precipitate the RNA. The aqueous phase mixed with the isopropanol was left 30 min on ice and, afterwards, centrifuged at 12,000 rcf for 15 min at 4ºC. Next, the supernatant was eliminated and 375 µL of 75% ethanol, which had been previously freshly prepared with RNase-free water, were added to the pellet. Another centrifugation step was performed at this point, at 12,000 rcf for 5min at 4ºC. Afterwards, the supernatant obtained was removed and RNA pellet was air dried for at least 25 min. Finally, when the RNA pellet was completely dried, 8 µL of nuclease-free water were added and the pellet was resuspended. The amount of RNA obtained was then quantified and its purity evaluated using the ND 1000 spectrophotometer (Thermo Fisher Scientific; Waltham, Massachusetts, USA).

Afterwards, the two-step RT-qPCR begins with first-strand synthesis using the qScript® cDNA SuperMix (Quantabio; Beverly, Massachusetts, USA). Firstly, a volume of 20 µL was needed so the following combination was performed: 4 µL of qScript cDNA SuperMix (5X), variable quantity of RNA template and variable RNase/DNase-free water volume. The resulting mix was vortexed gently to homogenize the contents and a centrifugation step was performed. After the supernatant was discarded, the rest of the components at the bottom of the reaction micro-tube were incubated in the thermal cycler (Techne; Staffordshire, UK) according to the following temperature and time cycles: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and finally kept at 4°C.

For the qPCR, SYBR Green Master Mix (FastStart Essential DNA Green Master. Roche diagnostics. Indianapolis, IN, USA) was used. The reaction volumes used to get a final volume 10 µL are specified in Table 2. For primers detail consult the annex.

LightCycler 480 (Roche; Basel, Switzerland) was used as the fluorescence detection system. The program used consists of a pre-incubation for 5 min at 95ºC. Then, there are 40

amplification cycles consisting of 3 stages (10 s at 95ºC, 10 s at 58ºC and 10 s at 72ºC) and the final obtaining of the melting curve (5 s at 95ºC and 1 min at 65ºC).

Table 2. Reagents and reagents' volumes needed to get 10 µL of final volume.

Reagent	Volume
Forward primer (10 µM)	0.5 µL
Reverse primer (10 µM)	0.5 µL
cDNA (1:10)	2.0 µL
SYBR Green Master Mix	5.0 µL
Mili Q water	2.0 µL

For each sample, three replicates were made and once the program was finished, they were normalized with respect to the value of *rp49* (ribosomal protein L32), a structural constituent of the ribosome, expressed in adult head, heart and organism and used as a reference since it is constitutive. The calculations to evaluate the relative gene expression levels were performed using the $2^{-\Delta\Delta CT}$ algorithm also known as the *ddCt* algorithm. It was one of the first methods to calculate qPCR results. It is a convenient method that requires the assumption that the housekeeping genes used are uniformly and constantly expressed in all samples. Therefore, other sample expressions are compared to that in the reference sample (Zhang *et al*., 2021).

3.3 DATA ANALYSIS

GraphPad Prism 9.0.0 program (GraphPad Software, Inc, California) and Excel 2016 (Microsoft, Albuquerque) were used for the graphical representations and statistical analysis of the different assays carried out. The normal distribution of the data was analyzed using the Kolmogorov-Smirnov test or Shapiro-Wilk test if the sample size is at most 50. If the sample suited a normal or Gaussian distribution we can use two-tailed Student's t-test or ANOVA, depending on whether we have 2 groups or more than 2 groups. Conversely, if the sample did not adjust to a normal distribution we can use Mann-Whitney test or Krustal-Wallis depending on whether there are 2 groups or more than 2 groups. ANOVA and Krustal-Wallis are the preferred tests because they reduce first species risk and they will be performed for the analysis of the test results. The statistical significance of all analysis was measured with the *P-value* parameter, taking as reference the value of alpha 0.05 (95% confidence interval).

4. RESULTS

Previously, in the lab where I am developing my current research, there was a project with the objective of searching for susceptibility modifiers of epileptic crisis. They decided to conduct a targeted screening of human candidate genes based on a review of the then recent literature available on genes related to the formation of neural circuits in epilepsy, mainly genes modifying the genes *SCN1A* and *SCN2A*, and genes regulating the formation of neural signaling circuits and networks. The initial 21 genes selected were analyzed to determine the homology degree of their proteins with those of *Drosophila melanogaster*. After this review, 13 genes remained to carry out the phase based on blocking their expression using RNAi and observing the variations in the epileptic phenotype that this insufficiency produces (Ñungo, 2018).

However, the results obtained were inconclusive in some cases due to the low number of individuals obtained. Therefore, Hernández (2019) developed her project studying 11 of the 13 genes selected by Ñungo (2018). But the control chosen to study those 11 genes was not appropriate because it conditioned the fly performance in the different locomotion assays due to its curvy wings (Cy dominant mutation in the CyO balancer chromosome). Taking into account these previous results, I selected the six genes that seemed to be involved in the modification of epileptic crisis susceptibility (Table 3). I also used a different control group from Hernández (2019) which is obtained by crossing virgin *para*^{bss1} females with males carrying an empty RNAi.

Table 3. The six human genes selected for the project, their homologous D. melanogaster genes and their corresponding function.

Human gene	*D. melanogaster* homologous gene	Protein Function (UniProt)
CACNA1A	*cac*	α1A subunit of the P/Q type calcium voltage-dependent channel.
CLCN1	*ClC-α*	Chloride voltage-gated channel protein 1.
KCNQ3	*KCNQ*	Member number 3 of the KQT subfamily of potassium voltage-gated channels.
CHRNB2	*nAChRα1*	β2 subunit of neuronal acetylcholine receptor.
CHRNA4	*nAChRα4*	α4 subunit of the neuronal acetylcholine receptor.
PAX6	*toy*	Transcriptional regulator involved in the embryo development of the eye, nose, central nervous system and pancreas.

The individual analysis that we will perform for each gene specified in the previous table (Table 3) is as follows. First, we will discuss for each gene whether gene silencing was previously known to enhance or suppress the seizure phenotype, based on the data available in the laboratory and the work cited above. Second, by means of the RT-qPCR, we will validate that there has been a decrease in transcript levels. Subsequently, we will characterize each

phenotype and for that, we will analyze the results of the negative geotaxis and flight tests of each gene with respect to the control. For the negative geotaxis assay we used the reflex of the flies to climb after being knocked down to the bottom of the vial, while for the flight test, we checked in a vertical plastic tube at what height the flies were able to produce enough flapping force to stabilize and stick to the wall. Finally, and only in the case of the *toy* gene, we will add and comment on the results obtained in the locomotion test. The reason for the choice of just this phenotype for this test is detailed below. The discussion and biological significance of the results shown here will be discussed in the next section.

4.1 *cac* GENE

We will start with *cac* gene of which we have previous seizure results indicating that its silencing is suppressive of the *bss1* mutation in *para* gene. We can also confirm the decrease in its expression when performing the RT-qPCR (Fig. 6A). cac^{RNAi} flies' behavior does not show remarkable differences when performing the negative geotaxis locomotion assay (Fig. 6B). As we can observe in Fig. 6C, cac^{RNAi} *Drosophila* take more time to contact the cylinder walls, although the uncertainty of the reported measurement is higher than in the case of the control group as the error bars indicate so. The number of cac^{RNAi} flies in each distance interval is more distributed along the 90 cm length than in the case of the control group (Fig. 6D) .

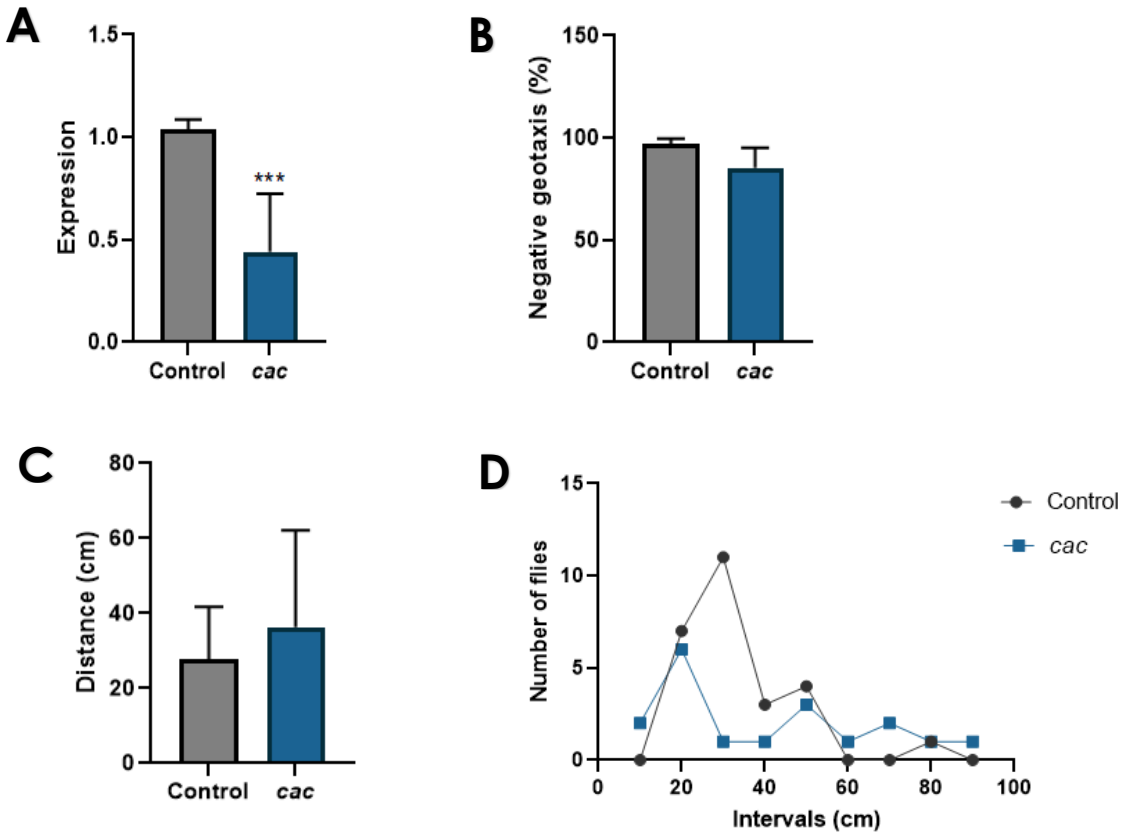

Figure 6. Effect of the cac gene silencing. *(A) Expression of the cac gene in the cac^{RNAi} fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall grouped by distance intervals. For A, B and C values represent the mean ± SEM. For A: *** p-value < 0.001 compared to rp49 expression. For B, C and D: no significant differences were found compared to control group. (A, B: ANOVA; C, D: Krustal-Wallis; for sample size, refer to materials & methods).*

4.2 *ClC-α* GENE

Contrary to the previous gene, our previous data points out that its silencing acts as an enhancer of the *para*^{bss1}. Also, there is the expected expression decrease of the gene in the *ClC-α*^{RNAi} *Drosophila* (Fig. 7A). This result is followed up by the non-significant results of the climbing locomotion assay (Fig. 7B). What is more, in this case the flight test performance of the RNAi engineered flies is also non-significant, as it is possible to observe in Fig. 7C and as it is given by the statistical results. The impossibility to find significant differences in the flight assay could be since the flies follow a similar tendency that the control group when producing the necessary thrust to stabilize themselves and contact the cylinder wall (Fig. 7D). However, the statistical analysis of the distribution by intervals has not shown significant results for any gene so we can only hypothesize.

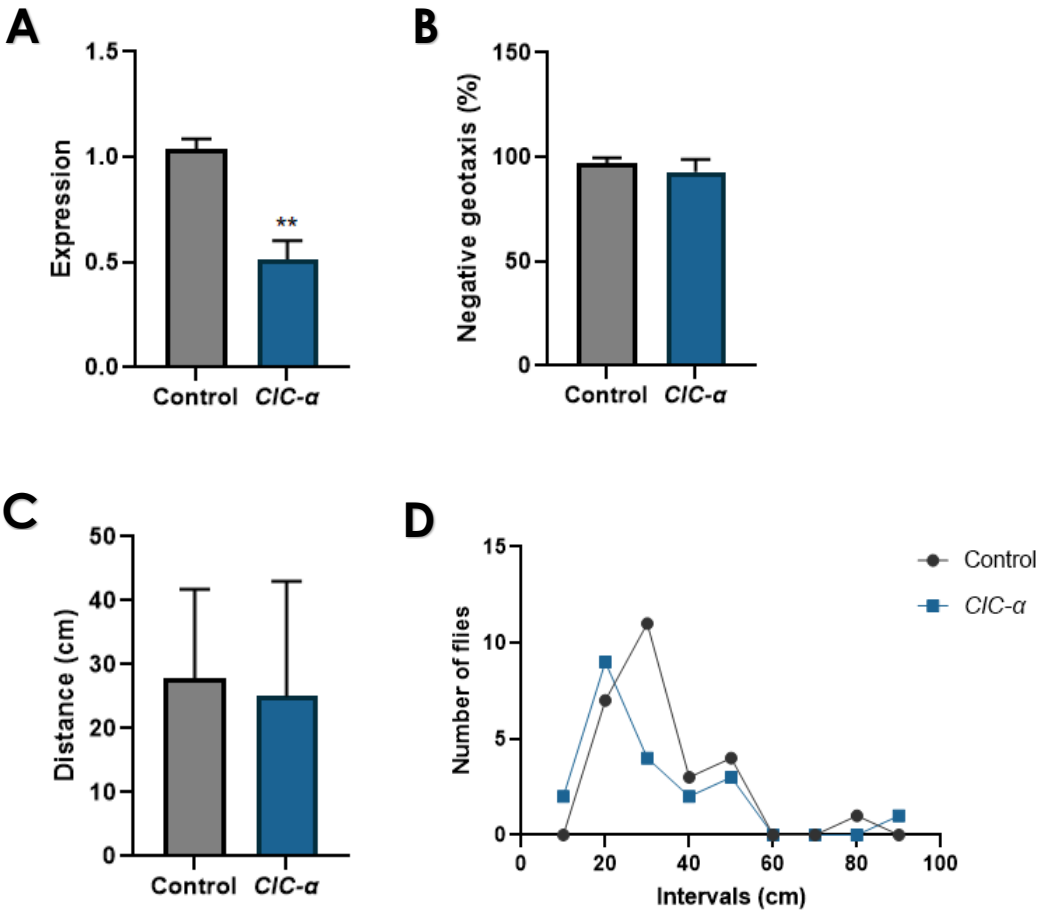

Figure 7. Effect of the ClC-α gene silencing. *(A) Expression of the ClC-α gene in the ClC-α*^{RNAi} *fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall grouped by distance intervals. For A, B and C values represent the mean ± SEM. For A: ** p-value < 0.01 compared to rp49 expression. For B, C and D: no significant differences were found compared to control group. (A, B: ANOVA; C, D: Krustal-Wallis; for sample size, refer to materials & methods).*

4.3 *KCNQ* GENE

According to the seizure data of our lab, its silencing acts as a suppressor of the *bss1* mutation of the *para* gene. Regarding to the present project, the results obtained after the RT-qPCR show that the expression level of the gene is lower than the control gene, therefore indicates that the level of transcripts has decreased with respect to those of the control gene (Fig. 8A). In addition, if we now look at the negative geotaxis assay, it does not show a significant difference between both groups (Fig. 8B). Conversely, the flight test shows that the *KCNQ*^{RNAi} flies are able to stabilize themselves in the air earlier than the control *Drosophila* (Fig. 8C), being able to make contact in 84.62% of the cases with the first third of the cylinder surface (Fig. 8D).

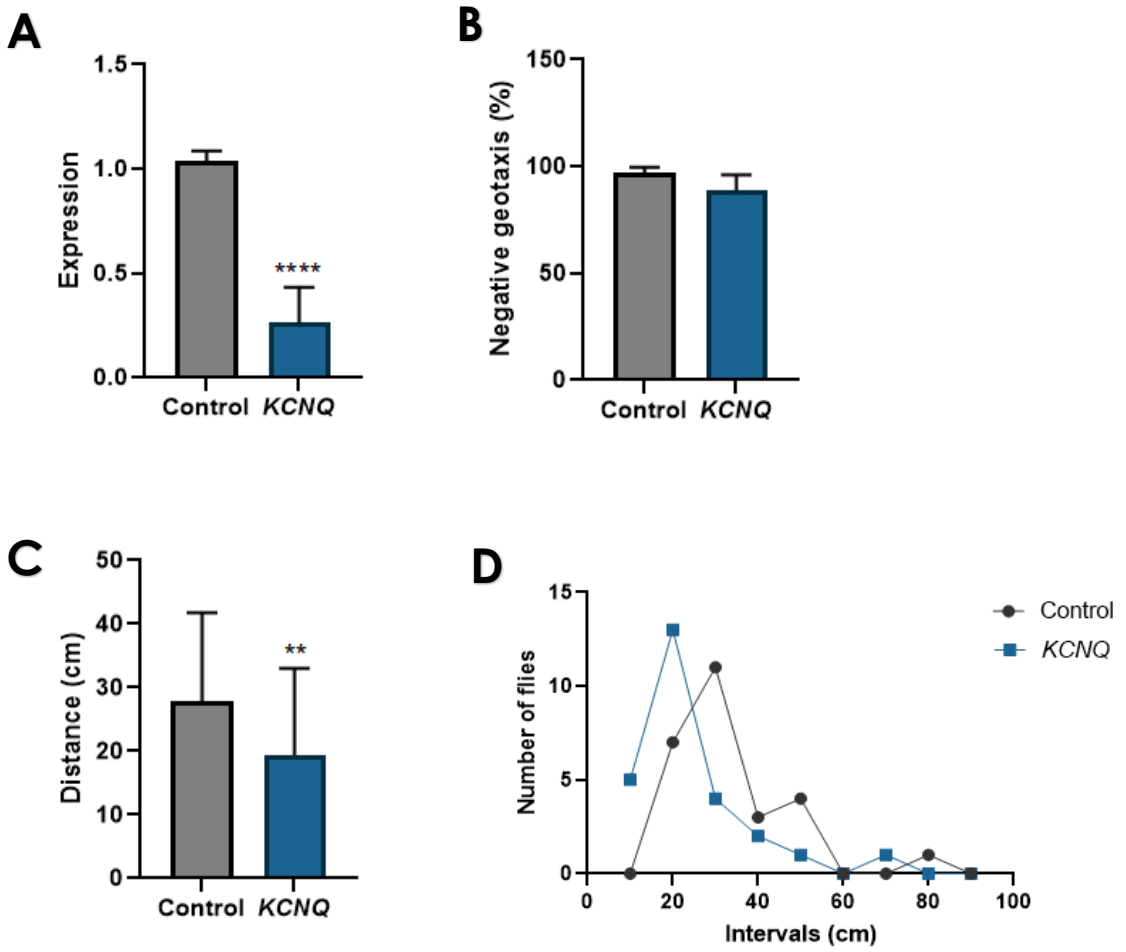

Figure 8. Effect of the KCNQ gene silencing. *(A) Expression of the KCNQ gene in the KCNQ*^{RNAi} *fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall grouped by distance intervals. For A, B and C values represent the mean ± SEM. For A: **** p-value < 0.0001 compared to rp49 expression. For B: no significant differences were found; for C: ** p-value < 0.01 and for D: no significant differences were found compared to control group. (A, B: ANOVA; C, D: Krustal-Wallis; for sample size, refer to materials & methods).*

4.4 *nAChRα1* GENE

According to the previous findings, the silencing of the *nAChRα1* gene functions as an enhancer of the seizure phenotype. Regarding the results of this project, (Fig. 9), the expression of *nAChRα1* decreases significantly compared to that of the control gene, which is indicative that the RNAi technique has been successful and the expression of the target gene has been regulated (Fig 9A). For the negative geotaxis assay both groups present very similar results regardless of whether *nAChRα1* has been repressed or not (Fig. 9B). However, we can remark that control flies take more time to produce enough thrust to contact the wall of the flight tester. On the contrary, *nAChRα1*^{RNAi} *Drosophila* are able to stabilize themselves earlier in the air (Fig. 9C), in the 92% of cases making contact with the adhesive wall in the first third of the flight tube, this is in the first 30 cm (Fig. 9D).

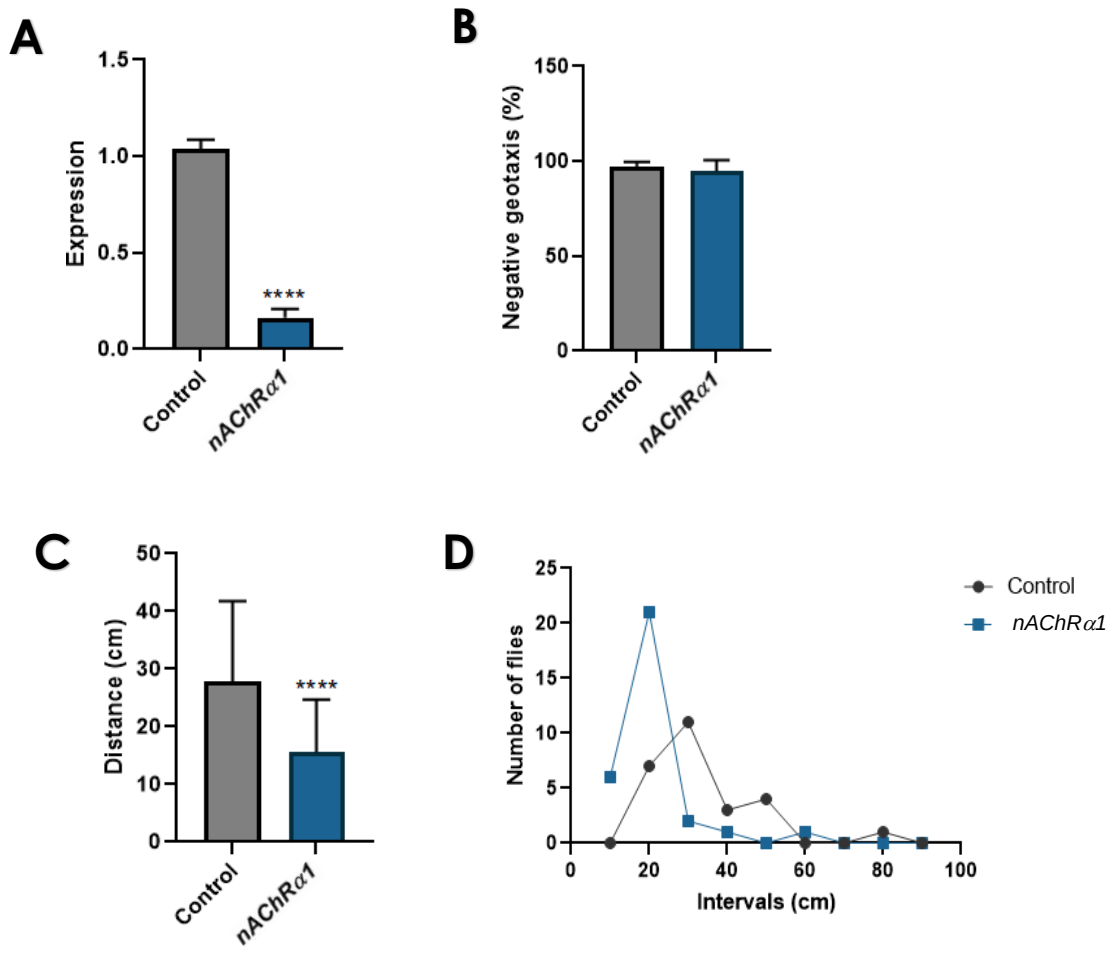

Figure 9. Effect of the nAChRα1 gene silencing. *(A) Expression of the nAChRα1 gene in the nAChRα1*^{RNAi} *fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall measured by distance intervals. For A, B and C values represent the mean ± SEM. For A: **** p-value < 0.0001 compared to rp49 expression. For B: no significant differences were found; for C: **** p-value < 0.0001; for D: no significant differences were found compared to control group. (A, B: ANOVA; C, D: Krustal-Wallis; for sample size, refer to materials & methods).*

4.5 *nAChRα4* GENE

Now we are going to proceed with the second last gene of our analysis whose silencing works as a suppressor of the *para*^{bss1} phenotype in previous projects. As it can be observed (Fig. 10A), the *nAChRα4* gene reports a significant difference regarding the level of expression as it is lower than the control gene level, thus indicating that the RNAi technique has been successful, and the expression of the target gene has been regulated. According to Fig. 10B, the negative geotaxis assay gives a significant difference between the control group and the *nAChRα4*^{RNAi} *Drosophila.* Nevertheless, control flies take more time to produce enough thrust to contact the wall of the flight cylinder. On the contrary, RNAi-silenced flies can stabilize themselves earlier, in 92.85% of cases contacting the wall in the first 30 cm of the tube (Fig. 10C and 10D).

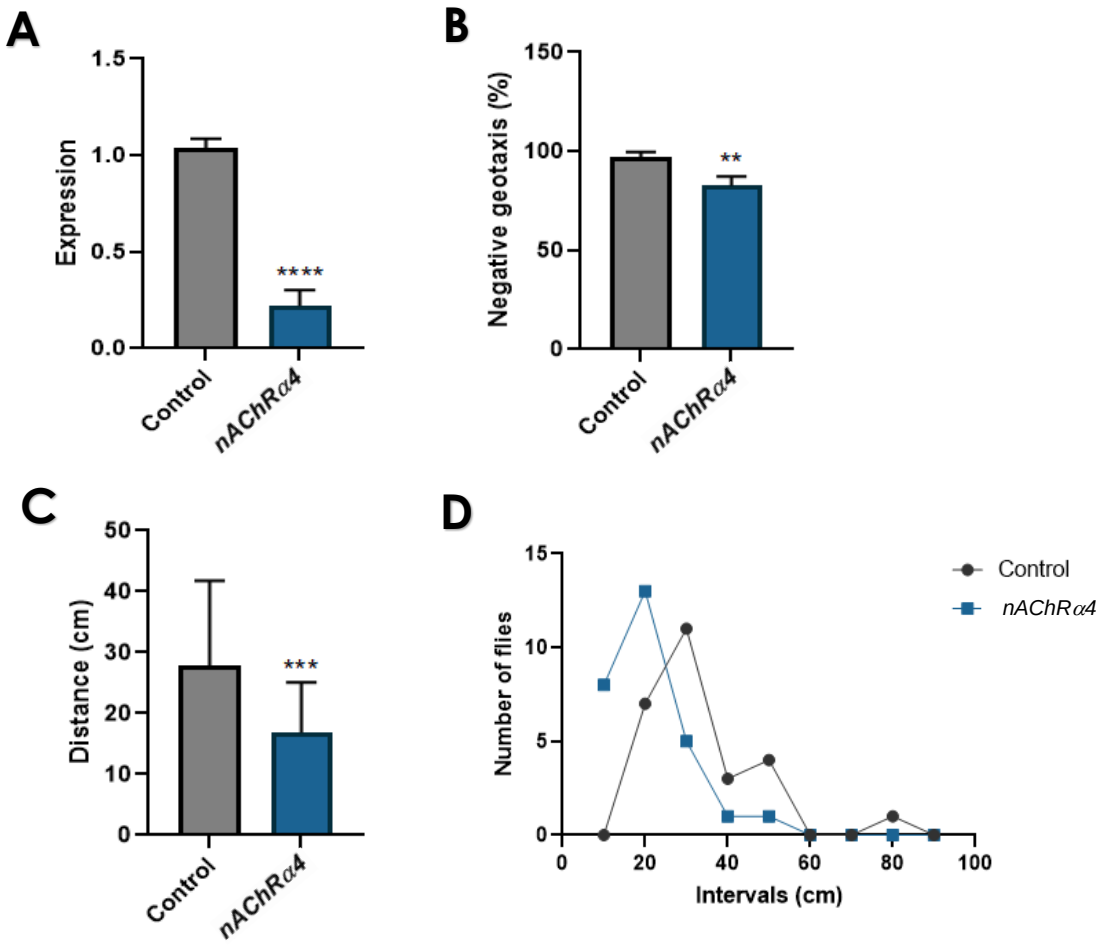

Figure 10. Effect of the nAChRα4 gene silencing. *(A) Expression of the nAChRα4 gene in the nAChRα4*^{RNAi} *fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall grouped by distance intervals. For A, B and C values represent the mean ± SEM. For A: **** p-value < 0.0001 compared to rp49 expression. For B: ** p-value < 0.01; for C: *** p-value < 0.001 and for D: no significant differences were found compared to control group. (ANOVA; for sample size, refer to materials & methods).*

4.6 *toy* GENE

Finally, we conclude with the last gene of our project. Regarding *toy* gene, significant results when compared to the control group are obtained in every assay. We already knew that the toy^{RNAi} phenotype is an enhancer of the *bss1* mutation of the *para* gene. In the RT-qPCR the *P* value obtained after the statistical analysis shows differential expression, indicating that *toy* expression has been decreased, thus regulated (Fig. 11A). This downregulation of the expression was expected due to the fundamentals of the RNAi technique. Second, the toy^{RNAi} flies' locomotion level in the negative geotaxis test has diminished when compared to the control flies (Fig. 11B). This motor affection also shows up when performing the flight assay although muscles involved are different; the *Drosophila* with the *toy* gene suppressed fall lower in the tube because they are unable to quickly produce enough thrust to stick to the cylinder walls (Fig. 11C and 11D).

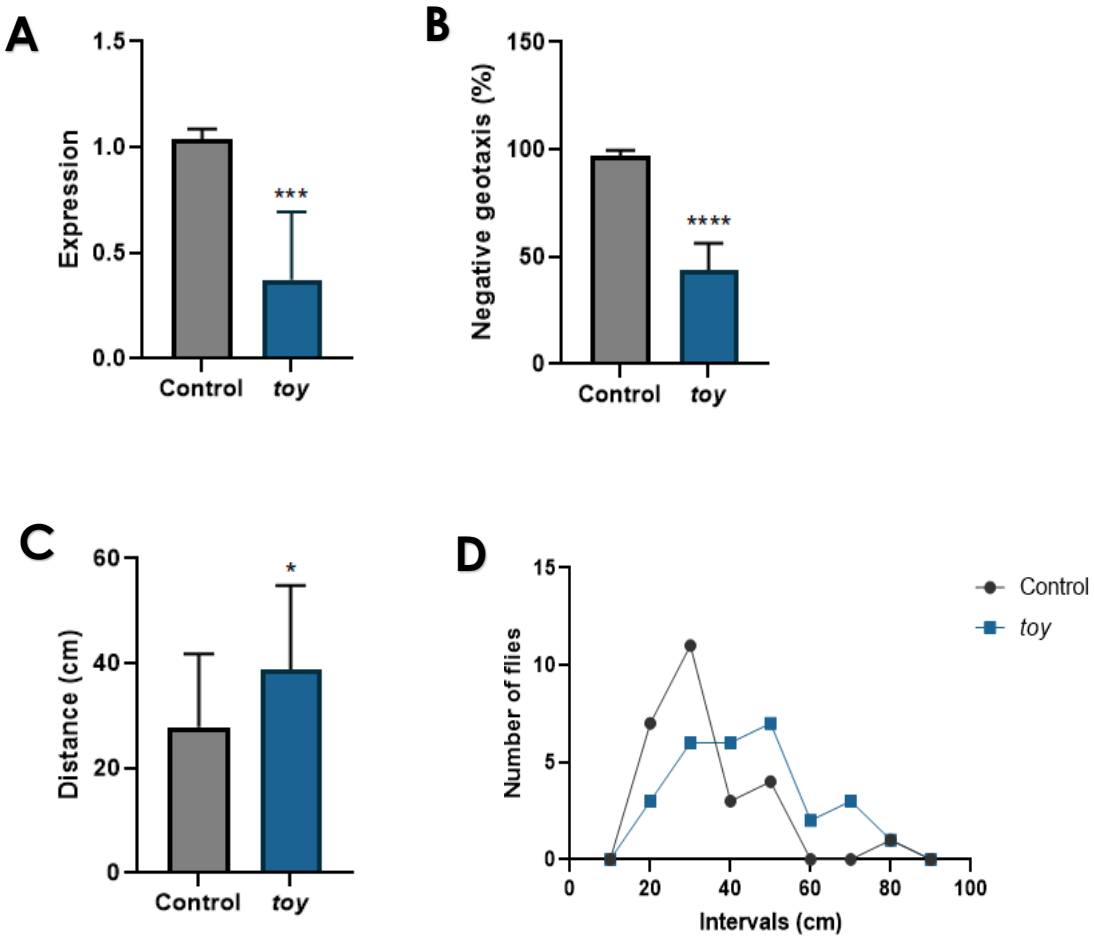

Figure 11. Effect of the toy gene silencing. *(A) Expression of the toy gene in the toy^{RNAi} fly phenotype, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. (D) Number of flies that got to the flight tester wall grouped by distance intervals. For A, B and C values represent the mean ± SEM. For A: *** p-value < 0.001 compared to rp49 expression. For B: **** p-value < 0.0001; for C: * p-value < 0.05 and for D: no significant differences were found compared to control group (ANOVA; for sample size, refer to materials & methods).*

As the previous results (Fig. 11) obtained were significant in every assay, the *toy*^{RNAi} flies were subjected to an additional locomotion test in an arena. Worsening all the results of the control group may not necessarily be a sign that we have found a modifier gene of *para*^{bss1}, but worsening all the results may be nonspecific. Hence, we needed to look for another experiment that gives us additional information. Therefore, we decided to use a locomotion test because it has multiple associated parameters that can give us information on several variables (i.e. time in movement or if this movement is erratic or not). The locomotion differences between the control group and *toy*^{RNAi} flies also are remarkable in the aforementioned arena locomotion assay. Considering the whole locomotion arena, both time in motion (Fig. 12A) and distance traveled (Fig 12B) are lower in the RNAi *Drosophila*. It was found that the *toy*^{RNAi} group spent more time in the internal ø 4.5 cm zone of the arena (Fig. 12C, Fig. 13), therefore their accumulative movement in this area was higher than the control movement (Fig. 12D).

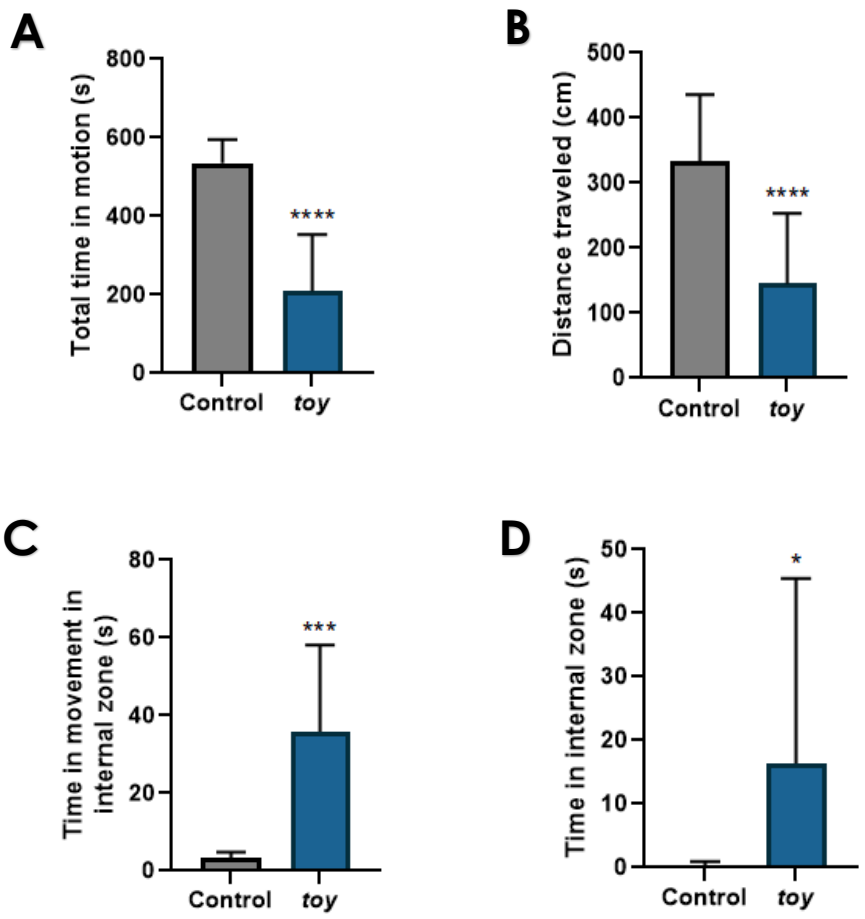

Figure 12. Effect of the toy gene silencing on locomotion. *(A) Total time spent in movement. (B) Distance traveled in the whole arena. (C) Time in movement in the internal ø 4.5 cm zone (D) Time spent by the flies in the internal ø 4.5 cm zone. Values represent the mean ± SEM. For A: **** p-value < 0.0001, for B: **** p-value < 0.0001; for C: *** p-value < 0.001 and for D: * p-value < 0.05 when compared to control group. (Mann-Whitney test; for sample size, refer to materials & methods).*

In addition to the results obtained and their statistical analysis shown on the previous page, we decided to complement it with other more visual representations (Fig. 13). For this purpose, we used the same program used for the analysis of the videos (EthoVision XT 15). Thus, we obtained a heat map of the arena used. In addition, we decided to extract a heatmap only representing the inner zone of ø 4.5 cm. This makes it much easier to identify the movement pattern of the *toy*^{RNAi} flies in front of the control. We can say that the movement of the *toy*^{RNAi} *Drosophila* is much more erratic (Fig. 13A), exploring a larger surface area of the ø 8.5 cm sand because, if we look at the Fig. 13C, we see that the control is more focused on moving around the edges of the plate rather than spending time exploring the inner surface (Fig. 13D). It is very simple that if we compare Fig. 13B and Fig. 13D we see why the difference in time inside the area (Fig. 12D) is so abysmal, and is that the control practically does not step inside. So it is also logical that its movement is less in this inner zone (Fig. 12C) because, although in all the other trials (Fig. 11, Fig. 12A and 12B) it is seen that its mobility is greater than the *toy*^{RNAi}, it almost does not enter the ø 4.5 cm area .

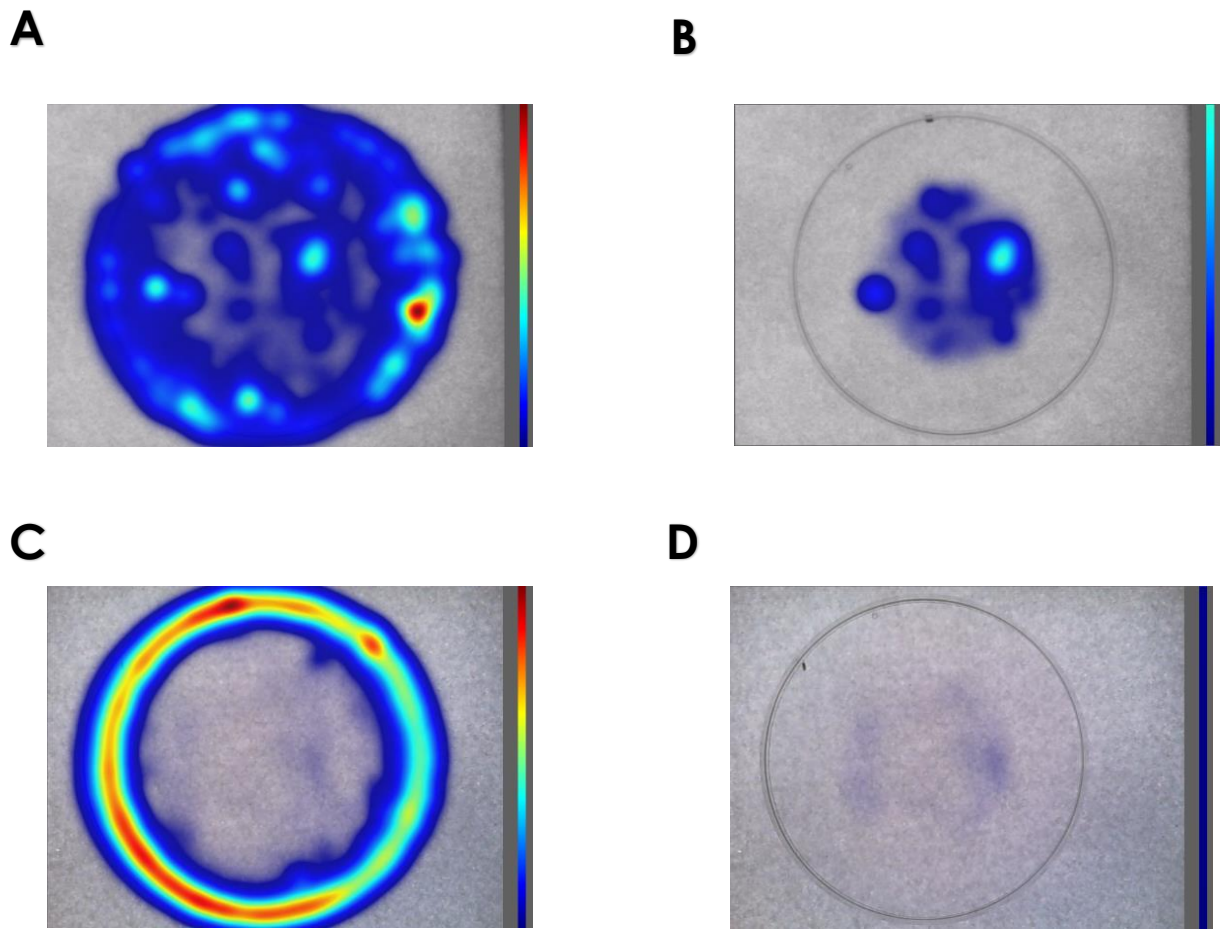

Figure 13. Locomotion arena heatmaps for the Drosophila toy^{RNAi} and control group. *(A) toy^{RNAi}: time in movement in the whole arena. (B) toy^{RNAi}: time spent in the internal ø 4.5 cm zone. (C) Control: time spent in movement in the whole arena. (D) Control: time spent in the internal ø 4.5 cm zone. Legend: from dark blue (little to no time) to dark red (considerable amount of time). Images B and D are normalized to the general scale of A and C.*

4.7 COMPILATION OF RESULTS FOR EACH GENE

Although each gene was analyzed in different individuals, a figure of all the previous results presented for each gene is shown below (Fig. 14) as a summary and to have a clear and quick visualization of the behavior of each phenotype:

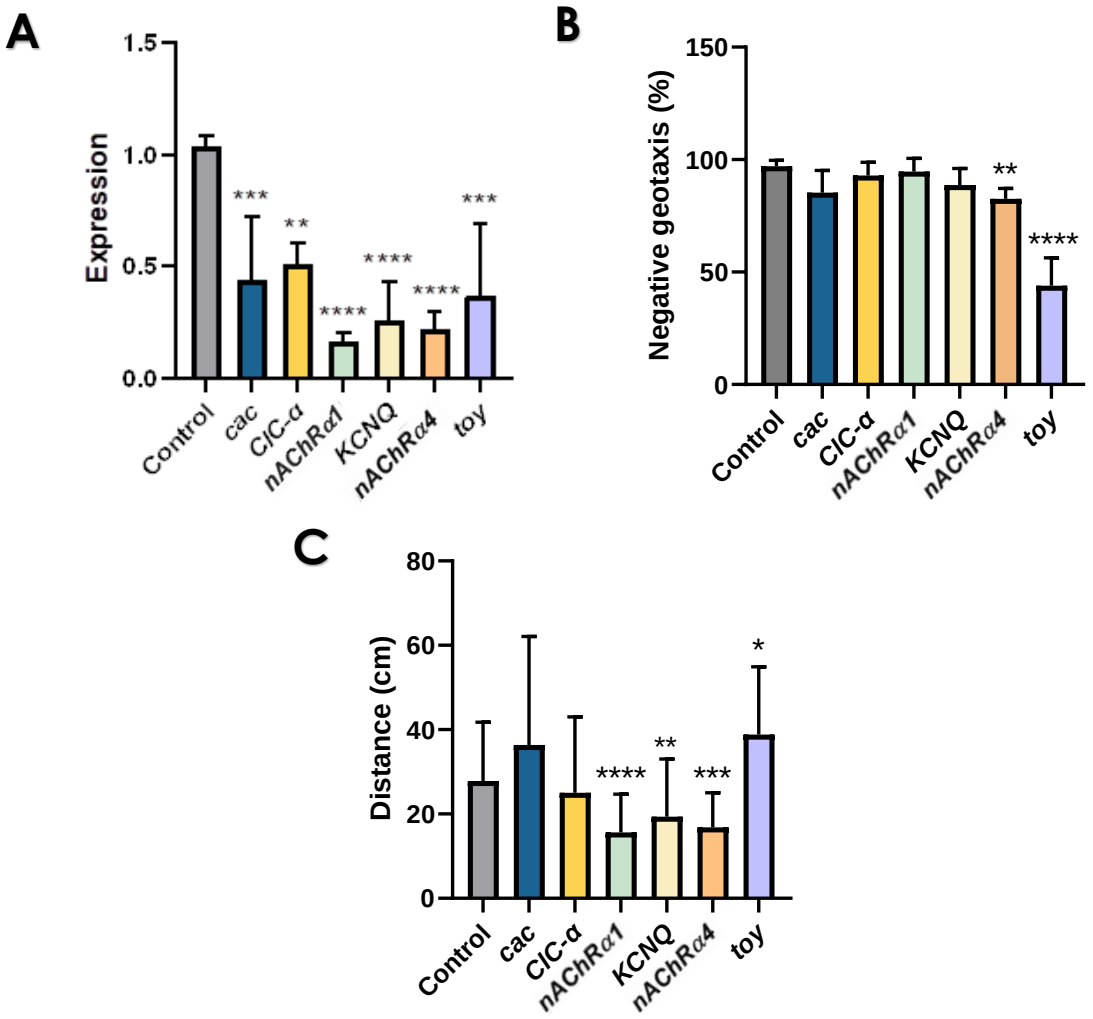

Figure 14. Effect of the silencing of the candidate modifier genes. *(A) Expression of the possible modifier genes in the different phenotypes, normalized and compared to the constitutive rp49 gene expression. (B) Percentage of flies which surpassed the 8 cm mark in the negative geotaxis assay. (C) Distance needed by the flies to produce enough thrust and get glued to the cylinder wall. For A, B and C values represent the mean ± SEM. **** p-value < 0.0001, *** p-value < 0.001, ** p-value < 0.01. * p-value < 0.05 (A, B: ANOVA. C: Krustal-Wallis; for sample size, refer to materials & methods).*

5. DISCUSSION

Dravet syndrome is a severe type of epilepsy, mostly myoclonic but with a characteristic tonic-clonic component, which begins during the first year of life and is associated with mutations in the voltage-gated sodium channel *SCN1A.* However, although the study of this gene is considered crucial for understanding the basis of the disease, it is known that in 20% of the cases tested it does not present any alterations. Furthermore, it has also been proven that, although there are patients with similar mutations in *SCN1A* that result in a similar loss of function, there may be large differences both phenotypically and in terms of clinical outcome, thus complicating an effective clinical prognosis. Therefore, as it has been established for several other genetic disorders, it is crucial to find modifier genes that influence the progression and outcome of the disease.

For this work, we have taken as a basis the projects of Hernández (2019) and Ñungo (2018), from which, through homology tests and other assays, 11 possible modifier genes of the syndrome phenotype were obtained. We selected the 6 most promising target genes according to these previous works and we regulated their expression in epileptic *para*^{bss1} *D.melanogaster* using interference RNAi and the *GAL4*/UAS system.

As we already have described in previous sections of this project, in order to ensure and validate the silencing of the target gene by means of the *GAL4*/UAS system, an RT-qPCR was performed. Afterwards, in order to characterized each phenotype by means of testing their physical aptitudes, they were subjected to negative geotaxis and flight assays. In addition, a locomotion test in an arena was designed for the *toy*^{RNAi} phenotype because, as mentioned before, the fact that this phenotype exacerbates the results of the control group in all the tests is somewhat unspecific, so this additional test was designed to obtain more information.

The first experiment carried out was RT-qPCR to check whether the selected genes were adequately silenced in the offspring of the crosses. Therefore, regarding the validation of the silencing technique, we would like to remark that the silencing of the target genes using the *GAL4*/UAS system was correctly achieved in the 6 phenotypes because the transcripts level has decreased when compare to those of the control gene *rp49*, as shown by the statistical analysis of the RT-qPCR results. Nevertheless, it is also shown that this gene silencing system involves considerable variability since not all genes have been silenced in the same proportion (Fig. 14A). In addition, it should be considered that the level of expression of certain genes may have been underestimated because they are not only present in neurons. The silencing is specifically directed to neuronal tissue, where *elav* is expressed, as we have already mentioned, so if any of the genes is also expressed, for example, in muscle tissue of the head of the fly, the RT-qPCR result would not be giving us the real level of gene repression in the brain.

Now, we will continue with the interpretation of the results of the fitness tests performed to characterize each phenotype. To note that the results vary depending on which gene has been silenced. We will first start with those genes which have a suppressor behavior regarding *para*^{bss1} phenotype. Here we have the *cac*, *KCNQ* and *nAChR*α*4* and we will proceed to discuss the results obtained for each one of them.

We will begin with *cac* which encodes the α1A subunit of the P/Q type calcium voltage-dependent channel. We already knew from the data of the other projects of our lab that the silencing of *cac* decreases the tendency to suffer seizures, working as a *para*^{bss1} suppressor. As commented its silencing in our project is correctly achieved (Fig. 14A). Otherwise, it does not present significant differences with respect to the control neither in the negative geotaxis test nor in the flight test (Fig. 14 B and C), although in this last one worsens the performance of the control flies but not enough to give a statistically significant difference. Since the error bar associated with the flight results is so high, indicating that there is a large uncertainty associated with the data, it could be hypothesized - not inferred - that *cac* silencing may function as an enhancer of the *para*^{bss1} phenotype, although this experiment would have to be repeated and supplemented with additional evidence that we will explain later.

With regards to *KCNQ*, it encodes for member number 3 of the KQT subfamily of potassium voltage-gated channels and according to our knowledge its silencing is also a suppressor of seizure predisposition in the *para*^{bss1} phenotype. Individuals with silenced *KCNQ* result in statistically significant differences in the case of the flight test, where they outperform the control group (Fig. 14C), acting as a suppressor of the *para*^{bss1} phenotype. However, the performance of the flies with this phenotype in the negative geotaxis test is very similar to that of the control flies, so although it is slightly lower as can be seen in (Fig. 14B), the difference is not such as to be considered significant.

Next, we go to the last gene that we consider a seizure suppressor, the *nAChRα4* gene. This gene encodes α4 subunit of the neuronal acetylcholine receptor. It gives significant results in all fitness tests performed in this project: negative geotaxis and flight test (Fig. 14). In the negative geotaxis test, the mean of results obtained is slightly lower than that of the control, enough to be statistically significant. However, in the flight test it is shown that this phenotype has a better physical fitness when flying than the control group. So, looking at the negative geotaxis result we could consider the silencing of this gene an enhancer of the *para*^{bss1} phenotype but if we look to the flight test, we will consider it has a suppressor effect. Thus, we hypothesize that the difference between the negative geotaxis and flight test can be given because the muscles involved in both activities are different.

Once we have discussed the crisis suppressor genes, it is time to focus on those genes that have the power to increase their frequency, i.e. that act as enhancers of the *para*^{bss1} phenotype. Among them we find *ClC-*α, *nAChRα1* and *toy*.

With respect to *ClC-*α, this is a gene that codes for protein 1 of chloride voltage-gated channel. In the case of the negative geotaxis test, it can be observed that it behaves very similarly to the control group (Fig. 14B). However, in the case of the flight test, this phenotype practically equals the results obtained by the control group (Fig. 14C). And as we have already commented the repression of this gene expression is a seizure intensifier, so our point of view is that its silencing would be a specific seizure enhancer, since in the other tests it behaves extremely similar to the control group.

Next, we find the gene *nAChRα1* which encodes the β2 subunit of neuronal acetylcholine receptor. The characterization of the *nAChRα1*^{RNAi} phenotype gives significant differences in the flight test where it acts as a suppressor of the *para*^{bss1} phenotype, but this does not happen

in the negative geotaxis test (Fig. 14B and C). The result of this last test is extremely close to the performance obtained by the control *Drosophila* (close to 100%), being the *nAChRα1*^{RNAi} flies the most similar to the control group (Fig. 14B). However, flies from both phenotypes produce enough thrust to contact the cylinder wall of the flight test earlier than the control group, showing higher physical fitness. It truly is surprisingly that the silencing of this gene that acts as a seizure intensifier and therefore as an enhancer of the *para*^{bss1} phenotype, causes the flies to be the best performer in all fitness tests (Fig. 14). Further on, we will give some reasons why we believe this is the case and what further complementary tests we could carry out.

Finally, there is the *toy* gene which encodes a transcriptional regulator involved in the embryo development of the eye, nose, central nervous system and pancreas. According to the data of our lab, silencing of this gene causes *toy*^{RNAi} phenotype to be more prone to seizures, therefore it enhances *para*^{bss1} phenotype. The most relevant aspect of this phenotype are the results of the fitness tests that have been developed to characterize it. We find that this phenotype worsens the performance of the control group in each of the tests (Fig. 14B and C). When these results were obtained both in the negative geotaxis test and in the flight test, we had to look for a different test to circumvent the possible unspecificity associated with the previous results, as I have already mentioned on several occasions. Therefore, a locomotion test was developed in an arena to which only this phenotype was subjected, thus obtaining the results shown in (Fig. 12 and 13). Then, we found that the flies with this phenotype were less mobile than those in the control group, and their behavior in exploring the space in which they were located was different; while the control flies walked around the Petri disk sticking to its plastic wall, the flies with the silenced *toy* gene explored the inner area that delimited the Petri disk and did not focus as much on walking around sticking to the plastic wall as the control flies. Due to all the above, we have come to the conclusion that the silencing of the *toy* gene is a clear enhancer of the *para*^{bss1} phenotype and that worsens the characteristics of the phenotype obtained with respect to the control group.

Below is a summary table (Table 4) showing how each phenotype behaves depending on the gene silencing that has occurred and there which would be potential candidates for *para*^{bss1} modifiers.

Table 4. Behavior of each phenotype upon the para^{bss1} phenotype according to the epileptic seizures test, negative geotaxis test and flight test. *S: suppressor of para^{bss1} phenotype; E: enhancer of para^{bss1} phenotype; - : no effect upon para^{bss1} phenotype.*

Phenotype	Crisis behavior	Negative geotaxis test	Flight test
cac^{RNAi}	S	-	-
ClC-α^{RNAi}	E	-	-
KCNQ^{RNAi}	S	-	S
nAChRα1^{RNAi}	E	-	S
nAChRα4^{RNAi}	S	E	S
toy^{RNAi}	E	E	E

As we have seen throughout this section and as summarized in Table 4, not all the results are equally conclusive in determining whether the silencing of a given gene has a suppressor or enhancer effect on the *para*^{bss1} phenotype.

We can clearly conclude that silencing of the *toy* gene functions as an enhancer of the *para*^{bss1} phenotype while *KCNQ* gene silencing has a suppressive effect on this phenotype. We could also consider suppressor the effect of the silencing upon of *nAChRα4* gene expression attending to seizures and flight test; however, it has a role as an enhancer of *para*^{bss1} in negative geotaxis. In the end, we have not been able to reach a firm conclusion about this gene, so more parallel tests should be developed in order to check its possible modifying action on the *para*^{bss1} phenotype.

Now, moving on to the more controversial gene results we have *nAChRα1, cac and ClC-*α. We will start with the latter, since it acts as an enhancer of the *para*^{bss1} phenotype only in the case of epileptic seizures and does not differ from the control in the other tests, we consider it a specific seizure enhancer modifier. The genes that give less conclusive results are *cac* and *nAChRα1* since the silencing of the former has a suppressive effect on seizures but an enhancing effect on the flight test - although it is not significant - and in the case of the latter gene the opposite results are produced: enhancing in seizures but suppressive in the flight test.

Thus, we have the following hypothesis. In humans there are several sodium channels ranging from $Na_v1.1$ to $Na_v1.9$, the tissue expression profile of each of them being different in the organism (Catterall *et al*., 2010). The point is that each is encoded by different genes, but in the case of *Drosophila* there is only one gene homologous to all of them, which is the *para* gene. Therefore, if this gene is located in GABAergic neurons, gene silencing will influence seizures, but if it is located in other neurons, the effects caused by this silencing could affect flight. In order to test this hypothesis, we have decided that it would be necessary to perform RNAi silencing in *Drosophila* individuals that do not express *para*^{bss1} to see if in the absence of the mutated gene, the flight of the tested flies is still affected.

Once we have explained why we consider or not that each gene can be a modifier of the *para*^{bss1} phenotype, we would like to comment on a couple of considerations related to the experiments and that we should take into account when interpreting the results obtained.

First, we would like to emphasize that the performance of the control phenotype in the geotaxis test was clearly close to 100% performance (Fig. 14B). Almost all flies crossed the line marked 8 cm from ground level in all repetitions. Regarding the interpretation of results, we must bear in mind that improving the result is impossible and that flies with regulated target genes can only give equal performance or cause a worsening of the results of control flies. Now, both the *toy* gene and the *nAChRα4* gene are significant in this test, worsening the performance of the control group; however, the performance of *nAChRα4*^{RNAi} flies is much better and closer to that of the control than that of Drosophila *toy*^{RNAi} (Fig. 14B).

Focusing now on the flight test, although an updated protocol by Babcock & Ganetzky (2014) was followed in which a drop tube is added to reduce the variability in handling, it is observed that the error bar of the graphs is considerably larger than in the rest of the graph of the other

tests. This indicates that the variability of the data and the uncertainty associated with a certain measure in this test is greater than in the rest, despite the addition of the drop tube.

What stands out about this project is that *D.melanogaster* has been used as a model, making use of the high homology existing between the nervous system and its genes with respect to the human system to study Dravet syndrome (Table 3). Now, we will extrapolate the interpretation of the results obtained in *Drosophila* genes to their human counterparts. So, with respect to the crisis suppressor genes, we can conclude that the silencing of *KCNQ3* appears to have a suppressor impact on the DS phenotype since the repression of its *Drosophila* homolog in *para*^{bss1} flies decreases the tendency to epileptic crisis, improves the performance of the control group in the flight test and equals it in the negative geotaxis test. Therefore, this gene could function as therapeutic target. Another gene that could function as target for drugs in DS is *PAX6* because the silencing of its homologue is the only one that is clearly postulated as an enhancer of the *para*^{bss1} phenotype. Next, the analysis of the results obtained from the silencing of the homologue of the human *CHRNA4* gene does not clarify the possible effect of this gene on the phenotype since it seems to depend on the muscle type involved, so the results obtained would have to be supplemented with additional tests. Now, the for the crisis enhancer genes, we will start with *CLCN1* which, as the silencing of its homolog only affects seizures, postulates as a crisis specific enhancer of DS. To conclude, the results of *CACNA1A* and *CHRNB2* homologs give them an ambiguous character as modifiers of certain features of the DS phenotype, so another complementary gene silencing test will have to be developed in individuals without the *para*^{bss1} mutation.

Therefore, throughout this project we have demonstrated that the *GAL4*/UAS system in *Drosophila* is an optimal procedure to achieve gene silencing despite the variability associated with it. More importantly, we have concluded that, as in other genetic and neurodegenerative diseases, modifier genes play a relevant role in the progression and outcome of Dravet syndrome. In this project, the *Drosophila* homolog genes identified that seem to have a greater relevance on the syndrome are *ClC-*α, *KCNQ* and *toy*. The specificity of *ClC-*α makes it a perfect candidate as an epileptic seizure modifier and the silencing of *KCNQ* is a tool for suppressing the *para*^{bss1} phenotype while the repression of *toy* gene expression potentiates the phenotype. Therefore, the human homologs of these genes -*CLCN1*, *KCNQ3* and *PAX6*, respectively- could be used in the future in the diagnosis and as therapeutic targets of Dravet syndrome.

6. CONCLUSSION

Dravet syndrome is a severe type of child-hood onset epilepsy associated with loss-of-function mutations in the voltage-gated sodium channel *SCN1A.* However, although the study of this gene is considered crucial for understanding the basis of the disease, there are patients with similar mutations in *SCN1A* which result in large differences both phenotypically and in terms of clinical outcome, thus complicating an effective clinical prognosis. So, as it has been established for several other genetic and neurodegenerative disorders, it is crucial to search for modifier genes that influence the diagnosis, progression, treatment and outcome of the drug-resistant syndrome.

Therefore, the main objective of this project was to identify candidate modifier genes for Dravet syndrome in a *Drosophila melanogaster* model. Hence, from the current work we can conclude that:

- *GAL4*/UAS system validation in *para*^{bss1} *Drosophila melanogaster* has been achieved.
- The gene *ClC-*α is a genetic modifier of *para* and its silencing has a specific enhancer effect on *para*^{bss1} epileptic seizures.
- The gene *KCNQ* is a genetic modifier of *para* and its silencing has a suppressive effect on the convulsive *para*^{bss1} phenotype.
- The *toy* gene is a genetic modifier of *para* and its silencing has an enhancing effect on the convulsive *para*^{bss1} phenotype.

7. REFERENCES

ALI, Y. O.; ESCALA, W.; RUAN, K. & ZHAI, R. G. (2011). Assaying locomotor, learning, and memory deficits in Drosophila models of neurodegeneration. *Journal of Visualized Experiments : JoVE*, *49*. https://doi.org/10.3791/2504

ASAKAWA, K. & KAWAKAMI, K. (2008). Targeted gene expression by the Gal4-UAS system in zebrafish. *Development, Growth \& Differentiation*, *50*(6), 391–399. https://doi.org/https://doi.org/10.1111/j.1440-169X.2008.01044.x

BABCOCK, D. T. & GANETZKY, B. (2014). An improved method for accurate and rapid measurement of flight performance in Drosophila. *Journal of Visualized Experiments : JoVE*, *84*, e51223. https://doi.org/10.3791/51223

BOUTROS, M. & AHRINGER, J. (2008). The art and design of genetic screens: RNA interference. *Nature Reviews Genetics*, *9*(7), 554–566. https://doi.org/10.1038/nrg2364

CAO, W.; SONG, L.; CHENG, J.; YI, N.; CAI, L.; HUANG, F. & HO, M. (2017). An Automated Rapid Iterative Negative Geotaxis Assay for Analyzing Adult Climbing Behavior in a Drosophila Model of Neurodegeneration. *Journal of Visualized Experiments : JoVE*, *127*. https://doi.org/10.3791/56507

CATTERALL, W. A. (2000). From Ionic Currents to Molecular Mechanisms: The Structure and Function of Voltage-Gated Sodium Channels. *Neuron*, *26*(1), 13–25. https://doi.org/https://doi.org/10.1016/S0896-6273(00)81133-2

CATTERALL, W. A.; KALUME, F. & OAKLEY, J. C. (2010). NaV1.1 channels and epilepsy. *The Journal of Physiology*, *588*(Pt 11), 1849–1859. https://doi.org/10.1113/jphysiol.2010.187484

CELNIKER, S. E. & RUBIN, G. M. (2003). The Drosophila Melanogaster Genome. *Annual Review of Genomics and Human Genetics*, *4*(1), 89–117. https://www.annualreviews.org/doi/10.1146/annurev.genom.4.070802.110323

CLAES, L.; DEL-FAVERO, J.; CEULEMANS, B.; LAGAE, L.; VAN BROECKHOVEN, C. & DE JONGHE, P. (2001). De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy. *American Journal of Human Genetics*, *68*(6), 1327–1332. https://doi.org/10.1086/320609

DE LANGE, I. M.; MULDER, F.; VAN 'T SLOT, R.; SONSMA, A. C. M.; VAN KEMPEN, M. J. A.; NIJMAN, I. J.; ERNST, R. F.; KNOERS, N. V. A. M.; BRILSTRA, E. H. & KOELEMAN, B. P. C. (2020). Modifier genes in SCN1A-related epilepsy syndromes. *Molecular Genetics \& Genomic Medicine*, *8*(4), e1103. https://doi.org/https://doi.org/10.1002/mgg3.1103

DRAVET, C. (2011). Dravet syndrome history. *Developmental Medicine and Child Neurology*, *53 Suppl 2*, 1–6. https://doi.org/10.1111/j.1469-8749.2011.03964.x

DRAVET C. Les épilepsies graves de l'enfant. Vie Med 1978; 8:543–8.

EPILEPSY ACTION (2021) *Epileptic seizures explained.* Viewed May 20th, 2021, https://www.epilepsy.org.uk/info/seizures-explained

FALCO-WALTER, J. J.; SCHEFFER, I. E. & FISHER, R. S. (2018). The new definition and classification of seizures and epilepsy. *Epilepsy Research*, *139*, 73–79. https://doi.org/https://doi.org/10.1016/j.eplepsyres.2017.11.015

FISHER, R. S.; ACEVEDO, C.; ARZIMANOGLOU, A.; BOGACZ, A.; CROSS, J. H.; ELGER, C. E.; ENGEL JR, J.; FORSGREN, L.; FRENCH, J. A.; GLYNN, M.; HESDORFFER, D. C.; LEE, B. I.; MATHERN, G. W.; MOSHÉ, S. L.; PERUCCA, E.; SCHEFFER, I. E.; TOMSON, T.; WATANABE, M. & WIEBE, S. (2014). ILAE Official Report: A practical clinical definition of epilepsy. *Epilepsia*, *55*(4), 475–482. https://doi.org/https://doi.org/10.1111/epi.12550

FISHER, R. S.; BOAS, W. VAN E.; BLUME, W.; ELGER, C.; GENTON, P.; LEE, P. & ENGEL JR.; J. (2005). Epileptic Seizures and Epilepsy: Definitions Proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia*, *46*(4), 470–472. https://doi.org/https://doi.org/10.1111/j.0013-9580.2005.66104.x

FISHER, R. S.; CROSS, J. H.; FRENCH, J. A.; HIGURASHI, N.; HIRSCH, E.; JANSEN, F. E.; LAGAE, L.; MOSHÉ, S. L.; PELTOLA, J.; ROULET PEREZ, E.; SCHEFFER, I. E. & ZUBERI, S. M. (2017). Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. *Epilepsia*, *58*(4), 522–530. https://doi.org/https://doi.org/10.1111/epi.13670

GASTAUT, H.; CAVENESS, W.F.; LANDOLT, W.; LORENTZ DE HAAS, A.M.; MCNAUGHTON, F.L.; MAGNUS, O.; MERLIS, J.K.; POND, D.A.; RADERMECKER, J. & STORM VAN LEEUWEN, W.; 1964. A proposed international classification of epileptic seizures. Epilepsia 5, 297–306.

GASTAUT, H. (1970). Clinical and Electroencephalographical Classification of Epileptic Seizures. *Epilepsia*, *11*(1), 102–112. https://doi.org/https://doi.org/10.1111/j.1528-1157.1970.tb03871.x

GUO, T.; CHUNG, J. H.; WANG, T.; MCDONALD-MCGINN, D. M.; KATES, W. R.; HAWUŁA, W.; COLEMAN, K.; ZACKAI, E.; EMANUEL, B. S. & MORROW, B. E. (2015). Histone Modifier Genes Alter Conotruncal Heart Phenotypes in 22q11.2 Deletion Syndrome. *The American Journal of Human Genetics*, *97*(6), 869–877. https://doi.org/https://doi.org/10.1016/j.ajhg.2015.10.013

HALES, K. G.; KOREY, C. A.; LARRACUENTE, A. M. & ROBERTS, D. M. (2015). Genetics on the Fly: A Primer on the Drosophila Model System. *Genetics*, *201*(3), 815–842. https://doi.org/10.1534/genetics.115.183392

HAMMER, M. F.; ISHII, A.; JOHNSTONE, L.; TCHOURBANOV, A.; LAU, B.; SPRISSLER, R.; HALLMARK, B.; ZHANG, M.; ZHOU, J.; WATKINS, J. & HIROSE, S. (2017). Rare variants of small effect size in neuronal excitability genes influence clinical outcome in Japanese cases of SCN1A truncation-positive Dravet syndrome. *PLOS ONE*, *12*(7), 1–16. https://doi.org/10.1371/journal.pone.0180485

HARVARD MEDICAL SCHOOL (2021) *in vivo fly RNAi (TRiP*). DRSC/TRiP Functional Genomics Resources & DRSC-BTRR. Viewed on May 12th, 2021, http://www.flyrnai.org/trip

HAWKINS, N. A. & KEARNEY, J. A. (2016). Hlf is a genetic modifier of epilepsy caused by voltage-gated sodium channel mutations. *Epilepsy Research*, *119*, 20–23. https://doi.org/https://doi.org/10.1016/j.eplepsyres.2015.11.016

HEDRICH, U. B. S.; LIAUTARD, C.; KIRSCHENBAUM, D.; POFAHL, M.; LAVIGNE, J.; LIU, Y.; THEISS, S.; SLOTTA, J.; ESCAYG, A.; DIHNÉ, M.; BECK, H.; MANTEGAZZA, M. & LERCHE, H. (2014). Impaired Action Potential Initiation in GABAergic Interneurons Causes Hyperexcitable Networks in an Epileptic Mouse Model Carrying a Human NaV1.1 Mutation. *Journal of Neuroscience*, *34*(45), 14874–14889. https://doi.org/10.1523/JNEUROSCI.0721-14.2014

HERNÁNDEZ, M. (2019). Estudio de genes modificadores para el Síndrome de Dravet en Drosophila melanogaster. Trabajo Fin de Grado.Universitata Politècnica de València.

HIROSE, S. (2006). A new paradigm of channelopathy in epilepsy syndromes: Intracellular trafficking abnormality of channel molecules. *Epilepsy Research*, *70*, 206–217. https://doi.org/https://doi.org/10.1016/j.eplepsyres.2005.12.007

HOWLETT, I. C.; RUSAN, Z. M.; PARKER, L. & TANOUYE, M. A. (2013). Drosophila as a Model for Intractable Epilepsy: Gilgamesh Suppresses Seizures in parabss1 Heterozygote Flies. *G3 Genes|Genomes|Genetics*, *3*(8), 1399–1407. https://doi.org/10.1534/g3.113.006130

INDIANA UNIVERSITY BLOMNINGTON. (2019) Bloomington Drosophila Stock Center (BDSC). Viewed on May 12th, 2021, http://flystocks.bio.indiana.edu/

KALUME, F.; WESTENBROEK, R. E.; CHEAH, C. S.; YU, F. H.; OAKLEY, J. C.; SCHEUER, T. & CATTERALL, W. A. (2013). Sudden unexpected death in a mouse model of Dravet syndrome. *The Journal of Clinical Investigation*, *123*(4), 1798–1808. https://doi.org/10.1172/JCI66220

KANO, S.; YUAN, M.; CARDARELLI, R. A.; MAEGAWA, G.; HIGURASHI, N.; GAVAL-CRUZ, M.; WILSON, A. M.; TRISTAN, C.; KONDO, M. A.; CHEN, Y.; KOGA, M.; OBIE, C.; ISHIZUKA, K.; SESHADRI, S.; SRIVASTAVA, R.; KATO, T. A.; HORIUCHI, Y.; SEDLAK, T. W.; LEE, Y.; RAPOPORT, J.L, HIROSE, S.; OKANO, H.; VALLE, D.; O'DONNELL, P.; SAWA, A. & KAI, M. (2015). Clinical utility of neuronal cells directly converted from fibroblasts of patients for neuropsychiatric disorders: studies of lysosomal storage diseases and channelopathy. *Current Molecular Medicine*, *15*(2), 138–145. https://doi.org/10.2174/1566524015666150303110300

KEARNEY, J. (2013). Sudden unexpected death in dravet syndrome. *Epilepsy Currents*, *13*(6), 264–265. https://doi.org/10.5698/1535-7597-13.6.264

KEARNEY, J. A. (2011). Genetic modifiers of neurological disease. *Current Opinion in Genetics & Development*, *21*(3), 349–353. https://doi.org/10.1016/j.gde.2010.12.007

KEARNEY, J. A.; & MEISLER, M. (2009). Genetics: Single Gene Mutations in Inherited and Sporadic Epilepsy. In *Encyclopedia of Basic Epilepsy Research* (pp. 369-374). Elsevier Inc. https://doi.org/10.1016/B978-012373961-2.00127-2

KOUSHIKA SP.; LISBIN MJ. & WHITE K. (1996) *ELAV*, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. *Current Biology*, 1;6(12):1634-41. https://doi.org/10.1016/s0960-9822(02)70787-2.

LIU, H.; HAN, M.; LI, Q.; ZHANG, X.; WANG, W.-A. & HUANG, F.-D. (2015). Automated rapid iterative negative geotaxis assay and its use in a genetic screen for modifiers of Aβ(42)-induced locomotor decline in Drosophila. *Neuroscience Bulletin*, *31*(5), 541–549. https://doi.org/10.1007/s12264-014-1526-0

LOUGHNEY, K.; KREBER, R. & GANETZKY, B. (1989). Molecular analysis of the para locus, a sodium channel gene in Drosophila. *Cell*, *58*(6), 1143–1154. https://doi.org/https://doi.org/10.1016/0092-8674(89)90512-6

MILLER, A. R.; HAWKINS, N. A.; MCCOLLOM, C. E. & KEARNEY, J. A. (2014). Mapping genetic modifiers of survival in a mouse model of Dravet syndrome. *Genes, Brain and Behavior*, *13*(2), 163–172. https://doi.org/https://doi.org/10.1111/gbb.12099

MOHR, S. E. (2014). RNAi screening in Drosophila cells and in vivo. *Methods (San Diego, Calif.)*, *68*(1), 82–88. https://doi.org/10.1016/j.ymeth.2014.02.018

MOSHÉ, S. L.; PERUCCA, E.; RYVLIN, P. & TOMSON, T. (2015). Epilepsy: new advances. *The Lancet*, *385*(9971), 884–898. https://doi.org/https://doi.org/10.1016/S0140-6736(14)60456-6

NASHEF, L.; SO, E. L.; RYVLIN, P. & TOMSON, T. (2012). Unifying the definitions of sudden unexpected death in epilepsy. *Epilepsia*, *53*(2), 227–233. https://doi.org/10.1111/j.1528-1167.2011.03358.x

NATIONAL INSTITUTE OF GENETICS (2021). *Fly Stocks of National Institute of Genetics (NIG)-Fly*. Viewed on May 12th, 2021, http://www.shigen.nig.ac.jp/fly/nigfly/

ÑUNGO, N.C. (2018). Búsqueda de modificadores de susceptibilidad a crisis epilépticas en un modelo de Síndrome de Dravet en Drosophila melanogaster con la mutación parabss1. Trabajo de Fin de Máster en Biotecnología biomédica. Universitat Politècnica de València.

PAN, X.; LI, Z.; JIN, X.; ZHAO, Y.; HUANG, G.; HUANG, X.; , SHEN, Z.; CAO, Y.; DONG M.; LEI, J. & YAN, N. (2021). Comparative structural analysis of human Nav1.1 and Nav1.5reveals mutational hotspots for sodium channelopathies. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(11). https://doi.org/10.1073/PNAS.2100066118

PARKER, L.; HOWLETT, I. C.; RUSAN, Z. M. & TANOUYE, M. A. (2011). Seizure and epilepsy: studies of seizure disorders in Drosophila. *International Review of Neurobiology*, *99*, 1–21. https://doi.org/10.1016/B978-0-12-387003-2.00001-X

PEARSON-SMITH, J. N. & PATEL, M. (2017). Metabolic Dysfunction and Oxidative Stress in Epilepsy. *International Journal of Molecular Sciences*, *18*(11). https://doi.org/10.3390/ijms18112365

PERRIMON, N.; NI, J.-Q. & PERKINS, L. (2010). In vivo RNAi: today and tomorrow. *Cold Spring Harbor Perspectives in Biology*, *2*(8), a003640. https://doi.org/10.1101/cshperspect.a003640

RAGSDALE, D. S. (2008). How do mutant Nav1.1 sodium channels cause epilepsy? *Brain Research Reviews*, *58*(1), 149–159. https://doi.org/10.1016/j.brainresrev.2008.01.003

RANA, A. & MUSTO, A. E. (2018). The role of inflammation in the development of epilepsy. *Journal of Neuroinflammation*, *15*(1), 144. https://doi.org/10.1186/s12974-018-1192-7

SCHEFFER, I. E. & NABBOUT, R. (2019). SCN1A-related phenotypes: Epilepsy and beyond. *Epilepsia*, *60*(S3), S17–S24. https://doi.org/https://doi.org/10.1111/epi.16386

SHNEKER, B. F. & FOUNTAIN, N. B. (2003). Epilepsy. *Disease-a-Month*, *49*(7), 426–478. https://doi.org/10.1016/S0011-5029(03)00065-8

STEEL, D.; SYMONDS, J. D.; ZUBERI, S. M. & BRUNKLAUS, A. (2017). Dravet syndrome and its mimics: Beyond SCN1A. *Epilepsia*, *58*(11), 1807–1816. https://doi.org/https://doi.org/10.1111/epi.13889

STONE, B.; BURKE, B.; PATHAKAMURI, J.; COLEMAN, J. & KUEBLER, D. (2014). A low-cost method for analyzing seizure-like activity and movement in Drosophila. *Journal of Visualized Experiments : JoVE*, *84*, e51460. https://doi.org/10.3791/51460

TRAVEN, A.; JELICIC, B. & SOPTA, M. (2006). Yeast Gal4: a transcriptional paradigm revisited. *EMBO Reports*, *7*(5), 496–499. https://doi.org/10.1038/sj.embor.7400679

UGUR, B.; CHEN, K. & BELLEN, H. J. (2016). Drosophila tools and assays for the study of human diseases. *Disease Models & Mechanisms*, *9*(3), 235–244. https://doi.org/10.1242/dmm.023762

UNIPROT (2021) UniProt Consortium European Bioinformatics Institute. Protein Information Resource. SIB Swiss Institute of Bioinformatics. Viewed on April 10th, 2021 https://www.uniprot.org/

VÉLEZ, J. I.; LOPERA, F.; SEPULVEDA-FALLA, D.; PATEL, H. R.; JOHAR, A. S.; CHUAH, A.; TOBÓN, C.; RIVERA, D.; VILLEGAS, A.; CAI, Y.; PENG, K.; ARKELL, R.; CASTELLANOS, F. X.; ANDREWS, S. J.; SILVA LARA, M. F.; CREAGH, P. K.; EASTEAL, S.; DE LEON, J.; WONG, M. L.; LICINIO, J.; MASTRONARDI, C A. & ARCOS-BURGOS, M. (2016). APOE*E2 allele delays age of onset in PSEN1 E280A Alzheimer's disease. *Molecular Psychiatry*, *21*(7), 916–924. https://doi.org/10.1038/mp.2015.177

VIENNA BIOCENTER CORE FACILITIES (2021) *Vienna Drosophila Resource Center (VDRC)*. Viewed on May 12th, 2021, https://stockcenter.vdrc.at/control/main

YAO, K.-M. & WHITE, K. (1994), Neural Specificity of *elav* Expression: Defining a *Drosophila* Promoter for Directing Expression to the Nervous System. Journal of Neurochemistry, 63: 41-51. https://doi.org/10.1046/j.1471-4159.1994.63010041.x

YU, F. H. & CATTERALL, W. A. (2003). Overview of the voltage-gated sodium channel family. *Genome Biology*, *4*(3), 207. https://doi.org/10.1186/gb-2003-4-3-207

YU, F. H.; MANTEGAZZA, M.; WESTENBROEK, R. E.; ROBBINS, C. A.; KALUME, F.; BURTON, K. A.; SPAIN, W. J.; MCKNIGHT, G. S.; SCHEUER, T. & CATTERALL, W. A. (2006). Reduced sodium current in GABAergic interneurons in a mouse model of severe myoclonic epilepsy in infancy. *Nature Neuroscience*, *9*(9), 1142–1149. https://doi.org/10.1038/nn1754

ZHANG, J. D.; RUSCHHAUPT, M. & BICZOK, R. (2021). ddCt method for qRT – PCR data analysis. *Bioconductor*, 1–8. https://doi.org/10.18129/B9.bioc.ddCt

8. ANNEX

8.1 DETAILED COLLECTION OF *Drosophila melanogaster*

The *Drosophila melanogaster* crosses necessary to obtain the six phenotypes, plus the control, that have been worked with throughout this project are detailed below.

Table 5. Drosophila melanogaster phenotype, genotype and cross performed to obtain the aforementioned traits. ♀: *virgin females.* ♂: *males.*

Phenotype obtained	Cross performed (Parental)	Genotype (F1)
cac^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ cac}{RNAi\ cac}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ cac}{+}$
ClC-α^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ ClC-\alpha}{RNAi\ ClC-\alpha}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ ClC-\alpha}{+}$
KCNQ^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ KCNQ}{RNAi\ KCNQ}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ KCNQ}{+}$
nAChRα1^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ nAChR\alpha1}{RNAi\ nAChR\alpha1}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ nAChR\alpha1}{+}$
nAChRα4^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ nAChR\alpha4}{RNAi\ nAChR\alpha4}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ nAChR\alpha4}{+}$
toy^{RNAi}	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNAi\ toy}{RNAi\ toy}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi\ toy}{+}$
Control	♀ $\frac{para^{bss1}}{para^{bss1}};\frac{elav}{CyO};\frac{+}{+}$ × ♂ $\frac{+}{Y};\frac{+}{+};\frac{RNA}{RNAi}$	♂ $\frac{para^{bss1}}{Y};\frac{elav}{+};\frac{RNAi}{+}$

8.2 CULTURE MEDIUM

The method of preparing 2 L of food or culture medium for the flies is as follows. First, prepare a mixture with the following ingredients: 20 g agar, 20 g soybean meal, 120 g corn flour and 100 g sugar and heat in the microwave 1,2 L of distilled water 5 min at 800 W. Add the mixture prepared and 60 g of dry yeast to the heated water and homogenize it with the mixer. Afterwards, autoclave the homogenized mixture to sterilize it and add 800 mL of distilled water heated in the microwave 5 min at 800 W. Next, add 6 g of Nipagin (methyl 4-hydroxybenzoate, antibacterial) dissolved in 20 mL of 96° ethanol and 10 mL propionic acid (antifungal). Now it is important to homogenize the autoclaved mixture, hot water, antifungal and antibacterial with

the blender. Dispense the culture medium into the tubes, 10 mL in each, with the Masterflex L/S Easy-Load III machine (Cole-Parmer; Vernon Hills, Illinois, Illinois, USA). Cover the tubes with a small sheet and let stand overnight at room temperature. The next morning cover the tubes with special anti-mite cellulose acetate caps and store them at 4°C for better preservation.

8.3 SEQUENCE OF THE PRIMERS USED

Table 6. Relation of the genes and the primer sequence used for the RT-qPCR. *RT-qPCR: retro transcriptase-quantitative polymerase chain reaction.*

Gene	Forward primer	Reverse primer
cac	TGACTGGATCACACAAGCAG	CATTATGTGCATTTTCTCTTCTTCC
ClC-α	CGAGATCGAGGCGTTCTACT	CGTCGCTTTTCGAGTATTTTG
KCNQ	CGTCCGTGGAGAATCTGG	TATGAACCTGATGGCGGTCT
nAChRα1	CGTTTCAGCGGCGACTAC	AGCCAAATCGAAGCGATG
nAChRα4	AAAAGAAGATTTATCGCAAAGCA	CTGGGCCATGAACCTGAC
toy	AACGGTTGCGTAAGCAAAAT	TCGAGGTTTTATCGATCCAGTT
rp49	CGTTTACTGCGGCGAGAT	GCGCTCGACAATCTCCTT