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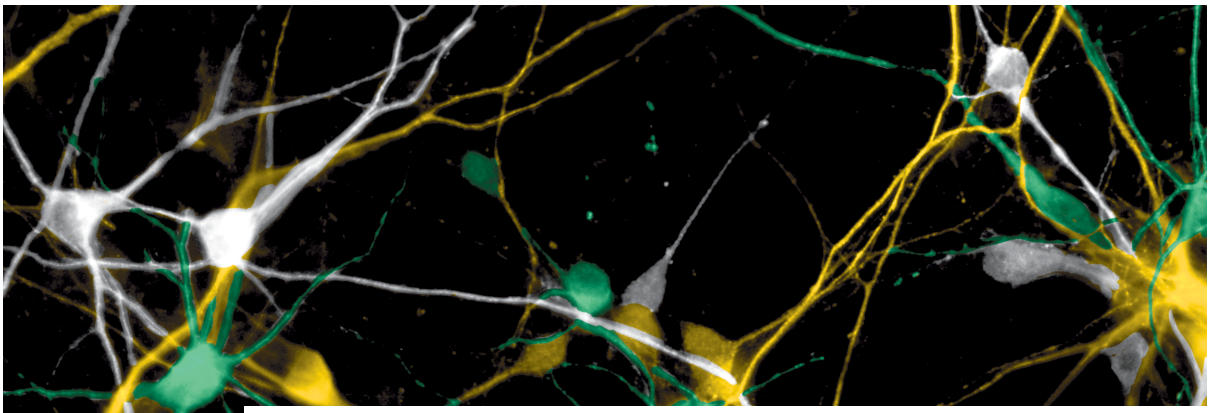
2022

Document Version:
Publisher's PDF, also known as Version of record



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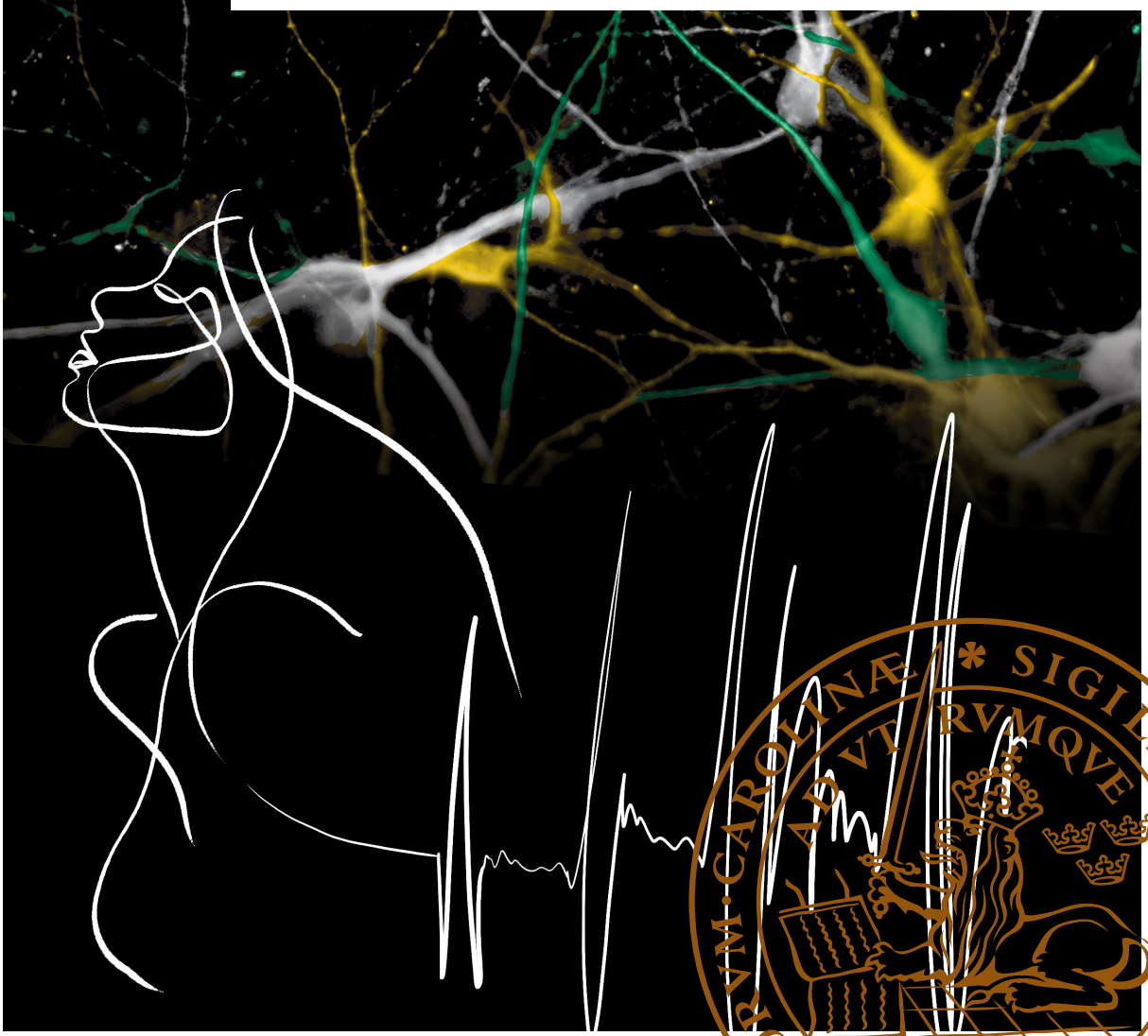

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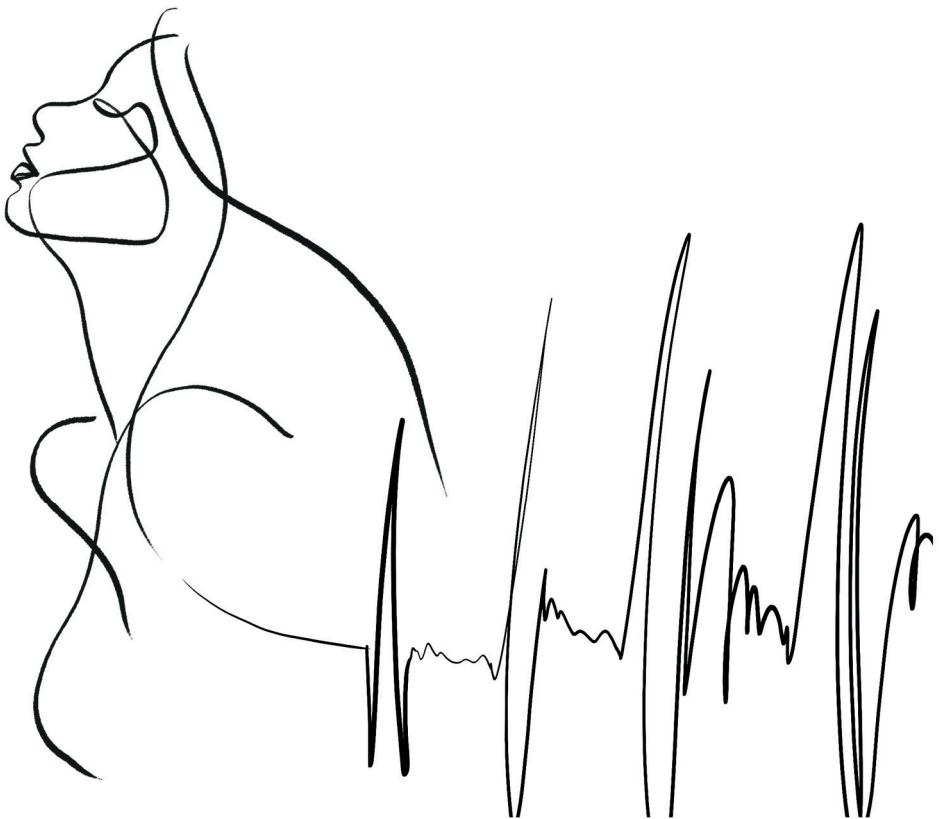

Enhancing neuronal inhibition by cell and gene therapy as a novel treatment for Epilepsy

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Segerfalksalen, BMC A10,
on Friday 8th of April 2022 at 13:15 h.

Faculty opponent

Professor **Jack Parent**, MD, PhD
University of Michigan Medical School, the United States of America

Organization LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences, Lund Epilepsy Center	**Document name** DOCTORAL DISSERTATION **Date of issue** 8th of April 2022
Author Ana Gonzalez Ramos	Sponsoring organization

Title and subtitle
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Abstract

Epilepsy is a family of heterogeneous and multifactorial neurological disorders, unified by the occurrence of spontaneous recurrent seizures. Overall, it affects 50 million people worldwide of all ages and genders. Available treatments are only symptomatic and have severe side effects, while they also fail to provide adequate seizure control in a third of the patients. Therefore, a cure is yet to be found. In this warrant for novel strategies to treat those refractory patients, this thesis evolved. The two approaches presented here based their goal on increasing inhibitory drive in the epileptic focus to reduce the pathological hyperexcitable neuronal network that characterizes epilepsy, and thus counteract seizures.

First, based on evidence of loss and/or alteration of gamma-aminobutyric acid (GABA)-ergic interneurons in the epileptic neuronal network, cell-based therapy has been developed and tested in three different scenarios for restoration of the excitatory/inhibitory balance. GABAergic interneurons (hdINs) were generated *in vitro* from human embryonic stem cells and proved to survive and integrate into both human and rodent epileptic environments. Host neuronal activity could be modulated by light-activation of hdINs using optogenetics. Finally, grafted hdINs were able to reduce the seizure frequency and total time spent in seizures in a rat model of temporal lobe epilepsy (TLE), the most common form of refractory epilepsy in adults. However, grafted hdINs failed to improve the pathology in a genetic mouse model of cortical dysplasia-focal epilepsy syndrome associated with autism spectrum disorder, which highlights the diversity of epilepsies and the need for gaining a better understanding of the mechanisms underpinning the disease.

Finally, direct inhibition of principal cells, similar to the one exerted by endogenous inhibitory interneurons, in the chronic epileptic hippocampus by using a chemogenetic approach delivered as gene therapy was also tested. Positive results were observed by decreasing ability to generate action potentials, although further investigation is required to evaluate the efficacy of this approach on seizures.

Collectively, the work presented here has moved the field forward in testing two different therapeutic strategies in a TLE model, and also one of them in a genetic epilepsy model.

Keywords
Epilepsy, interneurons, GABA, human pluripotent stem cells, cell therapy, gene therapy,chemogenetics, hippocampus, TLE, Cntnap2

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN** 978-91-8021-205-2
Recipient's notes	**Number of pages** 153	Price
	Security classification	

Signature  Date 2022-03-03

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Academic Dissertation
Lund 2022

Cover artwork by Jordi Rodó Morera and Ana Gonzalez Ramos

Faculty of Medicine
Department of Clinical Sciences, Lund

ISBN 978-91-8021-205-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2022

To my family and friends,
the stars that have walked through this journey with me.

‘I will love the light for it shows me the way,
yet I will endure the darkness for it shows me the stars.’
— *Og Mandino*

TABLE OF CONTENTS

LAY SUMMARY

The brain is the most unknown organ in the body, which might be caused by its remarkable complexity but also due to its limited accessibility for study. Nonetheless, it is known that the brain is composed of hundreds of billions of cells called neurons that connect to each other and send signals with information. Although all neurons work similarly, there are thousands of different kinds of neurons, with different shapes, molecular compositions, functions, and locations within the brain. They form a giant electrical network and are responsible for all the sensations, feelings, decisions, and actions that we make every day. Looking further into the complexity of this electrical network, other cell types help neurons to perform their function by cleaning the environment, giving support, and distributing factors that facilitate their survival. When focusing on this electrical network, neurons act as an electric device, so that each of them receives input from thousands of other neurons and at the same time it generates its outputs to other neurons. Inputs and outputs are usually chemical compounds called neurotransmitters, although the processing of them and the decision to send them is made electrically. That is, if the electric charge inside the neuron changes enough after receiving the inputs, it will send an output.

In epilepsy, this electrical network is altered giving rise to a hyperexcitable state in an area of the brain. Multiple reasons could lead to this alteration, as well as there are various manifestations depending on the area of the brain that is affected. For this reason, epilepsy is considered a family of heterogeneous and multifactorial disorders with the common hallmark of suffering from unprovoked seizures. A seizure could be understood as an excessive and synchronized activation of neurons that send signals at the same time, which may have functional manifestation. Importantly, epilepsy is a common neurological disorder since it affects one out of hundred people, so it sums up to 50 million people worldwide and includes patients of all ages and genders. Owing to the complexity of the disease a cure is yet to be found. Available medications, called anti-seizure medication, treat only the symptoms and may have unwanted side effects on mood, cognition, and memory. Moreover, anti-seizure medications are not effective in one-third of the patients, leaving them with uncontrolled seizures along with derived comorbidities and poor quality of life. An alternative treatment option is surgical resection of the brain region where seizures originate, although, in reality, it is only a feasible option for very few patients with 30-40 % of chances to not achieve seizure freedom. Hence,

there is an urge for improving our understanding, and developing new therapeutic approaches for refractory epilepsies.

Even though the diverse underlying processes underpinning the disease remain not fully understood, it is known that there is a decrease or alteration in inhibition affecting the whole network excitability. One could think of it as the fuses of the electric circuit of a house, which trip when the current is too high stopping the bulbs and protecting the electrical installation. In epilepsy, fuses do not trip by neuronal hyperexcitability, leading to seizures. In this line, new avenues aim to restore inhibition, so that it decreases hyperexcitability of the network and stops the generation of seizures. Due to the restricted inherent capacity to regenerate the brain neurons, an external source of inhibitory neurons could be used for this purpose, an approach called cell therapy. In this thesis (*Papers I and II*), inhibitory neurons were generated from human stem cells and proved to integrate into the brain electrical network, so that seizures were reduced in number and duration in a rodent model of the most common adult refractory epilepsy. The same approach was used in a model of pediatric refractory epilepsy associated with autism (*Paper III*), although with contrary results. Altogether, indicating the suitability of inhibitory cell therapy for certain epilepsies, and the need to gain a better understanding of others.

The work presented here also explored an alternative strategy, known as gene therapy, to increase inhibition in the epileptic neuronal network. This approach consists of the introduction of specific genetic information, a gene, in a desired neuronal population. In particular (*Paper IV*), the gene was used to produce a protein in the neuron that would act as a switch and could be controlled externally by a drug, a tool called chemogenetics. Thereby, gene therapy was directed to excitatory neurons in the epileptic region, so that we could inhibit their activity externally. Chemogenetics reduced the response to excitatory inputs in functional experiments outside the living animal, although further testing of the effect on seizures in living animals is desired.

In summary, this thesis confirms the suitability of inhibitory cell therapy for certain epilepsies and lays a foundation for further studies of using chemogenetics as an alternative avenue.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Hjärnan är det mest outforskade organet i kroppen, vilket kan bero på dess otroliga komplexitet men även på grund av dess begränsade tillgänglighet för studier. Likväl är det känt att hjärnan är sammansatt av hundratals miljarder celler som kallas neuroner som ansluter till varandra och skickar signaler med information. Även om neuroner fungerar på liknande sätt, finns det tusentals olika typer av neuroner, med olika former, molekyler, funktioner som återfinns i olika delar av hjärnan. De bildar ett gigantiskt elektriskt nätverk och är ansvariga för alla sinnesintryck, känslor, beslut och handlingar som vi utför varje dag. Om man tittar närmare på komplexiteten i detta elektriska nätverk, hjälper andra celltyper neuroner att utföra sin funktion genom att rengöra miljön, ge stöd och fördela faktorer som hjälper dem att överleva. När man fokuserar på detta elektriska nätverk fungerar neuroner som en elektrisk apparat, så att var och en av dem får ingående signaler från tusentals andra neuroner och samtidigt genererar sina egna utgående signaler till andra neuroner. In- och utgående signaler är vanligtvis kemiska föreningar som kallas neurotransmittorer, även om bearbetningen av dem och beslutet att skicka dem sker elektriskt. Det vill säga, om den elektriska laddningen inuti neuronen ändras tillräckligt efter att ha tagit emot signaler kommer den att skicka ut en signal.

Vid epilepsi förändras detta elektriska nätverk så att det uppstår ett överaktiverat tillstånd i ett område av hjärnan. Flera faktorer kan leda till denna förändring, såväl som en mängd olika uttryck beroende på vilket område av hjärnan som påverkas. Av denna anledning anses epilepsi vara en familj av heterogena och multifaktoriella sjukdomar med det gemensamma kännetecknet att av oprovocerade epileptiska anfall. Ett epileptiskt anfall kan förstås som en ökad och synkroniserad aktivering av nervceller som skickar signaler samtidigt, som kan uttrycka sig funktionellt. Nog så viktigt är att epilepsi är en vanlig neurologisk störning eftersom den drabbar en av hundra människor, det vill säga upp till 50 miljoner människor världen över och inkluderar patienter i alla åldrar och kön. På grund av sjukdomens komplexitet har man ännu inte hittat ett botemedel. Tillgänglig medicin som anti-epileptiska läkemedel behandlar endast symtomen och kan ha oönskade biverkningar på humör, kognition och minne. Dessutom är läkemedel mot epileptiska anfall inte effektiva hos en tredjedel av patienterna, vilket lämnar dem med okontrollerade epileptiska anfall tillsammans med samsjukligheter och nedsatt livskvalitet. Ett behandlingsalternativ är kirurgisk resektion av den hjärnregion där anfallen uppstår, även om det i verkligheten bara är ett möjligt alternativ för väldigt få patienter med 30-40 % chans att inte uppnå anfallsfrihet. Därför finns det ett behov av att förbättra

vår förståelse och att utveckla nya terapeutiska metoder för läkemedelsresistenta epilepsier.

Även om de olika underliggande processerna som ligger till grund för sjukdomen ännu inte är helt förstådda, är det känt att det finns en minskning eller förändring i inhibition som påverkar hela nätverkets aktivitet. Man skulle kunna förställa sig det som glödlamporna i ett hus på olika timers. Dessa timers startar och stoppar glödlamporna från att blinka när elektriciteten till dem är för hög och utan dem skulle hela huset blinka. I linje med detta kan nya sätt för att behandla läkemedelsresistent epilepsi sikta på att återställa den minskade inhibitionen, så att överaktiviteten i nätverket minskar och stoppar genereringen av anfall. På grund av hjärnans begränsade förmåga att regenerera, kan en extern källa av inhibitoriska neuroner användas för detta ändamål, ett tillvägagångssätt som kallas cellterapi. I denna avhandling (*Artikel I och II*) genererades inhibitoriska neuroner från mänskliga stamceller i labbet och visade integration i hjärnans elektriska nätverk, så att anfall minskade i antal och varaktighet i en gnagarmodell av den vanligaste typen av läkemedelsresistent epilepsi i vuxna. Samma tillvägagångssätt användes i en modell av pediatrisk läkemedelsresistent epilepsi förknippad med autism (*Artikel III*), men med motsatta resultat. Tillsammans indikerar det här lämpligheten av inhibitorisk cellterapi för vissa epilepsier, och behovet av att få en bättre förståelse för andra.

I arbetet som presenteras här undersöktes även en alternativ strategi känd som genterapi för att öka inhibition i det epileptiska neuronala nätverket. Detta tillvägagångssätt består av införandet av specifik information, en gen, i en önskad neuronpopulation. I synnerhet (*Artikel IV*) innehöll genen som användes information för att producera ett protein i neuronen som skulle fungera som en strömbrytare som kunde styras externt av ett läkemedel, ett verktyg som kallas kemogenetik. På så sätt riktades genterapi mot excitatoriska neuroner i den epileptiska regionen, så att vi kunde kontrollera deras aktivitet externt. Kemogenetik minskade responsen på excitatoriska inputs i funktionella experiment utanför det levande djuret men ytterligare undersökningar av effekten på anfall önskas.

Sammanfattningsvis bekräftar denna avhandling lämpligheten av inhibitorisk cellterapi för vissa epilepsier och uppmuntrar ytterligare studier av det kombinatoriska tillvägagångssättet för kemogenetik och genterapi som en alternativ väg.

RESUMEN EN ESPAÑOL

El cerebro es el órgano más desconocido del cuerpo humano, esto puede ser debido a su considerable complejidad, pero también a su limitada accesibilidad para estudiarlo. No obstante, se sabe que el cerebro está compuesto por cientos de billones de células llamadas neuronas que conectan las unas con las otras y envían señales con información. A pesar de que las neuronas funcionan de forma similar, hay mil clases diferentes de ellas con distintas formas, moléculas, funciones y localización en el cerebro. Las neuronas forman una red eléctrica gigante que es responsable de las sensaciones, sentimientos, decisiones y acciones que tomamos cada día. Si observamos con detalle la complejidad de esta red eléctrica, veremos que hay otras células que ayudan a las neuronas a hacer su función limpiando el ambiente, dando soporte estructural, y distribuyendo factores que ayudan a las neuronas a sobrevivir. Si nos centramos sólo en la red eléctrica, las neuronas actúan como un dispositivo eléctrico en el que cada uno recibe señales de otros miles de neuronas y a su vez genera respuestas que envía como señales a otras neuronas. Las señales de llegada y de respuesta acostumbran a ser compuestos químicos llamados neurotransmisores, aunque su procesamiento y la decisión de enviarlos se hace de forma eléctrica. Es decir, si la carga eléctrica en el interior de la neurona cambia lo suficiente después de recibir señales, esta enviará una respuesta.

En la epilepsia, esta red eléctrica se encuentra alterada, de forma que existe un estado de sobreexcitación en una zona del cerebro. Hay muchas razones que pueden llevar a esta alteración, a la vez hay muchas manifestaciones que pueden derivar de ella según la región del cerebro afectada. Por esta razón, la epilepsia se considera como una familia de enfermedades heterogéneas y multifactoriales que comparten el distintivo común de sufrir ataques epilépticos de manera no provocada. Un ataque epiléptico se puede entender como una excesiva y sincronizada activación de neuronas que envían señales a la vez, pudiendo causar manifestaciones funcionales. Esto es importante ya que la epilepsia es una enfermedad común del sistema nervioso que afecta a una de cada cien personas, sumando 50 millones de personas en el mundo, e incluye pacientes de todas las edades y géneros. Debido a la complejidad de la enfermedad, aún no se ha encontrado una cura. Los medicamentos disponibles, llamados anticonvulsivos, sólo tratan los síntomas y pueden tener efectos adversos en el humor, facultades cognitivas y la memoria. Además, estos medicamentos no son efectivos para un tercio de los pacientes, creándoles una dura realidad, con ataques epilépticos incontrolados, otras afectaciones asociadas y una baja calidad de vida. Una alternativa terapéutica es la cirugía resectiva de la región

del cerebro que causa los ataques, aunque en realidad sólo es una opción factible para muy pocos pacientes y con sólo un 30-40 % de probabilidades de éxito. Por ello, hay una necesidad de mejorar nuestro conocimiento y desarrollar nuevas estrategias terapéuticas para las epilepsias resistentes a los medicamentos.

A pesar de que los diversos mecanismos causantes de la enfermedad aún no se entienden por completo, se sabe que hay una disminución o alteración en la inhibición que afecta a toda la excitabilidad de la red. Se podría entender como los fusibles de la luz de la casa, que saltan cuando la corriente eléctrica es demasiado elevada y hace que se apaguen todos los aparatos eléctricos para que no se queme la instalación eléctrica. En la epilepsia no saltan los fusibles en situación de sobre excitación neuronal, causando ataques epilépticos. Por ello, las nuevas estrategias tienen como propósito restablecer los niveles de inhibición, lo cual a su vez disminuiría la sobre excitación de la red y evitaría la generación de ataques epilépticos. Debido a la limitada capacidad de regeneración de las neuronas del cerebro, es necesario usar una fuente externa de neuronas inhibitorias, estrategia conocida como terapia celular. En esta tesis (*Artículo I y II*), las neuronas inhibitorias han sido generadas a partir de células madre humanas en el laboratorio. Se ha demostrado que dichas células son capaces de integrarse en la red eléctrica cerebral y reducir el número y duración de los ataques epilépticos en un modelo animal de epilepsia resistente a medicamentos muy común en adultos. La misma estrategia se usó en un modelo pediátrico de epilepsia resistente a medicamentos asociada con autismo (*Artículo III*), aunque obteniendo resultados opuestos. En conjunto, estos resultados indican que la terapia celular usando neuronas inhibitorias es adecuada para ciertas epilepsias, si bien aún se necesita obtener un mayor conocimiento de ciertos tipos de epilepsia.

En esta tesis, también se explora una estrategia diferente conocida como terapia génica con la finalidad de incrementar inhibición en la red neuronal epiléptica. Esta estrategia consiste en la introducción de una información genética específica, un gen, en una población de neuronas deseada. En concreto (*Artículo IV*), el gen utilizado produce una proteína en la neurona que actúa como un interruptor y puede ser controlada externamente a través de un medicamento, una técnica que se conoce como quimiogenética. Por consiguiente, la terapia génica estaba dirigida a las neuronas excitatorias de la región epiléptica del cerebro, de forma que se pudiese inhibir (parar) su actividad de forma externa. La quimiogenética redujo la respuesta a estímulos excitatorios en los experimentos funcionales, aunque sería necesario investigar el efecto en ataques epilépticos en modelos animales.

En resumen, esta tesis confirma la idoneidad de utilizar terapia celular con neuronas inhibitorias para ciertas epilepsias, y establece los cimientos para futuros estudios utilizando quimiogenética como estrategia alternativa.

RESUM EN CATALÀ

El cervell és l'òrgan més desconegut del cos humà, la qual cosa pot ser deguda a la seva considerable complexitat, però també a la seva limitada accessibilitat per estudiar-lo. No obstant això, es coneix que el cervell està compost per centenars de bilions de cèl·lules anomenades neurones que conecten les unes amb les altres i envien senyals amb informació. Tot i que les neurones funcionen de manera similar, hi ha moltes classes diferents d'elles amb infinitat de formes, molècules, funcions i localitzacions en el cervell. Les neurones formen una xarxa elèctrica gegant que és responsable de les sensacions, sentiments, decisions i accions que prenem cada dia. Si mirem en detall la complexitat d'aquesta xarxa elèctrica, altres cèl·lules ajuden a les neurones a fer la seva funció netejant l'ambient, donant suport estructural, i distribuint factors que ajuden a les neurones a sobreviure. Si ens centrem només en la xarxa elèctrica, les neurons actuen com a un dispositiu elèctric en què cadascuna rep senyals d'altres milers de neurones i a la vegada genera respostes que remet a altres neurones. Els senyals d'arribada i de resposta acostumen a ser compostos químics que es coneixen com a neurotransmissors, tot i que el processament i la decisió d'enviar-los es fa de forma elèctrica. És a dir, si la càrrega elèctrica a l'interior de la neurona canvia prou després de rebre senyals, aquesta enviarà una resposta.

A l'epilèpsia, aquesta xarxa elèctrica es troba alterada, de manera que existeix un estat de sobreexcitació en una zona del cervell. Hi ha moltes raons que poden portar a aquesta alteració, tanmateix hi ha moltes manifestacions que poden derivar d'aquesta, segons la regió del cervell afectada. És per això que l'epilèpsia es considera una família de malalties heterogènies i multifactorials, els pacients de les quals tenen el distintiu comú de patir atacs epilèptics de forma no provocada. Un atac epilèptic es pot entendre com una activació excessiva i sincronitzada de neurones que envien senyals a la vegada, i que pot causar manifestacions funcionals. Això és important si bé perquè l'epilèpsia és una malaltia freqüent del sistema nerviós que afecta una de cada cent persones, sumant un total de 50 milions de persones al món, i inclou pacients de totes les edats i gèneres. A conseqüència de la complexitat de la malaltia, encara no s'ha trobat una cura. Els fàrmacs disponibles, coneguts com a anticonvulsius, només tracten els símptomes i poden tenir efectes adversos a l'humor, les facultats cognitives i la memòria. A més, aquests fàrmacs no són eficaços en un terç dels pacients, que continuen tenint atacs epilèptics incontrolats, altres afectacions associades i una baixa qualitat de vida. Una alternativa terapèutica és la cirurgia resectiva de la regió del cervell causant dels

atacs, tot i que a la pràctica només és una opció factible per a molt pocs pacients i amb una probabilitat d'èxit de només 30-40 %. Tanmateix, hi ha una necessitat de millorar el nostre coneixement i desenvolupar noves estratègies terapèutiques per a les epilèpsies resistents als tractaments farmacològics.

Malgrat que els diversos mecanismes causants de la malaltia encara no s'entenen del tot, se sap que hi ha una disminució o alteració en la inhibició que afecta a tota l'excitabilitat de la xarxa. Es podria entendre com els fusibles de la llum en una casa, que salten quan el corrent elèctric és massa elevat i fa que s'apaguin tots els aparells elèctrics de la casa per tal que no es cremi la instal·lació elèctrica. En l'epilèpsia no salten els fusibles en situació de sobre excitació neuronal causant atacs epilèptics. Per aquesta raó, les noves teràpies intenten restablir la inhibició que alhora disminuiria la sobreexcitació de la xarxa neuronal i evitaria l'aparició d'atacs epilèptics. Degut a la capacitat limitada de regeneració de les neurones al cervell, és necessari utilitzar una font externa de neurones inhibitòries, estratègia coneguda com a teràpia cel·lular. En aquesta tesi (*Article I i II*), s'han generat neurones inhibitòries a partir de cèl·lules mare humanes i s'ha demostrat que aquestes cèl·lules són capaces d'integrar-se a la xarxa elèctrica cerebral i reduir el nombre i durada dels atacs epilèptics en un model animal d'epilèpsia resistent als fàrmacs comuna en els adults. La mateixa estratègia es va utilitzar en un model pediàtric d'epilèpsia resistent a fàrmacs associada amb autisme (*Article III*), tot i que els resultats obtinguts van ser oposats. En conjunt, aquests resultats indiquen que la teràpia cel·lular emprant neurones inhibitòries és adient per certes epilèpsies, si bé encara es requereix assolir un millor coneixement de les altres.

En aquesta tesi, també s'explora una estratègia diferent coneguda com a teràpia gènica amb la finalitat d'incrementar la inhibició en la xarxa neuronal epilèptica. Aquesta estratègia consisteix en la introducció d'informació genètica específica, un gen, en una població de neurones desitjada. En concret (*Article IV*), el gen emprat produeix una proteïna en la neurona que actua com un interruptor i que pot ser controlat externament a través d'un fàrmac, una tècnica coneguda com a quimiogenètica. La teràpia gènica estava dirigida a les neurones excitatòries de la regió epilèptica del cervell, de forma que es pogués inhibir (aturar) la seva activitat de forma externa. La quimiogenètica va reduir la resposta a estímuls excitatòris en els experiments funcionals. Tot i això, seria necessari investigar l'efecte en atacs epilèptics en models animals.

En resum, aquesta tesi confirma la idoneïtat d'utilitzar teràpia cel·lular amb neurones inhibitòries per a certes epilèpsies, i estableix els fonaments per futurs estudis utilitzant la quimiogenètica com a estratègia alternativa.

ORIGINAL ARTICLES

Original papers and manuscripts included in the thesis

Paper I

Human stem cell-derived GABAergic neurons functionally integrate into human neuronal networks.
Gonzalez-Ramos A, Waloschková E, Mikroulis A, Kokaia Z, Bengzon J, Ledri M, Andersson M, Kokaia M.
Sci Rep. 2021 Nov 11;11(1):22050. DOI: 10.1038/s41598-021-01270-x

Paper II

Human stem cell-derived GABAergic interneurons establish efferent synapses onto host neurons in rat epileptic hippocampus and inhibit spontaneous recurrent seizures.
Waloschková E, **Gonzalez-Ramos A**, Mikroulis A, Kudláček J, Andersson M, Ledri M, Kokaia M.
Int. J. Mol. Sci. 2021 Dec 8, 22, 13243. DOI: 10.3390/ijms222413243

Paper III

Early postnatal transplantation of human stem cell-derived GABAergic interneurons alters the adult epileptic phenotype of *Cntnap2* knock-out mice.
Gonzalez-Ramos A, Berglind F, Laurin K, Hatamian D, Hayatleh M, Vombergar A, Ledri M, Kokaia M, Andersson M.
Manuscript 2022

Paper IV

Chemogenetics with PSAM^4-GlyR decreases excitability of principal neurons in the epileptic hippocampus.
Gonzalez-Ramos A, Berglind F, Kudláček J, Melin E, Ledri M, Andersson M, Kokaia M.
Manuscript 2022

Publications outside of the thesis

Leaky opto-electrical fiber for optogenetic stimulation and electrochemical detection of dopamine exocytosis from human dopaminergic neurons.
Vasudevan S, Kajtez J, Bunea A-I, **Gonzalez-Ramos A**, Ramos-Moreno T, Heiskanen A, Kokaia M, Larsen NB, Martínez-Serrano A, Keller SS, Emnéus J.
Advanced Science. 2019 Dec 18; 6(24):1902011. DOI: 10.1002/advs.201902011

CONTEXT OF THIS THESIS

The thesis was carried out within the Experimental Epilepsy Group, Epilepsy Center, Lund University. Three out of the four projects included within the thesis (Paper I-III) were part of the Marie Skłodowska-Curie Innovative Training Network, project Training4CRM (Grant Agreement No. 722779), and therefore funded by the European Union Horizon 2020 Program (H2020-MSCA-ITN-2016).

The overall scientific goal of the Training4CRM was to address existing gaps within cell-based replacement medicine for the treatment of neurodegenerative disorders, including Parkinson's and Huntington's disease, as well as epilepsy. As one of the early-stage researchers within the network, my work was the generation of human-derived GABAergic progenitors whose neuronal activity could be modulated by light, and test their effect *in vitro* and *in vivo* in an epileptic model.

As part of the training network, I have been involved in another project that resulted in the publication cited in the section *Publications outside of the thesis*. Briefly, human neuronal stem cell-derived dopaminergic neurons genetically modified to express channelrhodopsin-2 were used to prove the capacity of a multifunctional leaky optoelectrical fiber to permit light leakage that stimulates the cells resulting in the release of dopamine and its real-time detection from the same fiber using chronoamperometry.

ABSTRACT

Epilepsy is a family of heterogeneous and multifactorial neurological disorders, unified by the occurrence of spontaneous recurrent seizures. Overall, it affects 50 million people worldwide of all ages and genders. Available treatments are only symptomatic and have severe side effects, while they also fail to provide adequate seizure control in a third of the patients. Therefore, a cure is yet to be found. To this end, this thesis takes on the challenge of pursuing novel approaches to treat those refractory patients. The two approaches presented here based their goal on increasing inhibitory drive in the epileptic focus to reduce the pathological hyperexcitable neuronal network that characterizes epilepsy, and thus counteract seizures.

First, based on evidence of loss and/or alteration of gamma-aminobutyric acid (GABA)ergic interneurons in the epileptic neuronal network, cell-based therapy has been developed and tested in three different scenarios for restoration of the excitatory/inhibitory balance. GABAergic interneurons (hdINs) were generated *in vitro* from human embryonic stem cells and proved to survive and integrate into both human and rodent epileptic environments. Host neuronal activity could be modulated by light-activation of hdINs using optogenetics. Finally, grafted hdINs were able to reduce the seizure frequency and total time spent in seizures in a rat model of temporal lobe epilepsy (TLE), the most common form of refractory epilepsy in adults. However, grafted hdINs failed to improve the pathology in a genetic mouse model of cortical dysplasia-focal epilepsy syndrome associated with autism spectrum disorder, which highlights the diversity of epilepsies and the need for gaining a better understanding of the mechanisms underpinning the disease.

Finally, direct inhibition of principal cells, similar to the one exerted by endogenous inhibitory interneurons, in the chronic epileptic hippocampus by using a chemogenetic approach delivered as gene therapy was also tested. Positive results were observed by decreasing ability to generate action potentials, although further investigation is required to evaluate the efficacy of this approach on seizures.

Collectively, the work presented here has moved the field forward in testing two different therapeutic strategies in a TLE model, and also one of them in a genetic epilepsy model.

ABBREVIATIONS

AAV	Adeno-associated viral vector
aCSF	Artificial cerebrospinal fluid
AD	*Ascl1* and *Dlx2*
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
AP5	D(−)-2-Amino-5-phosphonopentanoic acid
ASD	Autism spectrum disorder
ASM	Anti-seizure medication
BBB	Blood-brain barrier
CA	*Cornu ammonis*
CaMKIIα	Calcium/calmodulin-dependent protein kinase II alpha
CB	Calbindin
CDFE	Cortical dysplasia – focal epilepsy syndrome
CGE	Caudal ganglionic eminence
ChR2	Channelrhodopsin-2
CNO	Clozapine N-oxide
CNS	Central nervous system
Cntnap2	Contactin Associated Protein-like 2
CR	Calretinin
DG	Dentate gyrus
DIV	Days *in vitro*
Dox	Doxycycline
DREADD	Designer receptor exclusively activated by designer drugs
E	Embryonic day
EC	Enthorinal cortex
EEG	Electroencephalogram
E/I	Excitatory to inhibitory
EMA	European medicines agency
FDA	U.S. Food and Drug Administration
GABA	Gamma-Aminobutyric acid
GC	Granule cells
GFP	Green fluorescent protein
GW	Gestational week
hESC	Human Embryonic Stem Cell
hdIN	Human ESC-derived GABAergic Interneuron
HLA	Human leukocyte antigen
IED	Interictal epileptiform discharge
IHKA	Intrahippocampal kainic acid
ILAE	International League Against Epilepsy

iN	Induced neurons
IN	Interneuron
i.p.	Intraperitoneal
iPSC	Induced pluripotent stem cell
KA	Kainic acid
KO	Knock-out
LGE	Lateral ganglionic eminence
LGIC	Ligand-gated ion channels
LTM	Long-term memory
LV	Lentiviral vector
MGE	Medial ganglionic eminence
mTOR	Mammalian target of rapamycin
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NMDA	N-methyl-D-aspartate
NOR	Novel object recognition
NPY	Neuropeptide Y
NSPC	Neural stem/progenitor cells
OLT	Object location task
O/N	Overnight
P	Postnatal day
PFA	Paraformaldehyde
PHB	Phenobarbital
PSAM	Pharmacologically selective actuator modules
P/S	Penicillin / Streptomycin
PT	Post-transplantation
PTX	Picrotoxin
PV	Parvalbumin
RMP	Resting membrane potential
RT	Room temperature
RT-qPCR	Reverse-transcription quantitative polymerase chain reaction
Ri	Input resistance
SE	Status epilepticus
sPSC	Spontaneous postsynaptic current
sPSP	Spontaneous postsynaptic potential
SRS	Spontaneous recurrent seizures
SST	Somatostatin
STM	Short-term memory
SUB	Subiculum
SUDEP	Sudden Unexpected Death in Epilepsy
TEA	Tetraethylammonium chloride
TF	Transcription factor
TLE	Temporal lobe epilepsy
TTX	Tetrodotoxin
VIP	Vasoactive intestinal peptide
WT	Wild type
Y	ROCK inhibitor Y-27632

INTRODUCTION

Epilepsy

Conceptual evolution: from antiquity until today

Epilepsy is an ancient disease that has equally fascinated and frightened physicians and scientists since Mesopotamian and ancient Egyptian times (Labat, 1951). At that time, epilepsy was related to gods and demonic possession. In the 5th century BC, Hippocrates was one of the earliest to question the cause of epilepsy and he attributed it to the brain, as well as suggested that it was hereditary rather than contagious (Magiorkinis et al., 2010). However, it was not until the 17th century AD that the Hippocratic idea gained traction in Europe by the hand of two physicians Samuel Tissot (Eadie, 2019) and William Cullen (Cullen, 1792). Since then, different discoveries have brought the concept of epilepsy to the current one (Sidiropoulou et al., 2010). A breakthrough was the discovery of synapses by Santiago Ramon y Cajal, a finding that brought him to receive the Nobel prize in 1906 and move the scientific community to understand that neurons communicate by electric currents at synapses. This was followed by the invention of electroencephalography (EEG) in 1913, which was used in 1929 for the first time to record human brain activity by Hans Berger (Ahmed & Cash, 2013). EEG remains nowadays the main diagnostic tool together with the magnetic resonance imaging, available later in 1980 for epileptic seizures.

Despite the progress towards a better understanding of epilepsy pathology, patients suffered from stigma and still do. Patients were secluded and considered as mentally unstable and a shame for their families (de Boer, 2010). Luckily, in the 20th century, several organizations were founded to improve the view and care of epilepsy patients. Among them outstands the International League Against Epilepsy (ILAE) established in 1909 (Magiorkinis et al., 2010). Currently, in most societies, epilepsy is accepted as a neurological disorder as many others are, although there are still ignorance and stigma around this disease (Fiest et al., 2014). To change this situation more work needs to be done on educating people about the pathology of epilepsy across the globe.

Today, we know that epilepsy is a collection of heterogeneous diseases of different etiologies characterized by underlying mechanisms that predispose the brain to

generate spontaneous and unpredictable epileptic seizures (Engel Jr, 1995; Fisher et al., 2005). Therefore, an epileptic seizure is the main symptom and manifestation of the disease, and it was conceptually defined by the ILAE in 2005 as follows:

> "An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain."
>
> (Fisher et al., 2005)

Importantly, having a seizure does not imply suffering from epilepsy since it can be an acute symptom of a brain insult. Indeed, up to 10 % of people worldwide have one seizure during their lifetime. Those seizures are provoked and are called acute symptomatic seizures. They represent 40 % of all afebrile seizures in the community, although as mentioned they occur in close temporal relationship with an acute event to the brain i.e. within one week from an intracranial surgery, an active central nervous system (CNS) infection, or a subdural hematoma (Beghi et al., 2010). A few years ago, epilepsy was diagnosed when two or more unprovoked seizures occurred separated by more than 24 h. Albeit a more refined definition to the clinical situation is used nowadays, which is based on the probability of the patient suffering from a second epileptic seizure after suffering the first one. Thereby, epilepsy is diagnosed when clinicians evaluate that the probability of suffering a second recurrent seizure is higher than 60 % over the next 10 years, or whether the person is diagnosed with an epilepsy syndrome (Fisher et al., 2014; Hesdorffer et al., 2009). This increased propensity to suffer a second epileptic seizure, or lowered seizure threshold, reflects the chronic pathological alterations in the brain that make it different from a healthy one. This process of epilepsy development that includes various structural and/or biochemical changes in the brain that result in network reorganization, hyperexcitability, and reduction of the threshold for seizure generation, is called epileptogenesis.

Finally, epilepsy can develop and remain during the whole life of the patient, but it can also be considered resolved after a certain time. This second scenario happens when patients have been seizure-free for the last 10 years, with no anti-seizure medication (ASM) for 5 years; and in individuals who had an age-dependent epilepsy syndrome and are now past the applicable age (Fisher et al., 2014).

Epilepsy in numbers and its classification

Epilepsy is a chronic group of diseases of the brain affecting people all over the world of both genders and all ages, so it is one of the most common neurological disorders. The prevalence of people diagnosed with epilepsy is 1 out of 100 individuals, representing around 50 million people worldwide. Every day, there are approximately 10.000 new cases (World Health Organization, 2019). Even though it can occur at all ages, it is more prevalent during childhood and in the elderly

(Forsgren et al., 2005). Every year, 1 out of 1000 children is diagnosed with epilepsy. Importantly, epilepsy during the first years of life is frequently associated with numerous neurodevelopmental disorders such as intellectual disability and autism spectrum disorder (ASD) (Lo-Castro & Curatolo, 2014).

Overall, epilepsy represents an enormous financial burden for society. It represents the third largest neurological disorder globally, preceded only by Alzheimer's disease and headache disorders (Kyu et al., 2018). Not only the socio-economic burden is important, but how patients experience the disease. Epilepsy patients face a lower quality of life due to limitations of the disease itself, stigma, and adverse effects of the ASM. Moreover, epilepsy is associated with higher mortality rates, which could be explained by elevated suicide rates and the risk of sudden unexplained death in epilepsy (SUDEP) (Jallon, 2004). Every year, there are 60.000 cases of SUDEP in the world.

There are various reasons for having a predisposition to epilepsy i.e. head injury, stroke, brain tumors, infections, inherited genetic defects, but also a not apparent cause (idiopathic epilepsy) (Duncan et al., 2006; Scheffer et al., 2017). All these possible etiologies reflect the complexity of epilepsy, which regardless of the cause will manifest as epileptic seizures. Thus, seizures can have diverse symptomatic appearances depending on where the abnormal activity occurs in the brain. The wide range of manifestations includes from a momentary loss of awareness to short periods of unconsciousness, or even highly generalized convulsions. Normally, seizures terminate themselves without any kind of intervention after a few seconds or minutes. Although if it persists over time exceeding 30 minutes, it becomes harmful for the brain to a life-threatening state known as status epilepticus (SE) (Tatum Iv et al., 2001). Considering these vastly different etiologies and semiologies of epilepsies and seizures, efforts have been made to classify them with a common vocabulary to facilitate communication within the field (Fisher et al., 2017; Scheffer et al., 2017). This classification is also important when applying a treatment since the medication of choice will differ, as well as the prognosis (Duncan et al., 2006).

The current classification of seizures and epilepsies was issued in 2017 by ILAE and is divided into three levels: *seizure type*, *epilepsy type*, and *epilepsy syndrome* (see Figure 1) (Scheffer et al., 2017). The first level refers to the initial features of the seizure. So that *focal seizures* involve one confined brain area limited to one hemisphere, at least initially. While *generalized seizures* engage both hemispheres at the onset. Generalized seizures are usually associated with a loss of consciousness, and depending on if they include movement or not, can be further divided into "*motor*" and "*nonmotor*" respectively. On the contrary, during focal seizures, the patient can be "aware" of what is happening or "unaware" of it. Focal seizures are also further classified into "*motor*" or "*nonmotor*" as before. Furthermore, focal seizures can propagate to other parts of the brain which causes convulsive seizures, categorized as "*focal to bilateral tonic-clonic*" (Fisher et al.,

2017; Scheffer et al., 2017). This last type of seizure is very common in temporal lobe epilepsy (TLE), the model used in Paper II and IV in this thesis.

The second level of classification, see Figure 1, *Epilepsy type*, is made on the clinical ground, supported by diagnostic tools such as EEG and neuroimaging. Here the main categories are again *focal*, *generalized,* and *unknown*, with the addition of *combined generalized and focal* manifesting both seizure types. The *unknown* category is given when clinical information is insufficient. The third and final level of diagnosis relates to *Epilepsy syndrome* (Scheffer et al., 2017). Clinicians might use any of the three levels as a final level for diagnosis, depending on the resources available. Nonetheless, the etiology of the disease should be considered at all levels of classification. A structural etiology is a common finding in epilepsy, and malformations of the cortical development originating from a genetic mutation are considered within this group (Scheffer et al., 2017). For instance, structural etiology by hippocampal sclerosis is frequently found in TLE. Moreover, structural and genetic etiology is found in cortical dysplasia-focal epilepsy (CDFE) syndrome caused by a mutation in the contactin-associated protein-like 2 (*CNTNAP2*) gene, used in Paper III.

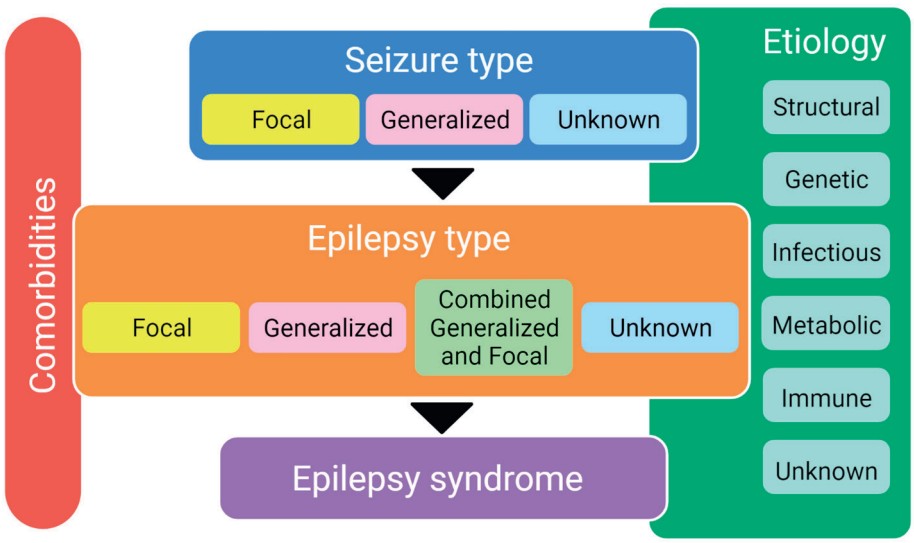

Figure 1. Classification of seizure and epilepsy
Flow chart of the classification of seizures and epilepsy issued by the ILAE in 2017. It illustrates the three main levels and the cross-level considerations of etiology and comorbidities. *Schematics adapted from Scheffer et al. (2017).*

Comorbidities are placed cross-levels since 84 % of epilepsy patients have at least one comorbidity, which can be somatic (medical or neurological), psychiatric, or social (Seidenberg et al., 2009). Frequently those comorbidities are non-diagnosed and therefore untreated, but they can have clinical implications at all levels, from diagnosis to treatment and prognosis. For instance, physical and psychiatric

comorbidities are associated with poorer health outcomes, increased health care needs, and reduced quality of life. The most prevalent comorbidities in adults are depression (23 %) and anxiety (20 %), while in children is intellectual disability (30-40 %) (World Health Organization, 2019). Some of them have a bidirectional relationship with epilepsy, in which epilepsy patients have an increased risk of developing particular comorbidity and vice versa. Some examples are migraine, dementia, psychiatric diseases, and sleep disorder. Moreover, the presence of comorbidities can determine the choice of an ASM.

It is important to keep in mind that in epilepsy, seizures are only the "tip of the iceberg" that we can see or detect.

Temporal lobe epilepsy

The most common form of epilepsy in humans is TLE, characterized by seizures arising from mesial temporal lobe structures which include the amygdala, hippocampal formation, and temporal neocortex (Engel Jr, 2001). Seizures are progressive, often starting with altered consciousness, followed by automatisms, and finally generalization into clonic motor symptoms (Blair, 2012). Due to the complex etiology of epilepsy mentioned before, it is difficult to isolate a precipitating event. Nevertheless, in many TLE patients, it is initiated by a traumatic event such as stroke, traumatic brain injury, infections, or febrile convulsions (O'Dell et al., 2012; Pitkänen & Sutula, 2002). This insult triggers epileptogenesis, which occurs silently during a latency period of 5-10 years before the first unprovoked seizure appears (O'Dell et al., 2012). Importantly, epileptogenesis is not restricted to the latency period, but contributes to the progression of the disease over the years since seizures *per se* are also insults to the brain (O'Dell et al., 2012; Pitkänen, 2010).

While the underpinning disease mechanism is not fully understood, TLE is often associated with various changes primarily occurring in the hippocampus, which include neuronal cell death, astrogliosis, inflammation, and aberrant mossy fiber sprouting from dentate granular cells in the dentate gyrus (O'Dell et al., 2012).

In this thesis, two animal models for TLE have been used, in Paper II and Paper IV. Both are acquired models meaning that there is an initial insult that triggers epileptogenesis and later the disease, for which kainic acid (KA) was used for both papers. More details on the animal models used in this thesis including the mechanism of action of KA are described under the *Key experimental procedures* section.

Cortical dysplasia-focal epilepsy syndrome

Childhood and early adolescence represent one of the peaks of incidence of epilepsy (Forsgren et al., 2005), which is one of the most common neurological disorders in children. Importantly, recurrent intractable seizures during development are detrimental for both their cognitive and neuropsychological development. An epilepsy onset within the first year of life is a strong risk factor for mental retardation. Moreover, epilepsy during childhood is frequently associated with several neurodevelopmental disorders such as intellectual disability, attention deficit hyperactivity disorder, and ASD (Lo-Castro & Curatolo, 2014). Even though focal epilepsies with onset during childhood are often self-limited (Berg et al., 2014) and of unknown cause, they should not be considered benign since the effect suffered during neurodevelopment and the comorbidities derived remain present in the individuals (Specchio et al., 2017). Within epilepsies in the young population, focal cortical dysplasia has a high prevalence and is the most common cause for refractory epilepsy with an estimated half of children undergoing epilepsy surgery diagnosed with focal cortical dysplasia (Aronica et al., 2012; Lee & Kim, 2013). It is also the second to third most common etiology of refractoriness in adults (Kabat & Król, 2012).

Regarding the relationship between pediatric epilepsy and other neurodevelopmental disorders such as ASD, 15-35 % of children with epilepsy exhibit ASD symptoms. At the same time, various studies have reported a range of 7-46 % of patients with ASDs that suffer from epilepsy. Altogether suggesting a strong relationship between both disorders (Danielsson et al., 2005; Jeste & Tuchman, 2015; Lo-Castro & Curatolo, 2014). A common feature between both pathologies is an excitation/inhibition (E/I) imbalance, presumably associated with gamma-aminobutyric acid (GABA)-ergic interneuron dysfunction (Lee et al., 2017; Sohal & Rubenstein, 2019). Although a decrease in GABA receptor subunit expression has also been reported (Fatemi et al., 2010; Guptill et al., 2007). This overlap and the presence of common features between both disorders, strongly suggest shared underlying mechanisms that could be explored as new therapy targets.

Variation in *CNTNAP2* has been identified, among other genes, in human genetic studies to have significant overlap in contribution to multiple neuropsychiatric disorders (Poot, 2015; Rodenas-Cuadrado et al., 2014). In particular, genetic variations in the *CNTNAP2* locus have been associated with childhood apraxia of speech, intellectual disability, ASD, epilepsy, and schizophrenia (Centanni et al., 2015; Friedman et al., 2008; Strauss et al., 2006). Although distinct functional phenotypes may be caused by different mutations within the same gene. However, the biological mechanism underlying *CNTNAP2* contribution to the pathologies remains unclear (Dennis et al., 2011; Scott-Van Zeeland et al., 2010). Strauss and colleagues characterized for the first time the homozygous inherited loss-of-

function mutation (3709delG; # 604569.0001) in *CNTNAP2* (OMIM # 610042) in an Old Order Amish family (Strauss et al., 2006). Loss of function of *CNTNAP2* causes a rare and severe neurodevelopmental syndrome called CDFE. CDFE is characterized by relative macrocephaly, autistic behavior, stereotypic movements, and early-onset epilepsy typically within the first 3.5 years of life, after which moderate to severe intellectual disability, hyperactivity and language regression are also present. Importantly, patients showed cortical dysplasia, and thus resective surgery was inefficient to treat their refractory epilepsy (Strauss et al., 2006). A similar constellation of symptoms has been described in other homozygous mutations in *CNTNAP2* outside the Amish population (Riccardi et al., 2019; Saint-Martin et al., 2018).

CNTNAP2 gene (7q35-q36.1) encodes for CASPR2, a transmembrane protein from the neurexin superfamily that is widely expressed throughout the brain already during embryonic development (Alarcón et al., 2008; Gordon et al., 2016). Functions of *Cntnap2* has been described mainly in rodent models and include roles in clustering of voltage-gated potassium channels (Kv1) in myelinated axons (Anderson et al., 2012; Horresh et al., 2010; Poliak et al., 2003), dendritic arborization (Anderson et al., 2012), synaptic development and function (Anderson et al., 2012; Varea et al., 2015), and neuronal migration (Penagarikano et al., 2011). Deficits in the distribution of inhibitory GABAergic neurons have been reported in the *Cntnap2* knock-out (KO) mouse model, which has been associated with a disruption of the E/I balance (Gdalyahu et al., 2015; Jurgensen & Castillo, 2015; Penagarikano et al., 2011; Varea et al., 2015). Importantly, the *Cntnap2* KO mouse model has been studied quite extensively as an ASD model reflecting several features of the human disease such as similar pathology in the cortex, and behavioral alterations with autistic features (Penagarikano et al., 2011), although less is known of the epileptic phenotype. In this thesis, the *Cntnap2* KO mouse model has been used, in Paper III, to further characterize the spontaneous recurrent seizures (SRS) reported to occur after six months of age (Penagarikano et al., 2011).

Current treatments and those in the pipeline

The treatment for epilepsy is widely dependent on the diagnosis including the type of seizures, as well as severity and frequency of those. Therefore, it is a tailored approach adjusted for each individual, similar to what we could call personalized medicine. In this scenario, a wrong treatment can worsen the situation. The first and most common therapeutic approach is ASM, which aims to diminish the hyperexcitability in the epileptic brain tissue, and therefore restore the E/I balance of the network. Typically, ASMs act by either increasing the net inhibitory drive by enhancing inhibitory GABAergic signaling, an approach that is refined in this thesis by two new advanced strategies, or by reducing excitatory transmission by interfering with sodium and calcium channels (Löscher & Schmidt, 2011), as

illustrated in Figure 2. Approximately 60-70 % of all epilepsy patients are successfully treated with ASMs, but, unfortunately, the rest of them fail to respond and are classified as drug-resistant or refractory epilepsy. Moreover, ASMs have certain limitations, they do not modify the pathology (not anti-epileptogenic) but only treat the symptoms derived from it. Moreover, as the drugs are administered systemically, mainly by oral intake, they affect the whole brain including regions that are not contributing to the pathology, which then, lead to side effects (Fisher et al., 2000). Those side effects, such as cognitive and mood dysfunction, may persist even in patients with complete seizure control (Rathouz et al., 2014).

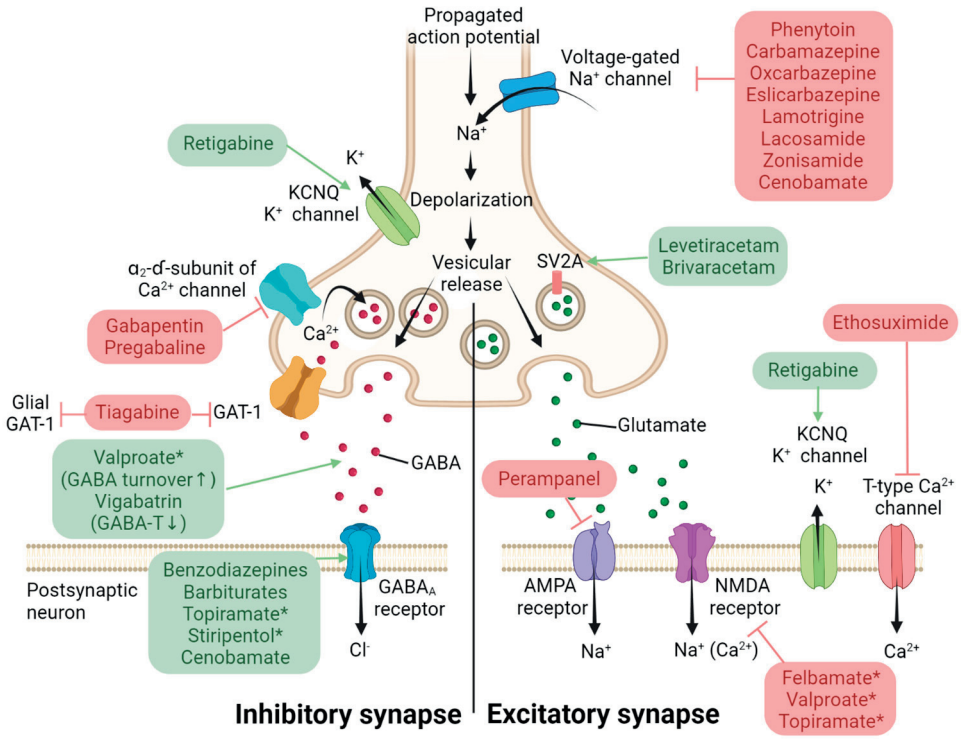

Figure 2. Strategies for controlling ictogenesis and mechanism of action of ASMs

Schematics of a zoom-in of a synapse, which at the same time is divided in the representation of an inhibitory synapse on the left and an excitatory synapse on the right. It illustrates the different mechanisms of action of clinically approved ASMs. Agonists or positive modulators are indicated in green and antagonists or negative modulators in red. Asterisks indicate that these compounds act in multiple mechanisms, although not all the mechanisms are shown here. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, γ-aminobutyric acid; GABA-T, GABA aminotransferase; GAT-1, GABA transporter 1; KCNQ, Kv7 potassium channel family; NMDA, N-methyl-D-aspartate; SV2A, synaptic vesicle protein 2A. *Schematics adapted from Löscher et al. (2020).*

From the first ASM used to treat epilepsy in 1853, the potassium bromide, it has passed 169 years. Besides recent development of new ASMs has improved some aspects of them such as simpler pharmacokinetics or fewer interactions, their efficacy is not superior to the previous ones. Indeed, 30-40 % of epilepsy patients

remain drug-resistant nowadays, therefore refractory epilepsy still constitutes a significant burden in healthcare (Duncan et al., 2006; Löscher & Klein, 2021). An individual is diagnosed with refractory epilepsy upon failure to achieve seizure freedom after using two tolerated and appropriately chosen drugs either as monotherapy or as a combination (Kwan et al., 2011) (Figure 3). For a small number of these refractory patients, fewer than 5 % (Fois et al., 2016; Jetté et al., 2016), surgical resection of the structure holding the seizure onset area may be a therapeutic option, without compromising important functions such as language or speech (eloquent areas). However, currently up to 40 % of the patients undergoing surgery show early or late failure in seizure freedom (Blumcke et al., 2013). A similar approach to surgical resection, the laser interstitial thermal therapy, is getting popular in some health systems.

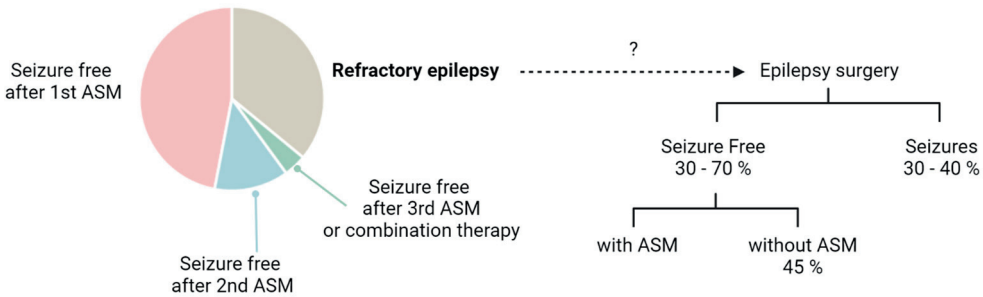

Figure 3. The therapeutic success of ASMs and outcomes from resective epilepsy surgery
Pie-chart illustrating the proportion of patients responding to the first ASM tried (47 %), to the seconds (13 %) or third (4 %), and finally the ones classified as refractory epilepsy (36 %). From the refractory epilepsy patients, few are candidates for surgical resection of the epileptic area. Outcomes from resective epilepsy surgery are illustrated in the flow chart on the right, being 30-40 % still suffering from seizures and 30-70 % seizure-free without (45 %) or with ASM.

Alternative or complementary treatments to ASMs to ameliorate refractory epilepsy also include dietetic therapy by ketogenic diet or modified Atkins diet (for GLUT1 deficiency), as well as vitamin supplementation. The ketogenic diet has been shown to decrease seizure frequency in children, although it is unpleasant and hard to maintain for a prolonged period (Kwan et al., 2011). Other surgical options involve vagus nerve stimulation or responsive neurostimulation, which are effective for some patients although do not produce seizure freedom in all (Ben-Menachem, 2002). In addition, some therapies are specific for a certain epilepsy type or syndromes such as anti-inflammatory or immunosuppression therapy, the rapamycin analog *Everolimus* for tuberous sclerosis complex (Krueger et al., 2013), or cannabidiol for Lennox-Gastaut syndrome (Thiele et al., 2018). Overall, the alternative treatments are not suitable for all patients due to either only moderate efficiency or undesirable side effects.

One major determinant for a good quality of life is seizure freedom (Birbeck et al., 2002), and this protracted situation of an immutable third of patients remaining

drug-resistant is problematic. Therefore, there is a need for effective disease-modifying and/or anti-seizure treatments to achieve seizure control in these refractory patients. Ideally, to limit side effects and reduce the impact on quality of life, the treatment should be administered only in the specific region of the brain involved in seizure generation or propagation, and preferably target only cell types responsible for seizure generation.

Currently, three clinical trials are testing advanced therapies for refractory epilepsy, and at least one more planning to start in the coming future. The first one is carried out at the University College London and it is currently in Phase I/IIa investigating the safety and efficacy of lentiviral gene therapy using an engineered potassium channel against refractory neocortical epilepsy in patients who are being evaluated for surgical resection of the seizure focus. The viral vectors will be administered via intracerebral infusion to the area scheduled for resection. The second trial is carried out by Neurona Therapeutics and it is a multi-center Phase I/II clinical trial as well, which started December 2021. Neurona Therapeutics is evaluating its cell therapy, NRTX-1001, in terms of safety, tolerability, and efficacy in people with mesial temporal lobe epilepsy. The third trial in the pipeline, carried out by Stoke Therapeutics, is a gene therapy based on antisense oligonucleotides, STK-001, to boost SCN1A protein production for treating Dravet syndrome patients, a severe form of genetic epilepsy, and potentially prolong survival for people with this condition (Wengert et al., 2022). In particular, STK-001 potentiates *SCN1A* mRNA levels and thus NaV1.1 expression, affecting the genetic mechanism of Dravet syndrome (Han et al., 2020). For more information about the ongoing trials, I kindly refer the reader to www.clinicaltrials.gov (NCT04601974, NCT05135091, and NCT04442295 together with NCT04740476, respectively). Finally, CombiGene's epilepsy project CG01, an AAV-based gene therapy approach to increase levels of neuropeptide-Y (NPY) and Y2 receptor, is now approaching the final stage of the preclinical phase.

The hippocampal formation

Organization and connectivity

The hippocampal formation, or hippocampus, is a pair of brain structures with a C-like shape located within the mesial temporal lobe, one on each cerebral hemisphere mirroring the other. The hippocampal formation is encompassed by the lateral ventricle and in close contact with the parahippocampal gyrus (Amaral & Lavenex, 2007).

Anatomically, the hippocampal formation is divided into subregions, which are dentate gyrus (DG); the hippocampus proper or also called *cornu ammonis* (CA)

including the regions CA1, CA2, CA3, and CA4; and the subiculum (SUB). This last subregion is located within the grey matter region of the parahippocampal gyrus near the entorhinal cortex (EC) which is the main gateway of communication between the hippocampus and the neocortex. The DG region also includes the hilar region or hilus, encompassed between its two blades (Amaral & Lavenex, 2007). The subregions are interconnected by a main excitatory neuronal pathway, also known as tri-synaptic circuit, that relies information mainly in one direction and where the primary cells are excitatory glutamatergic granule cells (GC) in the DG and pyramidal cells in the CA3 and CA1 regions (see Figure 4) (Amaral & Lavenex, 2007).

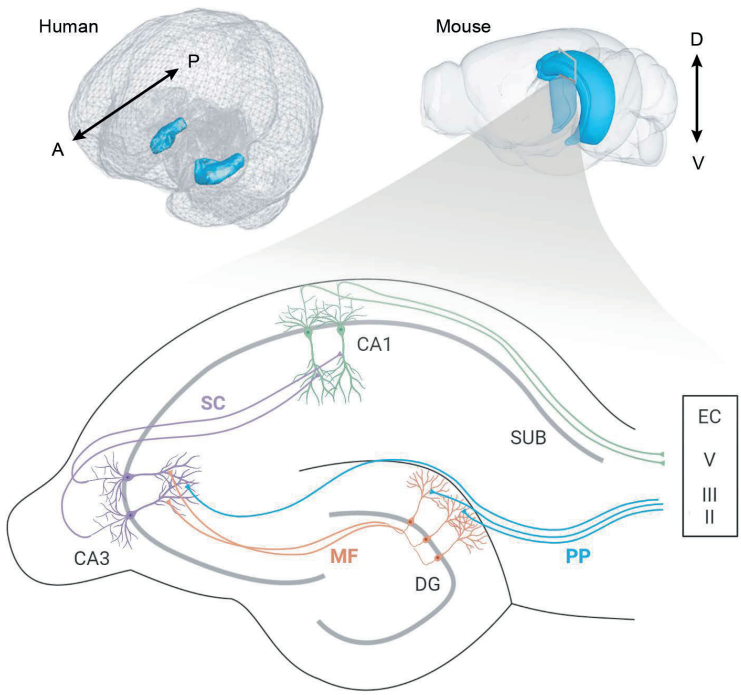

Figure 4. Hippocampal glutamatergic neuronal network organization and connectivity

Anatomical location of the hippocampal formation in humans (left) and mice (right), in cyan. Zoom in to the hippocampal formation with a representation of the glutamatergic connectivity circuit known as the trysinaptic circuit. The entorhinal cortex (EC) relays sensory information from layers II and III to the dendritic trees of granule cells (GC) in the dentate gyrus (DG) via the perforant pathway (PP) (synapse 1). Then, the GC's axons, called mossy fibers (MF), cross the hilus and form synapses on the proximal dendrites of principal cells in CA3 (synapse 2). In turn, principal cells in CA3 relay the signal by forming synapses on the dendrites of principal cells in CA1 via Shaffer collaterals (SC) (synapse 3). The circuit ends when principal cells in CA1 connect to cells in the subiculum (SUB) via the alveus, and thus projections from the SUB exit the hippocampus towards the deep layers (V) of the EC. A, anterior; P, posterior; D, dorsal; V, ventral. *Adapted from Madan et al. (2015).*

The hippocampus is a highly interconnected structure that receives sensory information from cortical areas such as the visual or somatosensory cortex, and integrates that information to form memories. Simultaneously, it connects this sensory information to deeper subcortical areas to exert emotional and behavioral responses to the received information. The sensory input is conveyed to the entorhinal cortex (layer II-III) and then further relayed to the input-area DG (Figure 4). It continues via CA3-CA1 through the tri-synaptic circuit to finally reach the output-area SUB that connects to deep layers (layer V-VI) of EC (Figure 4). Moreover, the DG is believed to contribute to the formation of new episodic memories, and it is noted for being one of the few locations in the brain with adult neurogenesis (Paredes et al., 2018). There is a functional distinction, although oversimplified, between the dorsal and ventral parts of the hippocampus. So that the dorsal part is more implicated in declarative memory and spatial orientation for navigation. While the ventral part is associated with emotional context memory. Those differences are caused and have implications in the network connections (Strange et al., 2014).

The EC is not the only gateway of the hippocampal formation, there are other intrinsic and extrinsic connections in the hippocampus. For instance, Schaffer collaterals from CA3 to CA1 also form lamellar layers interconnecting CA3 areas in the dorso-ventral axis (or septo-temporal in the case of humans). In parallel, pyramidal cells in CA3 also send projections back to DG, and even to the contralateral hippocampus via the commissures (Witter, 2007). Similarly, in the hilus reside mossy cells, considered as excitatory cells, which provide long-range ipsilateral and commissural projections into the DG, connecting the contralateral hippocampus although with a limited spread in the long axis (Amaral, 1978). Output fibers, from the SUB and EC, extend over the surface of the hippocampus and collect other fibers becoming a thicker fiber bundle (from ventral to dorsal in rodents) located in the lateral extreme of the hippocampus called fimbria. Once those fibers leave the hippocampus and continue to the forebrain, they are referred to as fornix. Importantly, fimbria and fornix carry both efferent fibers from the hippocampus, but also subcortical afferent fibers to the hippocampus. Besides receiving information from the EC, other various extrinsic inputs influence the hippocampus network such as cholinergic projections from the medial septum, noradrenergic projections from the locus coeruleus in the brain stem, serotoninergic projections from the raphe nuclei in the brain stem, and to less extent dopaminergic projections from the ventral tegmental area (Leranth & Hajszan, 2007; Witter & Amaral, 2004). Finally, the ventral hippocampus is recognized to be a critical player controlling motivated and emotional behavior, as already mentioned above. These functions are possible due to CA1 bidirectional projections to and from the basolateral amygdala, which at the same time sends projections to the medial prefrontal cortex, nucleus accumbens, and other regions (Gergues et al., 2020).

The hippocampal formation is also largely populated by various subtypes of GABAergic interneurons (INs), which are inhibitory neurons responsible for modulating the activity of both the principal cells and other INs (Freund & Buzsáki, 1996). Therefore, intrinsic input in the hippocampus is mediated by INs and some modulating neuropeptides (Carnahan & Nawa, 1995). INs make the hippocampal neuronal circuit more interconnected so that they play a crucial role in the regulation of synchronization and network oscillations, which is the foundation of normal hippocampal function (Buzsáki, 2001). Neural oscillations can be divided into frequency ranges with different implications. There are three main frequency bands highly relevant for hippocampal functions. The lowest oscillatory frequency in the theta band (4-8 Hz) occurs during learning, memory, and spatial navigation in many animals. It is followed by the gamma range (> 30 Hz), which can be subdivided into slow (30-70 Hz) and fast gamma (70-140 Hz) oscillations, being the fast associated with cognitive processing. Finally, the sharp-wave ripple complex, composed of 110-200 Hz ripples superimposed to 0.01-3 Hz sharp waves, which are observed during sleep, and it is believed to have an active part in both memory consolidation and memory retrieval for processes such as decision-making (Joo & Frank, 2018; Sullivan et al., 2011). Coupling between theta and gamma activity is believed to be crucial for memory functions (Buzsaki, 2006).

Imbalance between excitation and inhibition

In the brain, there are two main neuronal types, excitatory glutamatergic principal cells, and inhibitory GABAergic INs. Networks of excitatory and inhibitory neurons are organized hierarchically in complex brain circuitries. Within these networks, principal cells specialize in transmitting information among different areas, whereas INs provide inhibitory inputs to the local circuitries and shape synchronized oscillations (Klausberger & Somogyi, 2008). INs mainly act on glutamatergic neurons through the activation of the postsynaptic $GABA_A$ receptor, which is a chloride-permeable ion channel. Moreover, INs are activated by excitatory afferents or neighboring glutamatergic neurons, so that INs establish feedforwards and feedback inhibitory circuits. Therefore, the dynamic equilibrium holding the E/I balance is crucial for physiological brain functions (Haider et al., 2006; Yizhar et al., 2011).

INs are considered the main cellular element maintaining E/I balance. Despite they only represent 20-30 % of the overall neuronal population and are exquisitely heterogeneous (Kelsom & Lu, 2013), INs can form synapses with hundreds of neurons (Sik et al., 1995) that endow them with the ability to synchronize oscillations and act as pacemakers. Moreover, INs mediate information processing by precise spatiotemporal signal gating through specific pathways (Kremkow et al., 2010; Vogels & Abbott, 2009).

Considering the varied and crucial functions that INs perform, reduced or enhanced GABAergic transmission is associated with epileptiform activity or cognitive impairment, respectively (Kalueff & Nutt, 1996; Treiman, 2001). In particular, subtle IN deficits may cause aberrant signal processing in neuropsychiatric disorders such as schizophrenia, bipolar disorder, depression, and ASD, while a larger disruption of the inhibitory circuits might lead to epilepsy (Marín, 2012; Paterno, 2020; Ramamoorthi & Lin, 2011; Sohal & Rubenstein, 2019). Importantly, some of the disorders mentioned above arise during development, so that network dynamics are further complicated by opposite GABA actions at different neurodevelopmental stages, from depolarizing to hyperpolarizing (Ben-Ari et al., 2012). Hence, neurodevelopmental disorders' symptoms may vary over time. More information about the interneuron origin, maturation, and diversity, can be found in the dedicated section below.

In addition to predominant GABAergic dysfunction, other features might contribute to the increased excitability or the predisposition of certain brain areas to initiate self-sustained activity leading to seizures. The special structure of the hippocampus and the feed-forward tri-synaptic neuronal circuit that forms a feedback loop from the EC to the hippocampus and back to the EC creates a seizure sensitive network, so that it is proposed to be the reason for this increased proportion of seizures originating in the temporal lobe (Jefferys et al., 2012; Shuman et al., 2020). In addition, back projections from CA3 to DG and recurrent connectivity in the CA3 area make this structure prone to induction of epileptiform discharges (Strange et al., 2014; Witter, 2007). Nonetheless, in a physiological context, GCs in the DG are slightly hyperpolarized at resting membrane potential, so they are resistant to activation and act as a filter to the weak transmitted information, a function called pattern-separation (Hsu, 2007). However, during epileptogenesis in TLE models, GC's axons grow and form recurrent connections to other GCs (mossy fiber sprouting), contributing to increased excitability in the DG (Scharfman et al., 2003).

In contrast, some studies speculate that parvalbumin (PV)-positive INs may contribute to the generation of abnormal synchrony and maintenance of epileptic seizures (Sessolo et al., 2015; Yekhlef et al., 2015). The reasoning behind it includes that: (i) PV-positive INs are to some extent preserved in models of TLE (Wyeth et al., 2010), (ii) they receive contacts from mossy fiber collaterals (Kotti, 1997; Sloviter et al., 2006), (iii) PV axonal contacts are maintained in CA1 and DG of tissue from epileptic patients (Wittner et al., 2004; Wittner et al., 2001), and (iv) functional properties of PV INs in controlling action potential generation in principal cells.

This thesis will not describe further regulation of the E/I balance, albeit there are other factors influencing this dynamic equilibrium. For instance, other modulators are acting as extrinsic inputs to the hippocampal network by afferent projections that include monoaminergic synapses such as noradrenaline, serotonin, acetylcholine, and dopamine (Witter & Amaral, 2004). Furthermore, other cell types

are important for supporting neuronal function and homeostasis in the milieu, among other functions. As an example, it has been shown that astrocytic dysfunctions i.e. alteration in potassium channels such as Kir4.1, increase potassium extracellularly leading to lower firing threshold from the surrounding neurons and followed by hyperexcitability (Steinhäuser et al., 2012).

Interneurons

The denoted relevance of INs on the aforementioned maintenance of the E/I balance and their alteration in a wide range of neurological conditions including developmental disorders, highlight the importance to understand the origin, maturation, and circuit formation of INs. Owing to INs being remarkably heterogeneous and varying in the different brain regions, this section is mainly focused on the hippocampal formation.

Origin, maturation, and migration of INs

The INs originate from ganglionic eminences located in the embryonic subpallium, between embryonic day (E)11 and E17 in mice and 5-6 gestational week (GW) in humans (Pleasure et al., 2000; Wonders & Anderson, 2006; Zecevic et al., 2011). More specifically, the genesis of hippocampal INs, similarly to cortical INs, occurs from medial and caudal ganglionic eminences (MGE and CGE respectively), whose progenitors reach the hippocampus at E14.5-18 by tangential migration from ventral to dorsal areas (Danglot et al., 2006; Tricoire et al., 2011) (Figure 5 and 6). While lateral ganglionic eminence (LGE) precursors mainly give rise to olfactory interneurons and striatal projection neurons. Once in the hippocampus, IN progenitors differentiate through a complex maturation process influenced by the interaction of specific genetic and environmental factors. Therefore, progenitors from certain ganglionic eminence generate specific INs, i.e. MGE progenitors give rise mainly to PV- and somatostatin (SST)-positive INs, while calretinin (CR)-positive INs arise mainly from CGE precursors (Figure 5) (Lim et al., 2018). Additionally, several studies have suggested a secondary origin in the dorsal pallium for a subpopulation of cortical INs, primarily CR-positive INs, both in mice (Bellion et al., 2003; Götz et al., 1995) and humans (Letinic et al., 2002; Zecevic et al., 2011). Notwithstanding genesis of hippocampal INs occurs prenatally, their morphological maturation prolongs to the postnatal period.

Already at birth, INs contribute to synchronize neuronal activity known as spontaneous plateau assemblies by activation of membrane conductance in groups of neurons connected by gap junctions (Allene et al., 2008; Crépel et al., 2007). During the first postnatal week in the mouse hippocampus, owing to a gradual maturation of INs and formation of synaptic connections, early network activity is replaced by giant depolarizing potentials (Ben-Ari et al., 1989). At this time, GABA depolarizes postsynaptic cells via the $GABA_A$ receptor (Ben-Ari et al., 2012).

Importantly, some differences have been observed across brain regions leading to hypothesize that GABAergic influence on developing neural networks varies in different areas (Allene et al., 2008), so its alteration may cause region-dependent circuit outcomes.

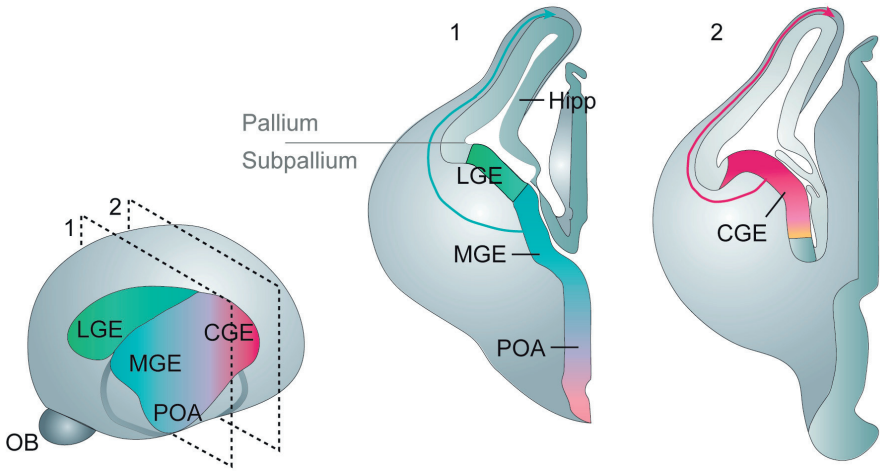

Figure 5. Developmental origin of hippocampal and cortical interneurons
On the left, interneuron origin on the subpallial ganglionic eminences during embryonic development in mice. On the right, there are two coronal sections (1 and 2) showing in more detail the MGE (blue) and CGE (magenta), and pathway followed during tangential migration (arrows) towards the pallium. OB, olfactory bulb; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; CGE, caudal ganglionic eminence; POA, preoptic area; Hipp, hippocampal primordium. *Adapted from Marín (2012).*

Excitatory GABA at this developmental period is crucial for several processes including neuronal differentiation, dendritic arborization, and activity-dependent regulation of neuropeptides (Ben-Ari et al., 2007; Ben-Ari et al., 2012; Marty et al., 1996). It is worth noticing that neuronal activity regulation of neuropeptide levels is conserved in the adult hippocampus and thus affected by seizures (Schwarzer et al., 1996). In parallel to the depolarizing GABA, there is a peak of synaptogenesis with a high number of glutamatergic synapses that lead to an inherently more seizure prone brain during this early postnatal developmental period (Ben-Ari et al., 2012; Dzhala et al., 2012; Gómez-Di Cesare et al., 1997).

Changes in chloride transporters render a switch of GABA from being depolarizing (excitatory) to hyperpolarizing (inhibitory) during the second postnatal week, postnatal day (P)10 in mice and 92–210 GW (1–3.3 years) in humans (Dzhala et al., 2005) (Figure 6). The expression of the potassium-chloride co-transporter KCC2 increases towards the end of the first postnatal week in mice and about 42 GW in humans (Dzhala et al., 2005). Together with a shift in the location of the sodium-potassium-chloride cotransporter NKCC1 from somatic to dendritic (Marty et al., 2002). In parallel with those changes, there is a decrease of primitive oscillations (Vanhatalo et al., 2005), and a substantial reorganization of IN bodies and axons,

and even INs death. Interestingly, similar patterns of expression are observed between the calcium-binding protein PV and the voltage-gated potassium channels of the Kv3 subfamily, which are necessary for the fast-spiking characteristics of some PV-expressing INs, at the end of the first postnatal week in mice (Martina et al., 1998; Tansey et al., 2002). Finally, in humans, inhibitory circuits continue to develop until adolescence (16-18 years old) when the activity-dependent pruning of excess synapses known as the refinement phase finalize (Semple et al., 2013; Watson et al., 2006), which in mice occur at P35-49 (as illustrated in Figure 6).

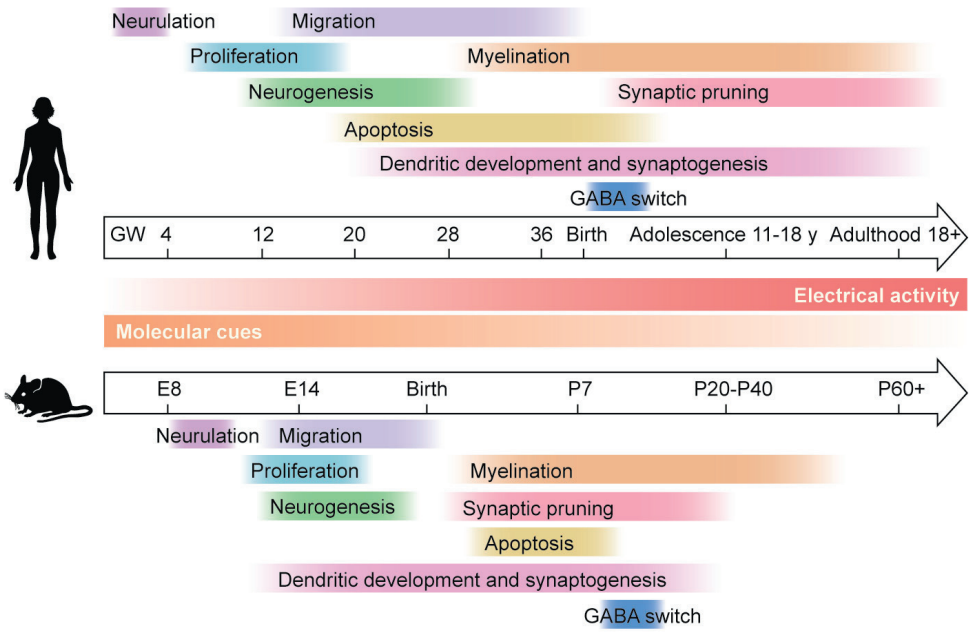

Figure 6. Neurodevelopment in humans and mice
Timeline with a representation of the main processes guiding the neuronal development in humans (top) and mice (bottom). Equivalent periods between humans and mice are represented at the same level of the timeline, so human birth would correspond to P7 in mice. GW, gestational week. *Adapted from Chini and Hanganu-Opatz (2021).*

In conclusion, INs display a protracted and progressive development with the sequential and overlapping acquisition of morphological and neurochemical characteristics by the interplay of several determinants that extends until adolescence and early adulthood in humans (Figure 6). On one hand, intrinsic factors defined by place of birth and transcription factors (TFs) expressed, determine neurochemical characteristics and connectivity after migration. On the other hand, extrinsic factors such as neuronal activity regulate the development and number of dendritic branches and axonal varicosities, as well as the level of expression of neuropeptides. Moreover, IN alterations during development, do not only include the loss of certain inhibitory mechanisms, but rather are crucial for the maturation of neuronal circuits and neural synchrony, especially in phases of activity-dependent

remodeling (Hensch, 2005; Uhlhaas et al., 2010). Consequently, the pathological phenotype would reflect the alterations in the maturation of neuronal circuits and the dynamic adaptation of those to the changes during development, so that most likely other neurotransmitter systems are also perturbed.

Classification of INs

In the adult brain, INs are a highly heterogeneous population with more than twenty different subtypes. The INs subtypes are distinguished by their morphology, location, biochemical profile, intrinsic electrophysiological properties, and connectivity pattern (Lim et al., 2018; Pelkey et al., 2017). As aforementioned, the function of INs is to provide basic inhibition and shape spatiotemporally the activity profile of principal neurons. Within the hippocampus, INs have specific projection patterns that are important because affect differently the activity of excitatory neurons. For instance, inhibitory synapses on cell bodies or axon initial segments control the genesis of action potentials, whereas INs targeting the dendrites may control dendritic calcium spikes and regulate the plasticity of other excitatory inputs from principal cells and remote excitatory afferents (Freund & Buzsáki, 1996; Miles et al., 1996). Moreover, some INs establish inhibitory synapses to other INs, leading to disinhibition. Overall, INs with specific projection patterns also express particular calcium-binding proteins or neuropeptides. Although INs can be classified depending on axonal projection, additional diversity can be found in their firing pattern and response to modulating transmitters. Thus, hippocampal INs cannot be easily ordered in a few well-defined groups when several criteria are taken into account. Despite that complexity, a basic illustration of the main biochemical INs subgroups in the adult hippocampus is described below since those are the main markers used for the analysis of grafted cell phenotype in transplantation studies.

Two main biochemical determinants can be used for this classification: calcium-binding proteins and neuropeptides. Neurons expressing calcium-binding proteins PV, CR, and calbindin (CB) represent largely nonoverlapping subpopulations of INs. PV-positive IN subpopulation is originated from the MGE and is divided into chandelier or axo-axonic cells and baskets cells, which form synapses to the axonal initial segment or the perisomatic region of principal cells, respectively. Then, CR-positive INs are generated from CGE and can be divided into spiny, which innervate dendrites of principal cells, and aspiny neurons that connect to other INs. Importantly, CR expression exhibits specie differences, and it is also expressed by Cajal-Retzius cells during development. Similarly, CB is expressed in INs generated both in the MGE and CGE with connections to the principal cell dendrites or distal projections to the medial septum, but also granule cells (negative for GABA). On the other hand, different neuropeptides have been identified to be expressed in INs, which are SST, cholecystokinin (CCK), vasoactive intestinal peptide (VIP), and NPY. SST-expressing INs originate from the MGE and are highly heterogeneous, although forming dendritic connections to the principal cells. Cholecystokinin-

expressing INs constitute another population of basket cells that form perisomatic synapses to the principal cells. Sometimes, a combination of those molecules can be found in subpopulations, such as CR/VIP, CR/SST, CB/SST, and NPY/SST (Danglot et al., 2006; Freund & Buzsáki, 1996; Kepecs & Fishell, 2014).

Different IN subtypes also have particular spiking patterns, that together with different axonal projections may be involved in shaping specific oscillatory activities. In addition, interconnection of GABAergic neurons may be crucial for their effect on oscillations, including within the local network by electrical and chemical synapses, and also GABAergic afferent and efferent connections outside the hippocampal formation.

Promising new avenues for epilepsy treatment

The complexity of the central nervous system and the presence of the blood-brain barrier (BBB) hinder traditional pharmacological treatments, which are proven to be often only symptomatic and insufficient in several neurological diseases, particularly in epilepsy. Therefore, new strategies are required to overcome this situation, and cell and gene therapies are spotlighted to hold a better promise.

Cell therapy

Most neurological disorders, such as epilepsy, are often associated with neuronal loss at some point during the disease course. That commonly derives to deterioration of the physical and intellectual function, and represents a significant health and economic burden. Besides the limited endogenous regenerative capacity of the adult human brain, current treatments for such disorders are largely symptomatic. However, those symptomatic treatments often only achieve transient and partial efficacy and do not offer substantial functional recovery. Alternative strategies optimally generating a disease-modifying effect are highly warranted (Grade & Götz, 2017; Steinbeck & Studer, 2015).

To this issue, cell therapy represents a promise for brain repair. Cell therapy aims to replace, repair, or enhance the biological function of a damaged tissue or organ by either transplanting cells, in sufficient number and quality, or by modulating specific cell populations in the target organ to ameliorate and/or restore the lost function (Upadhyay et al., 2015). For instance, in the case of epilepsy, the transplantation of cells into the seizure onset area may aim at replacing neurons injured/lost in the damaged region, or at releasing substances such as neuropeptides that modulate the microenvironment (Raedt et al., 2007). While the second approach holds great potential and may be achieved by encapsulated cell delivery of glial derived neurotrophic factor (GDNF) (Nanobashvili et al., 2019) or mesenchymal stem cells

secreting anti-inflammatory and trophic factors (Agadi & Shetty, 2015; Hlebokazov et al., 2017), this section will focus on the first strategy of replacing neurons lost in the damaged area since it is the one explored in this thesis.

Since the first successful bone marrow transplantation in 1968 (Bach et al., 1968), stem cells of various origin and commitment levels within lineages have emerged as promising cell sources for their use as cell therapy. Stem cells are cells able to self-renew unlimited times and generate differentiated daughter cells, although called stem cells, not all preserve the same potency. All cells in the body, and even the placenta, originate from the zygote, the ultimate totipotent stem cell. Along with development, stem cells undergo progressive specialization giving rise to different embryonic tissues, and therefore, narrowing down in stemness to be more determined to certain cellular lineages. First, when an embryo reaches the stage of the blastocyst, cells in the inner mass are only able to generate all cells in the body, but not the placenta anymore, and this capacity is called pluripotency. Importantly human embryonic stem cells (hESCs) are derived from the inner mass of blastocysts retaining the characteristic capacity of the tissue origin (Figure 7). This specialization continues during development, so that, for example, neural stem cells only give rise to cells in the neural lineage that includes neurons, astrocytes, and oligodendrocytes. Mature neurons are extremely fragile to detachment and transplantation, so neuronal progenitors/precursors are usually the choice for cell therapy. Importantly, to achieve optimal results and reconstruction of the neuronal circuits, it is critical a precise determination of the type of cells needed, as well as when and where to deliver them.

Generation of neural cell types from various sources and their use in epilepsy

Primary fetal cells

Pioneering studies performed during the 80's and beginning of the 90's using fetal brain tissue for grafting in preclinical models of Parkinson's disease (Brundin et al., 1986; Dunnett et al., 1981) followed by early transplantations in patients (Lindvall et al., 1990; Lindvall et al., 1989) and later on also in Huntington's disease patients (Bachoud-Lévi et al., 2000; Reuter et al., 2008) represented a breakthrough in the translational applications using neural cells. Results from these clinical trials represented a proof-of-principle that neuronal replacement is feasible in the adult human brain offering long-lasting improvements (Lindvall, 2015). Fetal midbrain dopaminergic precursors were able to survive and mature into dopaminergic neurons acquiring the correct subtype within the striatum of the host (Barker et al., 2013; Grealish et al., 2010).

When pursuing cell transplantation studies, it is important to keep in mind that the immune system of the host body that is responsible to protect it from any foreign threats. Even though the brain is considered a partially immune-privileged organ, stereotaxic transplantation surgery leads to temporary disruption of the BBB.

Therefore, patients were immunosuppressed for up to 12 months in the Parkinson's disease trial, a relatively short time although enough for the BBB to seal postoperatively (Barker & Widner, 2004).

Despite this remarkable achievement in patients from Lindvall and colleagues, posterior double-blind placebo-controlled trials aiming to reproduce the initial findings failed to reach the primary endpoints (Freed et al., 2001; Olanow et al., 2003). Those studies revealed and highlighted several critical factors conditioning the success of the study, from patient selection to immunosuppression including issues related to the donor cell population such as gestational age, purity, quality, and storage (Barker et al., 2013). Beyond those factors, logistical challenges and ethical concerns were raised considering that tissue from several aborted fetuses was used for just a single patient and it is a disease affecting millions of people.

In epilepsy, pathological changes enhance E/I balance towards excitatory processes in the epileptic focus. Among those alterations, selective neurodegeneration of interneurons, leading to a loss of functional inhibition has been described. Therefore, cell therapy for epilepsy might be based on transplanting inhibitory GABA-releasing cells into the epileptic focus or sites that contribute to the spread of seizures.

The first studies evaluating the effects of neural transplantation in epilepsy models were done by Björklund, Lindvall, and colleagues using rat fetal neural tissue (Lindvall et al., 1994). Indeed, grafting fetal GABAergic cells or inserting GABA-releasing matrices into the epileptic foci has shown to have anticonvulsant effects in numerous animal models (Figure 7) (Holmes et al., 1991; Kokaia et al., 1994; Miyamoto et al., 1993). However, the anti-seizure effect was transient or modest in most of the studies. Other studies reported that the grafts themselves might generate seizures under certain circumstances (Buzsáki et al., 1989; Buzsáki et al., 1988). Moreover, the effect of transplantation of fetal GABAergic neurons targeting other regions of critical importance for seizure propagation was also explored. In particular, intranigral grafts demonstrated to raise local GABA concentrations and have a transient pronounced anticonvulsant effect (Löscher et al., 1998).

Finally, the first clinical trial was approved by the Food and Drug Administration (FDA), and porcine fetal GABA-producing neural cells were transplanted in three patients with refractory focal epilepsy. All patients showed an improvement in seizure frequency, but unfortunately, the trial was stopped for potential risk for cross-species infection by retroviruses (Schachter, 1998).

Primary neural stem/progenitor cells

Limitations in tissue quality, availability, and ethical concerns of the use of primary fetal cells made the field find an expandable cell population that could be prepared for therapeutic use in standardized and quality-controlled conditions. In this line, human fetal neural stem cells were shown to be expandable *in vitro* both as

neurospheres and monolayer culture (Caldwell et al., 2001; Flax et al., 1998; Sun et al., 2008), as well as multipotent neural progenitor cells were expanded from several regions of the adult human brain (Nunes et al., 2003). Moreover, it has been demonstrated that neural stem/progenitor cells (NSPCs) survive transplantation into immunocompromised rodents, differentiate into mature neurons, and innervate the regions of interest. Although fate specifications and differentiation to certain mature neuronal subtypes, and the therapeutic potential of the derived neurons were quite limited. Nonetheless, many therapeutic principles have been first described using rodent primary donor cells.

The transplantation of progenitor cells from embryonic rat LGE (Hattiangady et al., 2008) and mouse/rat MGE (Hunt et al., 2013) have shown long-term survival in the host brain (up to 12 months), long-lasting suppression of SRS, and reduced behavior comorbidities in various rodent models of epilepsy. Moreover, rodent MGE progenitor cells have been used as a prophylactic treatment in genetic epilepsy models (Baraban et al., 2009) and maximum electroconvulsive model (Calcagnotto et al., 2010), as well as in post-SE model of epilepsy (Henderson et al., 2014; Hunt et al., 2013; Waldau et al., 2010). In all cases, no changes in histopathological features of TLE such as mossy fiber sprouting were seen, so the seizure-suppression effect was most likely due to enhanced inhibitory synaptic transmission. This was supported by the inability of grafts to exert such effect at early periods, but rather need protracted times for functional maturation.

Neural cells derived from pluripotent stem cells

Relatively recent discoveries have fueled the field pushing it forward by providing a human, illimited, expandable, and highly kneadable cell source. First, Thomson and colleagues isolated for the first time hESCs (Thomson et al., 1998), which have stemness capacity and can generate a variety of specialized neuronal subtypes relevant for cell therapy (Figure 7). In 2006, Yamanaka and Takahashi published a ground-breaking work proving that somatic cells, more specifically mouse fibroblasts, could be reprogrammed back to the pluripotent state by overexpression of certain TFs, named as induced-pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006). A year later, the same principle was proven with human adult fibroblasts using a minimal combination of four TFs: *Oct4, Sox2, Klf4,* and *cMyc* (OSKM) (Takahashi et al., 2007). Further studies proved that iPSCs were molecularly and functionally equivalent to ESCs (Figure 7). Moreover, iPSCs could be derived from the patient's fibroblasts, so that do not have ethical or immune rejection associated issues as ESC and fetal primary cells do (Okita et al., 2007; Wernig et al., 2007). The fact that iPSCs can be derived from patients' fibroblasts opens the possibility for disease modeling and drug screening since derived neurons recapitulate disease-specific features in a dish (Brennand et al., 2011; Dimos et al., 2008).

Various neural cell types (Reubinoff et al., 2001; Zhang et al., 2001) and developmentally distinct neural stem cell populations (Elkabetz et al., 2008; Koch et al., 2009) have been generated from those pluripotent stem cells. These derived cells have comparable properties to primary NSPCs in terms of expandability and differentiation potential, with the addition of a higher control for neuronal subtype specification. In synergy with these advances, numerous strategies were developed to generate region and subtype-specific neuronal fates from those human pluripotent stem cells without transition through the neural stem cell intermediate state. The strategy that started using embryoid body cultures to enhance neuronal induction and further rosette formation, evolved towards using recombinant morphogens and growth factors known to be involved in early mammalian CNS development (Pera et al., 2004). Lately, it also included small molecules interfering in key developmental pathways (Chambers et al., 2009; Smith et al., 2008). Altogether, these new strategies decreased the differentiation timings and provided an unprecedented efficiency and purity of the final populations.

When aiming for generating INs, usually sonic hedgehog agonists are used to pattern cells towards a ventral fate of the subpallial regions of the telencephalon and induce robust *NKX2.1* expression (Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013). Several IN subtypes are obtained, with SST-positive cells usually being more abundant. Difficulties in generating PV-positive cells are most likely related to its late developmental expression at postnatal stages. Overall, MGE-like cells derived from hESCs and hiPSCs require an extended time to maturate functionally *in vitro* (Nicholas et al., 2013) and *in vivo* (Cunningham et al., 2014). Nonetheless, it is critical to purify derived NSPCs before transplantation to avoid the possibility of teratoma formation. One solution could be to modify the cells to express the green fluorescent protein (GFP) under specific markers, such as *LHX6* which is a promoter active in postmitotic interneurons derived from MGE, so that they could be sorted for GFP expression before the transplantation (Maroof et al., 2010).

Recent studies proved that hESC- and iPSC-derived GABAergic INs can integrate into the neuronal circuitry in the epileptic hippocampus, remarkably reduce seizure frequency, alleviate comorbidities of epilepsy and improve memory (Cunningham et al., 2014; Upadhya et al., 2019; Waloschková et al., 2021).

The use of both cell sources, hESC and iPSC, for cell therapy, presents different pros and cons. On one side, the use of hESC is associated with ethical concerns, while hiPSC skip that matter. On the other side, the derivation of hiPSCs for each patient, so-called personalized medicine, requires a long time as well as quality controls, making it logistically and economically unfeasible (Stadtfeld & Hochedlinger, 2010). Another concern is the possibility of patient-derived iPSCs of having disease-specific genetic backgrounds. Moreover, both stem cell sources carry a certain risk of tumor formation (Miura et al., 2009). Currently, in the new planned clinical trials for Parkinson's disease, three different locations are involved in the transplantations, two using hESCs-derived NSPCs (Europe and US) (Kirkeby

et al., 2017; Studer, 2017), while the third one using iPSC-derived NSPCs (Japan). The iPSCs used are not patient-specific (autologous) but established from individual volunteers with homozygous human leukocyte antigen (HLA) haplotypes (allogenic) and stored in a bank of iPSCs that covers the most common haplotypes (Takahashi, 2017). In the clinical trial using the strategy of HLA-matching iPSCs to reduce immune response, no immunosuppressants are considered. Although for the other two trials, 9-12 months of immunosuppressants following the transplantation are planned to be used, as previously described to be efficient in offering graft survival up to 24 years (more than 20 years after withdrawn of the immunosuppressants) and without triggering side effects in the liver or the kidney (Barker & Widner, 2004; Li et al., 2016). Finally, in the NRTX-1001 clinical trial for epilepsy carried out by Neurona Therapeutics, it is planned to immunosuppress the subjects for 1 year as well since NRTX-1001 are derived from hESCs.

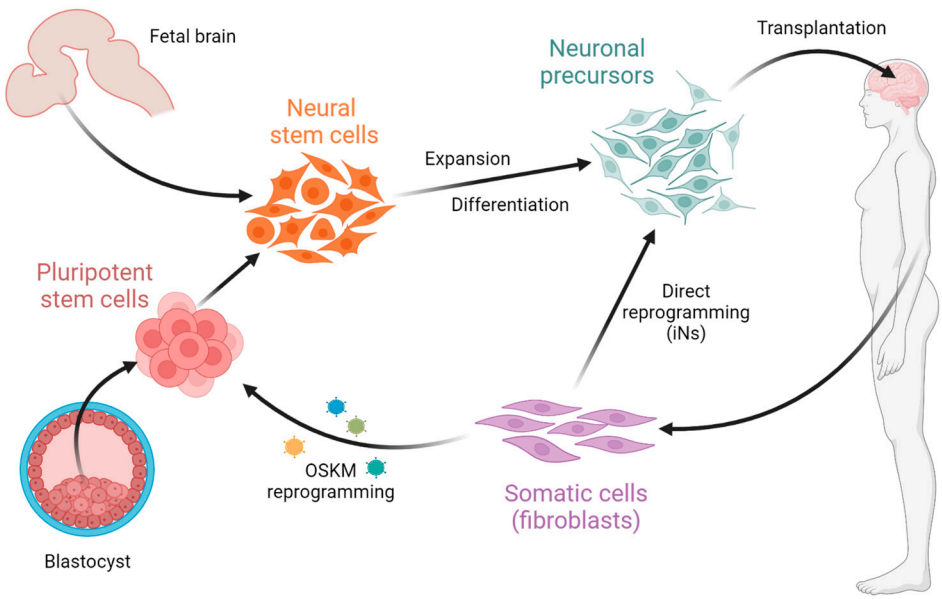

Figure 7. Alternative neuronal sources for cell therapy
Transplantable GABAergic interneurons can be isolated from developing fetal ganglionic eminences (the top left), differentiated from pluripotent stem cells including hESC and iPSC (bottom left), or generated via direct reprogramming of somatic cells to iNs. OSKM, *Oct4, Sox2, Klf4,* and *cMyc*.

Alternative sources and direct reprogramming

For many years, the unidirectional process of cell differentiation occurring during development was considered the dogma of cell fate determination (Waddington & Kacser, 1957). However, the epigenetic landscape illustrated by Waddington and colleagues changed after the nuclear transfer experiments in frogs by Gurdon in 1962 (Gurdon, 1962), and the more recent discovery that somatic cells could be

reprogrammed back to a pluripotent state in 2006 (Takahashi & Yamanaka, 2006). Both authors shared the Nobel Prize in Medicine in 2012 for those discoveries. The next big step in the reprogramming field was proving that somatic cells could be reprogrammed directly to another somatic cell from a different germ layer. In 2010, Wernig and colleagues showed direct reprogramming of mouse fibroblasts (mesodermal lineage) into induced neurons (iNs) (ectodermal lineage) by overexpression of three TFs (Vierbuchen et al., 2010), which opened new opportunities for regenerative medicine (Figure 7). Hence, direct conversion bypasses the pluripotent state, which decreases the risk of teratoma formation and shortens the differentiation time in culture. Besides increasing studies generating diverse human iNs for disease modeling and potential future use as cell therapy, there are many open questions and issues that need to be addressed. For instance, a low conversion rate, limited cell yield after the conversion compromising future translation, verification of a long-term sustainable genetic program after conversion, and limited functional maturation of the iNs often not acquiring appropriate membrane excitability or synaptic drive (Broccoli et al., 2015).

Inspired by the discovery of the *in vitro* direct conversion strategy, the same principle has been applied *in vivo,* reprogramming residing astrocytes and reactive microglia (Grande et al., 2013; Guo et al., 2014; Torper et al., 2013). Those studies have shown promising results in reducing seizures in a mouse model of TLE (Lentini et al., 2021). Moreover, the use of pro-neural and subtype-specific TFs and small molecules allied to pluripotent stem cells has demonstrated accelerated neuronal differentiation (Yang et al., 2017; Zhang et al., 2013).

Currently, there is one cell therapy based on neuronal replacement for refractory epilepsy in clinical trials. NRTX-1001 are hESC-derived MGE-like post-mitotic interneurons that release GABA, developed by Neurona Therapeutics (NCT05135091). Preclinical results have shown that NRTX-1001 cells expressed LHX6 (91 %), and also expressed SST (23 %), while did not express markers of proliferative (KI67), progenitor (NKX2.1), or glial (GFAP or OLIG2) cells.

Gene therapy

Some of the new therapies in the pipeline and in preclinical studies to address the unmet need of developing effective treatments for refractory epilepsy patients encompass gene therapy. The principle of gene therapy is to alter the gene expression profile of specific cells to treat or cure a disease. This approach can be achieved as *in vivo* gene therapy using a direct transfer of the desired gene by either viral or non-viral vectors into the living organism, or by *ex vivo* gene therapy so that cells are engineered to express a transgene outside the living body and then implanted into the tissue. Overall, gene therapy offers advantages over conventional treatments such as it only requires one administration for a long-lasting effect, so

that it can be delivered inside the brain overtaking limited BBB penetrance and interactions with targets outside the CNS (Simonato et al., 2013).

Extraordinary progress has been done in the past few years with more than 20 gene therapy products licensed by the FDA and/or the European Medicines Agency (EMA). Besides mostly available treatments are designed for hematology and oncological diseases, there is one directed to the CNS. Gene therapy for spinal muscular atrophy was recently approved for clinical use by both FDA and EMA (Mendell et al., 2017).

Delivery systems

The success of gene therapy depends to grand extent on effective gene delivery. Great effort has gone into the creation of nonviral gene delivery vehicles such as liposomes and nanoparticles due to their low immunogenicity, cost, and large size cargo, although they have yet only achieved a low and time-restricted gene expression. Therefore, viral vectors remain a more efficient delivery vehicle for genetic material since they have evolved and refined in that direction over centuries. Nonetheless, wild-type viruses need to be hijacked to be suitable for therapeutic purposes, so that most of the viral genome needs to be replaced by the desired genetic material to be introduced in the target cells. Thus, the viral particles are able to infect the cells and transfer the genetic material to them so they will express it, but the viral vectors cannot replicate themselves since they lack the necessary information to do so. All different viral vectors have advantages and disadvantages. The most widely used vector types for gene therapy are lentiviral vectors (LV) and adeno-associated viral vectors (AAV).

The LVs consist of a single-stranded RNA limited by LTR sequences, with a maximum size of 9-10 Kb. They are relatively easy to produce and are the method of choice for *in vitro* preparations since the genetic material carried by the vector can be integrated into the host cell genome, both in replicative and quiescent cells. However, the insertion of the genetic material could lead to oncogenesis depending on the location in the genome that it happens, resulting in a potential problem of these viral vectors in clinics (Mátrai et al., 2010). On the other hand, AAVs are single-strand DNA vectors, capable of infecting dividing and non-dividing cells. They are non-integrative vectors since they tend to remain episomal, altogether reducing the risk of mutagenesis (Hitti et al., 2019). Due to the small size, they can only accommodate much smaller constructs, 4.7 Kb, and require a high dose for effective gene expression. Nevertheless, AAVs are usually the choice for *in vivo* delivery since the smaller size (25 nm) affords a larger coverage of certain areas from single injection sites (Gonçalves, 2005). Importantly, there is a risk of some individuals carrying neutralizing antibodies towards specific types of AAVs since humans are normally exposed to wild type (WT) AAV viruses, and thus those individuals will not be able to receive the potential therapy (Sun et al., 2003).

In the case of AAVs, there are several WT capsid proteins known as serotypes resulting in different vector tropisms (Castle et al., 2016). Moreover, considerable advances have been made in terms of overall transduction efficiency, change tropism, susceptibility to antibodies, ability to cross the BBB, and even retrograde transport by mutagenesis of the capsid for the derivation of novel recombinant capsids (Castle et al., 2016). For instance, AAV9 has the ability to cross the BBB after intravenous injection. Further refinement of vector targeting may be done by the addition of microRNA target sequences downstream of the transgene. Those sequences are recognized by microRNAs highly expressed in certain tissues or cells and, therefore, the transgene will not be expressed in that cell type or tissue (Bennett et al., 2012).

The level of transgene expression and the specificity of the expression is determined by the choice of the promoter placed upstream to the transgene. For targeting principal (excitatory) cell populations, transgenes are usually driven by the calcium/calmodulin-dependent protein kinase type II subunit alpha (CamKIIα) promoter (Liu & Jones, 1996), which also provides high enough expression levels. Unfortunately, cell-type-specific promoters for GABAergic INs or IN subpopulations are less efficient, so gene expression is too weak. Moreover, it is worth keeping in mind that not all the neurons contribute in the same way to the hyperexcitability of the network for the seizure onset, not even all the excitatory CamKIIα-positive neurons do (Wenzel et al., 2017). To affect only the neurons contributing the most in these ictogenic and epileptic dynamics, there is the possibility to target the hyperexcitable neurons by using promoters for activity-dependent genes such as *cfos* (Kawashima et al., 2014; Sørensen et al., 2016).

In addition, there are different strategies to regulate gene expression in time. One is the use of inducible promoters such as Tet-On and Tet-Off system, or even the new splicing system X^{on} (Monteys et al., 2021), which allows to modulate the effect of the therapy on dosage and time by using an orally administered drug. Similarly, chemogenetics and optogenetics may be combined with gene delivery systems for on-demand control of the effect of the therapy. Optogenetics allows for temporal control in a resolution of milliseconds, although it is technically more challenging and less translational since it requires delivery of light in the target brain area (Boyden et al., 2005). Whereas chemogenetics has slower temporal resolution but it is easier applicable since it requires oral delivery of the effector molecule and it has longer-lasting effects (Armbruster et al., 2007). For more information regarding optogenetics and chemogenetics, I kindly refer the reader to the *Key experimental procedures* section of this thesis.

Candidate genes for epilepsy

Epilepsy represents an opportunity for testing gene therapy for neurological disorders. First of all, due to the huge therapeutic need for alternative therapeutic strategies to treat refractory epilepsy that represents large patient numbers. It also

has the unique possibility to test the efficacy of the therapy in a selected population, that being refractory epilepsy patients selected for surgery. Therefore, there is a rescue strategy in case of therapy failure since patients would then be treated with resective surgery as it was planned initially, and consequently, there are no significant ethical hurdles. Moreover, the therapy could be assessed by a clear, reliable, and objective readout, the EEG-recorded seizures.

The potential of gene therapy in epilepsy has been demonstrated in several preclinical studies mainly using transgenes to modify the cell function and control hyperexcitability (Kullmann et al., 2014). In that line, different transgenes have been used with promising results, from ion channels i.e. potassium channels (Colasante et al., 2020; Snowball et al., 2019), to neurotransmitters (Richichi et al., 2004) and neurotrophic factors i.e. BDNF and FGF2 (Paradiso et al., 2009), to finally receptors i.e. Y2 (Woldbye et al., 2010).

To have a temporal control of the activation or inhibition of certain cell populations, both for gaining understanding and as a potential therapeutic approach, optogenetics and chemogenetics have been combined with viral vector delivery. Optogenetics has shown seizure suppression effect both by inhibiting excitatory principal cells using halorhodopsin and by activating PV-expressing interneurons with channelrhodopsin (ChR2) (Krook-Magnuson et al., 2013). Similarly, chemogenetics has also been used to achieve seizure reduction in rodent models of epilepsy (Avaliani et al., 2016; Călin et al., 2018; Desloovere et al., 2019; Kätzel et al., 2014; Wang et al., 2018; Zhou et al., 2019). Most of these approaches are based on Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) that are derived from G-protein-coupled receptors and are activated by a designer drug called clozapine N-oxide (CNO). Besides the promising results obtained in epileptic models, DREADDs have the drawback that CNO cannot cross the BBB. Therefore, most likely its effect on the DREADDs expressed in the brain is by being back-converted to clozapine, a molecule that interacts with other receptors in the CNS and might lead to side effects (Gomez et al., 2017). To overcome this limitation in clinical translation, other chemogenetic tools with a more translational profile are being tested, such as synthetic ligand-gated ion channels (LGICs) (Lieb et al., 2018), including $PSAM^4$-GlyR presented in this thesis (Magnus et al., 2019).

In addition, gene therapy has also demonstrated positive results in a mouse model of a genetic epileptic encephalopathy, the *Wwox* KO mice, by restoring the expression of the missing gene (Repudi et al., 2021). Another study targeting a Dravet syndrome model, an intractable developmental and epileptic encephalopathy caused by *de novo* variants in the *SCN1A* gene, has used a non-conventional delivery system based on intracerebroventricular administration of antisense oligonucleotides (naked DNA) (Han et al., 2020). Results from this study showed an up-regulation in the expression of productive *Scn1a* transcript, which resulted in an increase in NaV1.1 expression, reduced seizures, and prevented epilepsy-associated death (Han et al., 2020; Wengert et al., 2022).

New approaches are also exploring the effect of modulating non-coding RNAs since they have been described to be altered in epilepsy as well. For instance, Reschke and colleagues have proven potent anti-seizure effect in rat models of epilepsy by intracerebroventricular administration of antagomirs targeting microRNA-134 (Reschke et al., 2017).

Finally, two gene therapies for refractory epilepsy have already reached clinical trials and are currently starting phase I/IIa. The first one consists of lentiviral gene therapy using an engineered potassium channel to treat patients scheduled for resective surgery for refractory neocortical epilepsy (# NCT04601974). The other one is based on the antisense oligonucleotides against the *SCN1A* mRNA transcript mentioned above (# NCT04442295 and NCT04740476).

AIMS OF THE THESIS

Despite the availability and development of new ASM in recent years, one-third of patients remain unresponsive and the ones responding must cope with side effects. Therefore, there is a need for the development of novel approaches to achieve seizure control in these drug-resistant patients. Having this scenario in mind, the overarching goal of this thesis has been to develop and test the suitability and seizure suppressing effects of novel approaches that aim to enhance neuronal inhibition in the epileptic foci.

The specific key research objectives addressed in the thesis can be divided according to the main strategy used:

- Cell therapy
  - I. To generate a pure population of hESC-derived GABAergic interneurons (hdINs) *in vitro* and characterize them functionally. **(Paper I)**
  - II. To investigate the capacity of hdINs to survive, maturate and integrate into:
    - Healthy cell culture of human fetal primary neurons. **(Paper I)**
    - Human adult chronic drug-resistant epileptic brain tissue. **(Paper I)**
    - Hippocampi of KA-induced chronic TLE adult rat model. **(Paper II)**
    - Hippocampus of *Cntnap2* KO mouse model for ASD and CDFE. **(Paper III)**
  - III. To elucidate the effect of grafted hdINs on:
    - Epileptiform discharges *in vitro* and SRS *in vivo* in a KA-induced chronic TLE adult rat model. **(Paper II)**
    - Epileptic phenotype and behavior of the *Cntnap2* KO mouse model. **(Paper III)**
- Gene therapy
  - IV. To assess the suitability of increasing chloride membrane permeability in principal cells by means of $PSAM^4$-GlyR to decrease excitability in the epileptic hippocampus. **(Paper IV)**

SUMMARY OF RESULTS AND DISCUSSION

Over the last 80 years, an exponential increase in the development of new ASMs has occurred (Löscher et al., 2020). Despite this remarkable expansion of therapeutic options for epileptic patients, these medications only treat the symptoms and are associated with adverse side effects (Chen et al., 2017a; Chen et al., 2017b; Cramer et al., 2011). Moreover, one-third of the patients remain unresponsive to the medication (Duncan et al., 2006; Engel Jr, 2002). Hence, this thesis evolves around developing and testing novel therapeutic approaches targeting patients with drug-resistant epilepsy. The rationale of these new approaches is to enhance the inhibitory component of the neuronal network to restore the imbalance between excitatory and inhibitory processes in the epileptic brain, as illustrated in Figure 8.

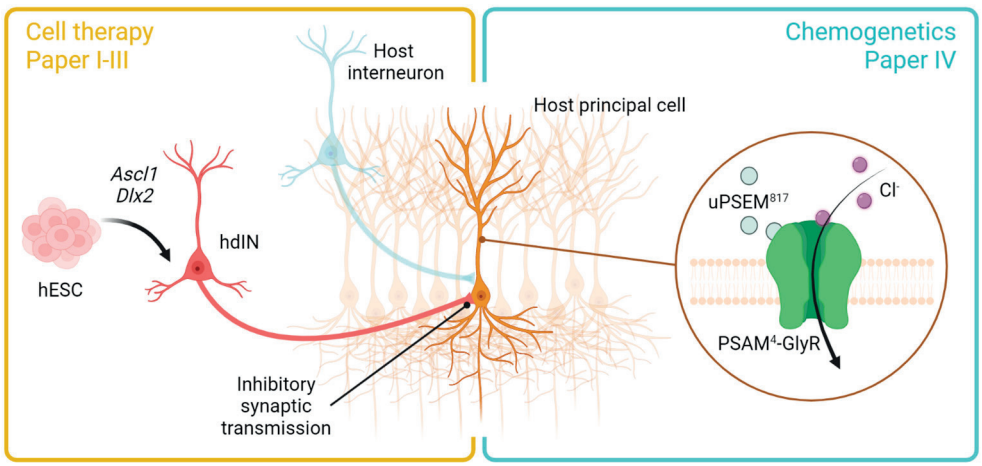

Figure 8. Overview of the two inhibitory approaches explored in this thesis
On the center, there are excitatory principal cells (orange) from the host tissue, and slightly to the left an endogenous inhibitory interneuron connecting to the principal cell (cyan). On the left, hdINs generated from hESC form efferent synapses to the principal cells. On the right, principal cells express a chemogenetic channel (PSAM^4-GlyR) on their membrane, which has been introduced by viral particles. PSAM^4-GlyR when activated by uPSEM^{817} allows chloride ions to flow across.

To this goal, both cell and gene therapy have been explored. In **Paper I**, a pure population of hESC-derived GABAergic interneurons (dhINs)[1] is generated and characterized *in vitro*. The functional synaptic integration of hdINs is tested in a human environment, both onto healthy human primary neurons and onto epileptic human brain tissue (**Paper I**). In addition, the survival, and seizure suppression effect of hdIN transplantation was assessed in a chronic rat model of TLE (**Paper II**) and in a genetic model of ASD with CDFE syndrome (**Paper III**) (Figure 8, left yellow). In **Paper IV**, the possibility to decrease the excitability of principal neurons by increasing chloride membrane permeability, as would result from the activation of $GABA_A$ receptors, was assessed. Chemogenetics were used to drive inhibition of the principal cells in the chronic epileptic hippocampus (Figure 8, right cyan).

Prominent GABAergic phenotype of hdINs

– Paper I

GABAergic INs are a key neuronal population in the brain that controls network excitability, among other functions such as modulating synchronous network oscillations and plasticity (Lehmann et al., 2012; Ouellet & de Villers-Sidani, 2014). Therefore, dysfunction or absence of interneurons has been associated with many neurological disorders, including epilepsy, schizophrenia, and ASD (Magloire et al., 2019; Steinecke et al., 2012). For these reasons, different studies have focused on generating specific subpopulations of GABAergic INs for either disease modeling or cell therapy (Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013; Yang et al., 2017).

Considering the limited endogenous regeneration capacity of the human brain (Paredes et al., 2018), and the implication of decreased numbers of INs in the hippocampus of TLE patients as a major factor orchestrating the hyperexcitable neuronal network and seizure occurrence (Liu et al., 2014; Sun et al., 2007; Zhang et al., 2009), transplantation of GABAergic INs, derived from different sources, in the epileptic brain has been suggested as an exogenous cell source to restore the E/I balance and ameliorate the network excitability. Indeed, several studies in epileptic animal models have demonstrated the beneficial effect of transplanting GABAergic neuronal precursors derived from various sources in the epileptic focus (Baraban et al., 2009; Casalia et al., 2017; Hattiangady et al., 2008; Henderson et al., 2014; Hunt et al., 2013; Waldau et al., 2010). Focusing on the variety of possible cell sources that fit the purpose, an initial classification could be the species of origin. Initial transplantation studies have used rodent fetal MGE neuronal progenitors, which although providing a proof-of-principle for the potential efficacy of this treatment

[1] Human ESC-derived GABAergic interneurons are named as hdINs in this thesis. The same name is used in Paper I and III, although notice that in Paper II they are referred to as hdInt.

strategy *in vivo*, have limited if any translational value since they cannot be applied clinically due to ethical concerns and variability in the quality of cell sources (Turner & Shetty, 2003). Similar concerns were arisen after the first transplantations in Parkinson's disease patients using human fetal tissue over the 1990s (Brundin et al., 2000; Lindvall et al., 1990). A more translational and viable source of neuronal precursors for a clinical application that would circumvent the issues exposed above is the use of human pluripotent stem cells. In that case, the host would then require immunosuppression to avoid rejection of the xenograft in the rodent or HLA and other antigen mismatching in human transplants. MGE-like neuronal precursors from both hESC and hiPSC have proven to survive and inhibit SRSs and behavioral comorbidities in various TLE models (Cunningham et al., 2014; Upadhya et al., 2019). From a clinical perspective, some limiting factors will determine which protocols are more suitable for this translation, such as the absence of proliferative cells at the time of transplantation to reduce the risk of teratoma formation. Another factor is the timing and simplicity of the protocol that increases sustainability, lowers the demand for resources, and increases reproducibility. In 2017, Wernig's lab developed a fast, simple, and efficient protocol to generate GABAergic neurons *in vitro* by using TF reprogramming (Yang et al., 2017). This protocol required a shorter time of differentiation and obtained a higher yield of INs compared to those published elsewhere based on the use of small molecules (Au et al., 2013; Liu et al., 2013; Maroof et al., 2013; Nicholas et al., 2013). Therefore, by adapting Yang *et al.* (2017) protocol, a pure population of GABAergic INs was generated *in vitro* in **Paper I**, with the perspective to be used as cell therapy.

Human ESCs were differentiated to GABAergic INs by using the *Ascl1* and *Dlx2* (AD) TF reprogramming approach, described in detail in the *Key experimental procedures* section of this thesis (Gonzalez-Ramos et al., 2021; Yang et al., 2017). The TF *Ascl1*, also known as *Mash1*, is expressed by subpallial progenitors in the ganglionic eminences. Consequently, *Ascl1* induces the expression of *Dlx1/2* in those cells, which thus induces the production of GABA by expression of GAD (65 and 67) (Anderson et al., 1999). This protocol induces a fast and dynamic switch from the expression of pluripotency markers (*POU5F1*) towards neuronal markers (*MAP2* and *SYN1*) around 4 and 7 days *in vitro* (DIV). These observations were the reason for choosing 7 DIV as the most suitable day for transplantation of the cells since they are already non-proliferative neuronal precursors, assessed by negative reactivity against the proliferative marker Ki67. Importantly, AD reprogramming did not generate astrocytes (*GFAP*) or oligodendrocyte precursors (*PDGFRα*) *in vitro*. At 35 and 49 DIV, most of the hdINs were positive for GABA (Figure 9A-B) and GAD65/67 markers (approximately 87 and 89 % respectively). The identity was confirmed by high *GAD1* expression levels. Within the GABAergic neuronal population, the most abundant subtypes were CB and CR expressing INs, representing approximately 36 and 29 % respectively (Figure 9C-D). Those results were consistent with the ones described in the initial publication, where 89 % of neurons were positive for GABA, and regarding different interneuron

subpopulations, 37 % were CR+, 28 % CB+ and 9 % SST+. Only the SST population differed in **Paper I** results compared to the initial ones. Finally, the GABAergic nature of hdINs was confirmed by electrophysiology as shown in Figure 9E, so that a significant decrease in amplitude and increase in inter-event interval of spontaneous synaptic currents were observed when adding picrotoxin (PTX), a $GABA_A$ receptor blocker, but not when blocking α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors by applying 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX) and 2-Amino-5-Phosphonovalerate (AP-5) respectively (Figure 9F).

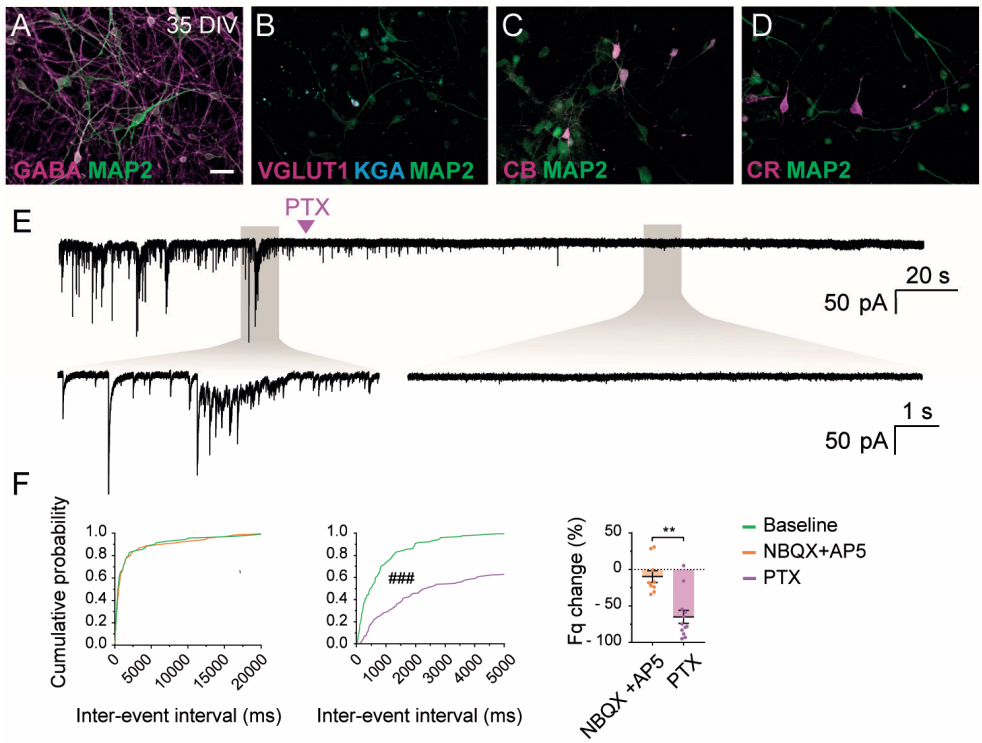

Figure 9. Biochemical and functional GABAergic phenotype of the hdINs
Immunocytochemistry of hdINs cultures at 35 DIV for the neuronal marker MAP2 together with **(A)** GABA, **(B)** the glutamatergic markers VGLUT1 and KGA, and the interneuron subpopulation markers **(C)** calbindin and **(D)** calretinin. **(E)** Spontaneous postsynaptic currents during baseline and after PTX addition. **(F)** cumulative distributions of the inter-event interval for spontaneous postsynaptic currents recorded from hdINs during baseline (green), and the addition of NBQX+AP5 (orange) or PTX (purple). On the right, average frequency change after the addition of each drug in comparison to the baseline. Mean ± SEM. CB, calbindin; CR, calretinin. Scale bar: 100 µm. *Adapted from Paper I.*

Functional integration of hdINs into human neuronal networks

– Paper I

Currently, most of the translational research is being focused on animal models, although we should not ignore the differences between species since this may have contributed to the failure of some therapies when tested in human clinical trials. Many shared features make rodents good models for human physiology and disease, however, differences between rodents and humans are extensively described including the rate of development and protein degradation, glial cell biology (Barinka et al., 2015; Han et al., 2013; Oberheim et al., 2009; Rayon et al., 2020; Zhang et al., 2016), neurotransmitter receptors and ion channels (Hodge et al., 2019; Kalmbach et al., 2018). Hence, the validation of animal data in human-derived tissue is crucial to ensure that outcomes are not specific just to the rodent brain, and thus increase the probability of translational success.

Survival and differentiation of hdINs in the rodent brain cortex *in vivo* have been shown in the initial publication by Yang *et al.* (2017). Nevertheless, survival and differentiation of those derived INs into a human environment were yet not proven. Moreover, it is believed that for cell replacement therapy, functional integration of transplanted neurons into the host brain circuitry is needed. To address this gap, the capability of hdINs to functionally integrate into human neuronal circuits was tested *in vitro*, in **Paper I**. For this purpose, hdINs were modified genetically to express *ChR2-mCherry* under the synapsin-1 promoter so that transplanted hdINs could be activated on-demand and thereby investigate their capability to generate efferent synapsis and to modulate the neuronal activity of the host neurons.

Optogenetics reveal efferent connections from hdINs to human primary neurons

First, human primary neuronal cultures from the brain cortex of eight-week aborted fetuses were used as an established *in vitro* model of human neuronal networks (Chalmers-Redman et al., 1997; Miskinyte et al., 2017), since those primary neuron cultures displayed spontaneous synaptic bursting. At 7 DIV, hdINs were detached and seeded onto the pre-established primary neuronal cultures, and co-cultured for four weeks (from 7 to 35 DIV). At this time point, hdINs received functional afferent connections from the human primary neurons, which had a strong glutamatergic component. Moreover, the induction of depolarizing currents to the hdINs using blue light (470 nm wavelength) (Figure 10, upper red) triggered postsynaptic currents in recorded human primary neurons with a given latency period from the light stimulation pulse onset (Figure 10, bottom blue). Moreover, the delayed light response was blocked by PTX, but not by NBQX and AP-5 (Figure 10, bottom

blue). Unlike, when the response is generated by the hdINs expressing ChR2 so that none of the drugs affects the light response (Figure 10, upper red). Therefore, these delayed light responses confirmed stable functional synaptic connections of GABAergic nature from hdINs onto human primary neurons after 4 weeks of co-culture.

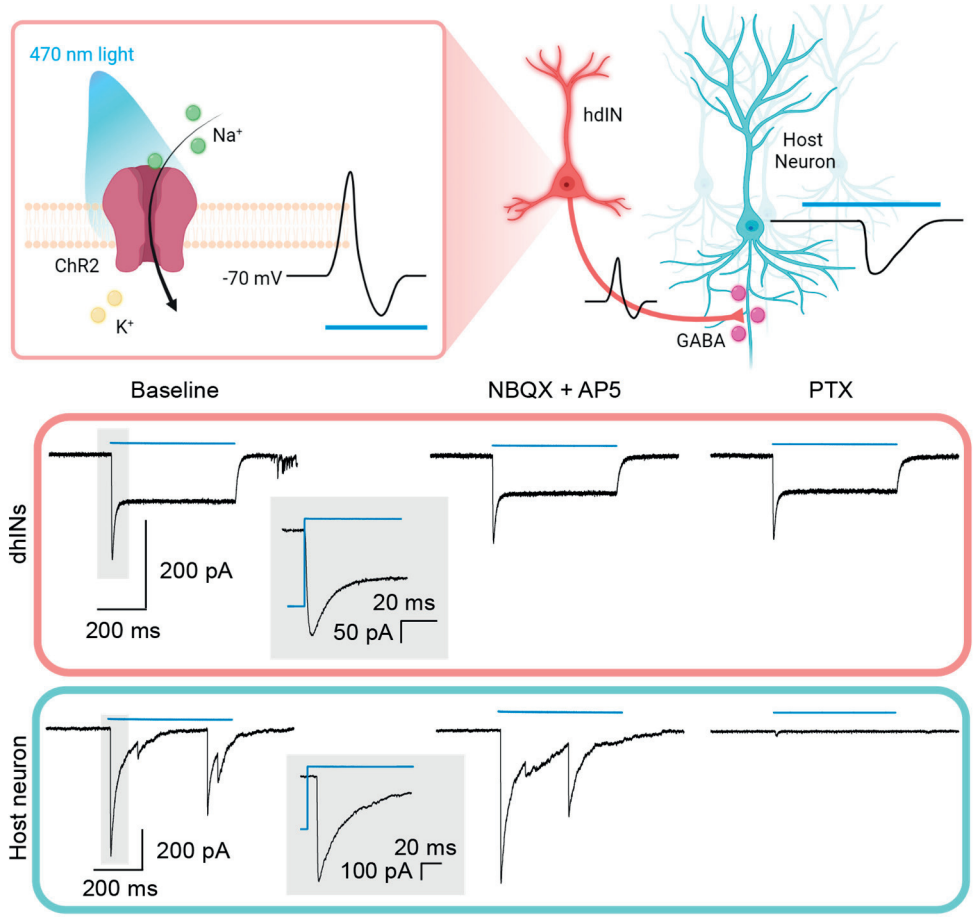

Figure 10. Effect of ChR2 activation on the hdINs and human primary neurons
On the top, schematics of hdINs expressing ChR2 on the membrane, which is activated by 470 nm wavelength blue light. The activation of ChR2 triggered an inward current leading to a membrane potential depolarization of the hdINs (red), which happened immediately after the initiation of the light pulse and was unaffected by either NBQX+AP5 or PTX. At the bottom, Light activation of ChR2 in hdINs was propagated resulting in a delayed postsynaptic response from the host cell (blue) regarding the initiation of the light pulse. This delayed postsynaptic light response was unaltered by the addition of NBQX+AP5 (middle column) but abolished by PTX (right column). Blue line, 470 nm light pulse stimulation. *Adapted from Paper I.*

Establishment of functional synapses that modulates the activity of host neurons in the epileptic human brain tissue

The final goal of hdINs was to be used as cell therapy for epilepsy. To determine the suitability of these cells for such purpose, organotypic cultures of brain tissue slices from the epileptic foci of refractory epilepsy patients undergoing resective surgery were used. This tissue represents one of the best models of refractory epilepsy for obvious reasons as being the real tissue to potentially treat in the clinics, although presenting certain constraints such as being an *ex vivo* approach and having a limitation in survival time.

The hdINs were transplanted at 7 DIV onto the organotypic human epileptic brain slices and kept in culture for 4 to 6 weeks on submerged membranes (Figure 11). After 4 and 6 weeks of culturing, hdINs were functional and presented intrinsic properties comparable to mature neurons. Moreover, hdINs responded with depolarizing currents to opto-stimulation and received afferent synaptic currents from the neighboring cells. In addition, like in the primary cultures, optogenetic activation of hdINs induced postsynaptic currents in host human neurons that were abolished when blocking $GABA_A$ receptors, but not glutamate receptors AMPA or NMDA.

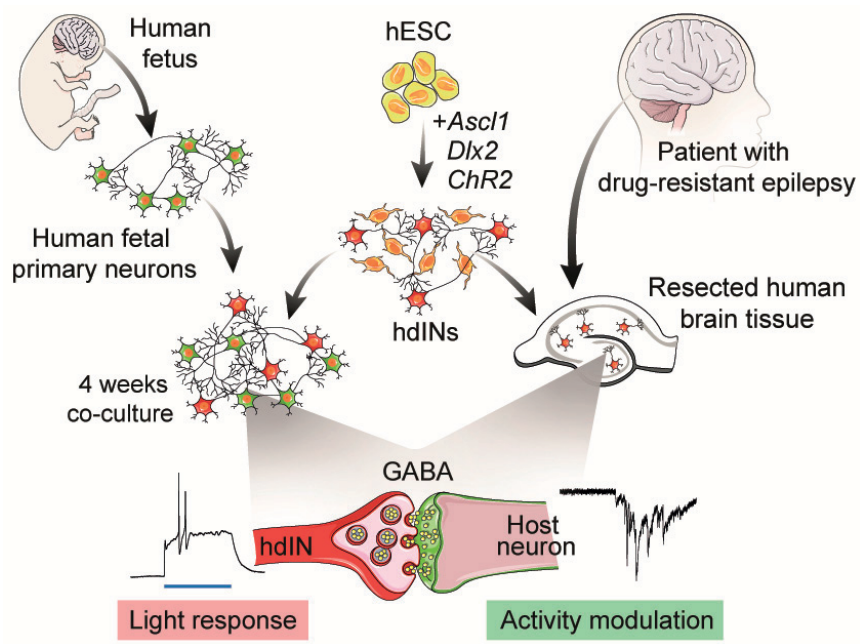

Figure 11. Graphical summary of the functional integration of hdINs onto human neuronal networks
In the center, there is the transcription factor reprograming to generate GABAergic interneurons from hESC. On the left, primary neurons are harvested from cortical brain tissue from an aborted fetus. On the right, brain slices from surgical resections of refractory epilepsy patients. At the bottom, functional demonstration of synaptic integration of the hdINs onto the human neuronal networks by activity modulation of the host neurons after stimulation of ChR2 expressed in the hdINs.

Overall, these results confirm that hdINs are able to survive and differentiate in the chronic epileptic tissue, as well as form functional connections with the host human neuronal network. It is the first evidence, to our knowledge, that hESC-derived INs are capable of forming functional efferent synaptic connections to human neurons, and thereby possess the potential to modulate activity and network excitability of the human neuronal network.

In addition, these two human environments presented here (Figure 11), the human primary fetal neurons and the *ex vivo* human epileptic organotypic cultures, respectively could be considered as a useful platform incorporated into the roadmap of clinical translation.

Survival and phenotype specification of grafted hdINs into chronic epileptic animal models

– Paper II and III

As a continuation of the encouraging results obtained in the previous human neuronal models (**Paper I**), the potential of hdINs as cell therapy was validated in living rodents. Two different animal models have been used to that goal. A commonly used chronic TLE adult rat model based on the application of a chemoconvulsive insult (**Paper II**), and a genetic mouse model of ASD and CDFE syndrome, the *Cntnap2* KO (**Paper III**).

The hdINs survive up to six months and express CR and CB markers in the chronic epileptic rat hippocampus

In **Paper II**, hdINs were transplanted at 7 DIV into the hippocampi of adult immunodeficient rats suffering from chronic SRSs after KA-induced SE, as illustrated in Figure 12A. The hdINs survived up to six months in this epileptic environment and expressed GABA marker, indicating that hdINs became GABAergic once grafted (Figure 12B), which was indeed confirmed by electrophysiology as before. A deeper characterization of the specific interneuron subpopulations indicated that hdINs matured mostly into CB and CR interneurons, approximately 38 % in both cases, a similar scenario as observed *in vitro* (Figure 12D).

Maturation and synaptic integration of hdINs post-transplantation into the chronic epileptic rat hippocampus

Electrophysiological properties of the grafted hdINs were analyzed to assess functional maturation and synapse formation. A progressive maturation of the intrinsic electrophysiological properties of the cells was observed between three and six months post-transplantation (PT). The hdINs decreased their input resistance, presented a more hyperpolarized resting membrane potential, and larger and faster action potentials at the later time point (Figure 12C).

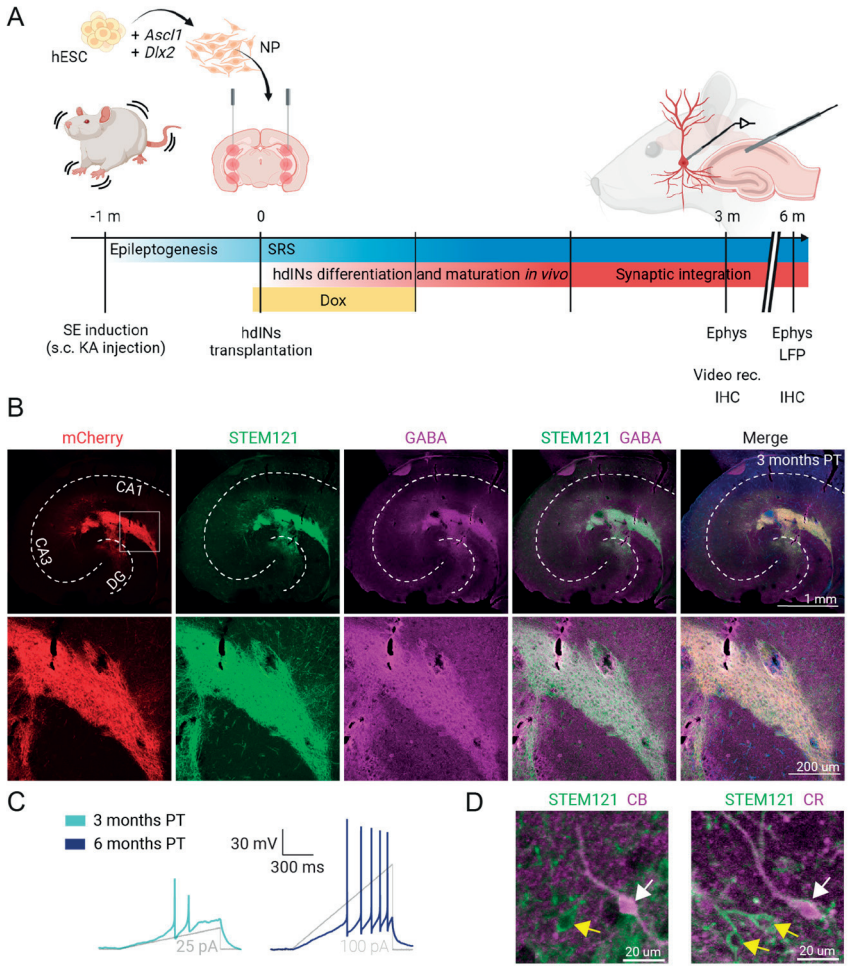

Figure 12. Overview of the hdIN transplantation study in the chronic TLE rat model
(A) Timeline of the study indicating the main procedures and analysis. **(B)** Immunohistochemistry of hippocampal brain slices with a visible hdIN graft core. The bottom row corresponds to the magnification of the square indicated in the upper row. **(C)** Differential response to ramps of depolarizing current from hdIN 3 and 6 months PT, light and dark blue respectively. **(D)** Immunohistochemistry for calbindin and calretinin markers together with the human cytoplasmatic marker STEM121. NP, neuronal precursors; m, month; s.c., subcutaneous; Dox, doxycycline; Ephys, whole-cell patch-clamp recordings; LFP, local field potential *in vitro* recordings; Video rec., video recording; IHC, immunohistochemistry. *Adapted from Paper II.*

Then, synaptogenesis of hdINs when grafted *in vivo* was studied by looking into spontaneous and evoked synaptic currents. Afferent synapses formed from the host cells onto the grafted hdINs were stronger (higher amplitude) and more frequent at the later time point. Thereafter, ChR2 expression in hdINs was used to determine whether grafted cells had the ability to form efferent synapses onto the surrounding neurons. Efferent synapses were observed onto both other hdINs and the host rat neurons as delayed light-induced postsynaptic responses in the cells upon opto-stimulation, which were readily blocked by PTX. Graft-to-graft and graft-to-host synapses were increased in proportion at six months PT. In conclusion, synaptic integration, by forming both afferent and efferent synapses, of the grafted hdINs into the host neural network was increased over time in the rat brain. Previous results from Cunningham and colleagues also support our findings, proving the capacity of hESC-derived GABAergic neurons to establish functional synaptic contacts onto the host rodent hippocampal neurons, which enhances inhibitory synaptic transmission (Cunningham et al., 2014).

Human dINs survive up to nine months after early postnatal transplantation into the hippocampus of *Cntnap2* KO mice

The perinatal and early postnatal periods are crucial times for brain development. Establishment, development, and consolidation of brain connectivity happen during the perinatal phase, and most of the developmental processes prolong into the postnatal days, such as dendrites and dendritic spine formation, synaptogenesis, and myelination (Kostović & Jovanov-Milošević, 2006). Along these periods, the developing brain evolves from an exuberance of connectivity after birth, due to a rapid arborization and synaptogenesis, to a refinement of connections through pruning that extends until late childhood, which consists in selective reshaping of axons, and elimination of dendrites (Innocenti & Price, 2005). Consequently, alterations in certain genes expressed during these periods lead to a predisposition to multiple neurodevelopmental disorders such as ASD and epilepsies. Several studies in neurodevelopmental diseases had pointed out genes active during early cortical patterning, establishment of neuronal connectivity (Parikshak et al., 2015), and tuning of physiological E/I balance (Marín, 2012). Considering the strong dynamics in the early postnatal brain milieu, the outcome of transplanting neuronal precursors might not be the same as in the adult brain, and therefore was investigated.

In **Paper III**, hdINs were transplanted at 7 DIV into the right dorsal hippocampus of *Cntnap2* KO mice at P2, an ASD and CDFE model that has been described to suffer SRS at six months of age and resembles most of the features of human patients (Penagarikano et al., 2011) (Figure 13A). Even though seizures arise in adult mice, alterations in INs have been described already during the postnatal period when the network starts to form. Therefore, hdINs were transplanted early

after birth when epileptogenesis might be ongoing. Human dINs survived up to nine months PT and were localized at the injection site (right dorsal hippocampus), although cells also dispersed across the ipsilateral hippocampus and to the contralateral one (Figure 13B). Importantly, no local inflammation or immune reaction against the transplanted cells were observed either at P14 or 2 months PT, as assessed by levels of astrogliosis (GFAP), inflammatory cytokines (IL-1), T lymphocytes (CD8), and reactive microglia (Iba1, CD68, and Gal3).

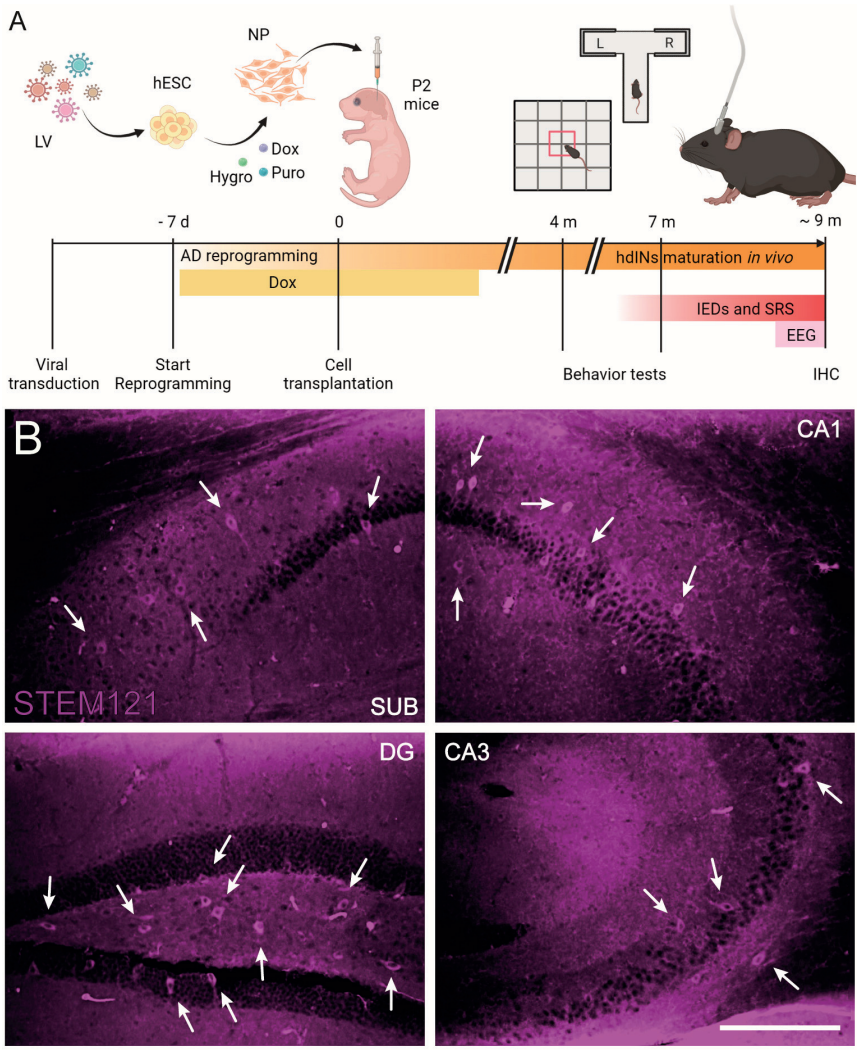

Figure 13. Overview of the hdIN transplantation study in early postnatal *Cntnap2* KO mouse model
(A) Timeline of the study indicating the main procedures and analysis. **(B)** Immunofluorescence for the human cytoplasmatic marker (STEM121) on different hippocampal regions from cell-transplanted *Cntnap2* KO mice at 9-months PT. Arrows point out at cells. LV, lentiviral particles including TetOn system; Dox, doxycycline; Puro, puromycin; Hygro, hygromycin; NP, neuronal precursor of hdINs; AD, *Ascl1* and *Dlx2*; P2, postnatal day 2; d, day; m, month; IED, interictal epileptiform discharges; SRS, spontaneous recurrent seizures; EEG, video-EEG recordings; IHC, immunohistochemistry. Scale bar: 200 µm. *Adapted from Paper III.*

Grafted hdINs into the hippocampus of *Cntnap2* KO mice demonstrate predominance of SST and PV phenotypes

Previous results using the AD reprogramming protocol *in vitro* in **Paper I** (Gonzalez-Ramos et al., 2021) and after transplantation into the hippocampi of adult epileptic rats in **Paper II** (Waloschková et al., 2021), have characterized and proven that hdINs are GABAergic with a high prevalence of CR and CB expressing INs. Nine months PT in the early developmental brain of *Cntnap2* KO mice (**Paper III**), hdINs mainly expressed PV (approx. 58 %) and SST (approx. 54 %) markers, and to a lower extent NPY, while only a few cells were positive for CR or CB (1.8 and 2 % respectively) (Figure 14A-C). The total percentage was exceeding 100 % due to possible cellular marker overlap and intra-animal variation. Within the hippocampal structure, hdINs were located mainly in CA1 and CA3 layers, surrounding principal cells bodies and projections (Figure 14D).

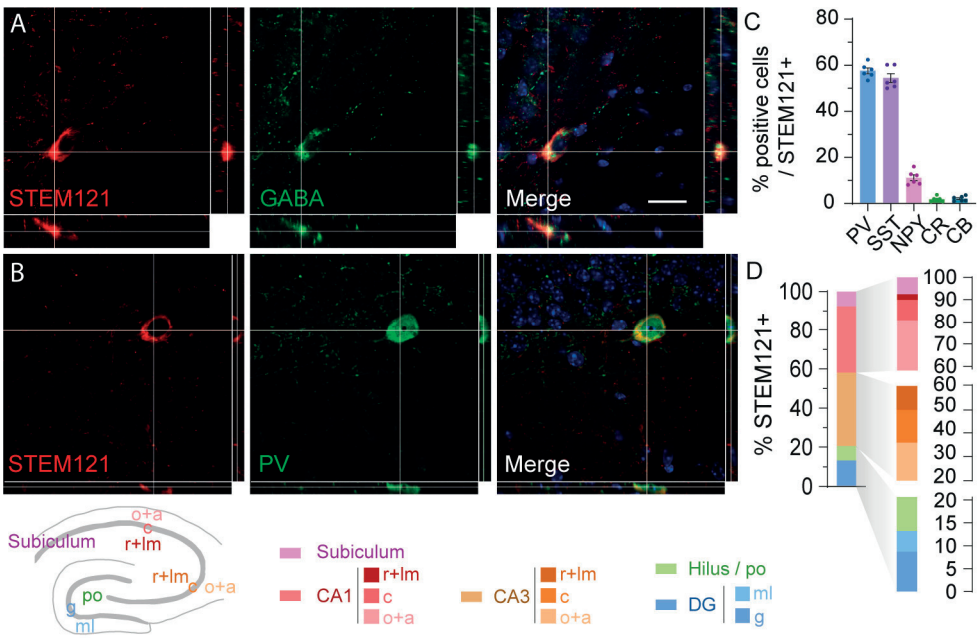

Figure 14. The hdINs differentiate to PV and SST expressing INs and distributed to principal layers throughout the hippocampus

Immunofluorescence of an example double-positive cell for STEM121 and GABA **(A)**, and for STEM121 and PV **(B)**. **(C)** Percentage of interneuron subtypes that hdINs became 9-months PT. **(D)** Location of the overall STEM121+ cells within the hippocampal architecture. CA, cornu ammonis; DG, dentate gyrus; r + lm, stratum radiatum and stratum lacunosum moleculare; c, pyramidal cell layer; o + a, stratum oriens and alveus; po, polymorphic layer; ml, molecular layer; g, granular cell layer; PT, post-transplantation. Mean (n = 6 mice). Scale bar: 20 µm. *Adapted from Paper III.*

The phenotype of hdINs nine months PT diverged from the one observed *in vitro* and six months PT in adult rats, which could be explained by the different milieu and/or the extended time *in vivo* that may help hdINs to acquire a certain identity

that is known to require protracted timings (Avaliani et al., 2014; Lim et al., 2018; Lu et al., 2017; Nicholas et al., 2013). In addition, a larger proportion of SST-positive cells was obtained using the same AD reprogramming protocol in another publication (Allison et al., 2021), where few CR-positive INs were also detected and no PV-expressing cells. The same authors did also compare AD reprogramming in organoids, a 3D system, where they observed a faster and more uniform maturation predominated by SST-positive cells, although few PV-positive cells were also detected using this model. These data confirmed that some IN subpopulations, such as PV-positive cells, require extended differentiation periods, and their maturation is dependent on network activity. Additionally, Yang and colleagues transplanted hdINs at 14 DIV into the subventricular zone and cerebral cortex of immunodeficient newborn mice (Yang et al., 2017). Most cells remained close to the injection site and expressed mainly SST, CR, and CB similarly to their results obtained *in vitro*, although some NPY and PV cells were also detected. The difference in the dispersion of the cells after transplantation in newborn mice could be due to the age of the cells at the time of transplantation. While in **Paper III** we transplanted at 7 DIV, Yang and colleagues did it at 14 DIV when the cells are more mature. The fact that cells dispersed and did not remain as a core, could facilitate the influence of the host environment, as well as the establishment of synapses between the host and the grafted cells, which may accelerate maturation. Notice that during development CB is also expressed a few days after the cessation of cell division and the beginning of the extension of neuronal processes, thus it is also associated with immature and migratory INs (Anderson et al., 2001; Andressen et al., 1993). Altogether, the early age of hdINs at the transplantation day, 7 DIV, might have led to a considerable dispersion of the cells instead of remaining as a core, which at the same time allowed for increased interaction from/to the host tissue, and reduced graft-to-graft cell interaction. Thus, more dynamic host tissue in the newborn stage and the extended period PT could have influenced the observed differential phenotypic outcome of the transplanted cells in **Paper III**.

In addition to the physiological developmental changes that may affect the survival and phenotype specification of the transplanted cells, the genetic alteration, in this case, loss of function of the *Cntnap2* gene, could also have exerted an effect on the hdINs. For instance, the absence of CASPR2 inhibits spine-stabilization (Gdalyahu et al., 2015), which might affect the integration and synaptogenesis of the hdINs in the host network. In addition, mammalian target of rapamycin (mTOR) signaling has been described to be hyperactivated in *Cntnap2* KO mice, which among other functions regulates differentiation of PV- and SST-expressing INs (Malik et al., 2019). Further investigation on the potential alteration of hdINs' phenotype after transplantation due to the host environment lacking CASPR2 function is needed.

Interneuron subpopulations differ from each other by differential molecular expression profiles, anatomical diversity including morphology and connectivity, and electrophysiological features both intrinsic membrane and temporal firing

properties (Kepecs & Fishell, 2014; Pelkey et al., 2017). Consequently, the impact of alterations or absence in the different subpopulations varies depending on the subtype affected, as well as the distinct outcome on the network activity that might be expected from the use of a specific subpopulation for transplantation. Various types of interneurons seem to be affected across different types of epilepsies (Liu et al., 2014). For instance, mutations of the *Cntnap2* gene are characterized by a reduced number and alteration of several interneuron subtypes including PV+, CR+, and NPY+ (Penagarikano et al., 2011). On the other hand, chemogenetic activation of both PV+ and SST+ interneurons have been shown to suppress epileptiform synchronization *in vitro*, while *in vivo* activation of PV+ neurons attenuated seizures (Călin et al., 2018). In human tissue from TLE patients, CB+ interneurons display an altered morphology and CR+ neurons are reduced in number and exhibit altered connectivity (Blumcke et al., 1996; Maglóczky et al., 2000). In rodent epilepsy models, CR+ neurons together with other interneuron subtypes, such as SST+ and NPY+, appear to be fewer in the hippocampus, while PV+ neurons are not affected (Sun et al., 2007; Tóth & Maglóczky, 2014). In addition, a variety of cells have been tested in preclinical models of epilepsy for their ability to suppress seizures after transplantation into different regions of the brain, although predominantly the hippocampus. Those studies included distinct cell sources such as primary NSPCs, GABAergic precursors from both LGE and MGE, and GABAergic precursor cells derived from hESC, and hiPSC (Shetty & Upadhya, 2016). Various grafted cell compositions have been described in those studies from a considerably high content of PV and SST expressing cells (Baraban et al., 2009; De la Cruz et al., 2011) to a predominance of CB and CR expressing cells (Hattiangady et al., 2008; Henderson et al., 2014), and interestingly all of the approaches have reported anti-seizure effect in preclinical models of TLE. In line with those observations, a recent study performing *in vivo* direct reprogramming by *Ascl1* and *Dlx2* expression from endogenous reactive glial cells into low-threshold spiking interneurons, mainly VIP, SST, CR, and NPY, also reported a reduction in SRS in an epileptic mouse model (Lentini et al., 2021).

Taken together, several IN subtypes might have a beneficial effect when transplanted into models of neuropsychiatric disorders and epilepsy. One could further speculate, based on the literature, that perhaps transplantation of a heterogeneous population of interneurons could result in a better outcome for ameliorating some symptoms of the disease.

Differential therapeutic effect of grafted hdINs into chronic epileptic animal models

– Paper II and III

Previously in **Paper I**, the effect of GABA release from the transplanted hdINs could not be explored due to the poor capability of inducing epileptiform activity in a human resected tissue after 4 to 6 weeks in culture, as well as the variability on surgery and other circumstances. Therefore, the effect of the grafted hdINs on network activity and epileptic phenotype was investigated in the animal models.

Suppression of epileptiform discharges *in vitro* and SRS *in vivo* by hdIN transplantation into a chronic TLE adult rat model

To evaluate whether acute GABA release from the transplanted hdINs might exert an effect on the network activity of the epileptic hippocampus, acute hippocampal brain slices were generated from rats at six months PT. Epileptiform discharges were induced on the slices by high-potassium or zero-magnesium levels in the artificial cerebrospinal fluid (aCSF). The hdINs in the tissue were activated by opto-stimulation. A small but significant decrease in epileptiform discharge rate was observed when a five seconds continuous blue light pulse was applied, suggesting that hdINs might have effects on seizure activity *in vivo* as well.

Then, behavioral motor SRSs were analyzed at four months post-SE by continuous video monitoring during seven days (Figure 15A), comparing non-transplanted (sham) and hdINs transplanted rats. Rats transplanted with hdINs presented a decrease of 87 % in SRS frequency and a shorter total time spent in seizures (Figure 15B), although the average duration and severity of the seizures were not affected. Hence, grafted hdINs exert a seizure-suppressant effect even without optogenetic activation.

In conclusion, grafted hdINs reduced the rate of epileptiform discharges *in vitro* by optogenetic stimulation, and decreased SRS frequency and total time spent in SRSs *in vivo* as compared to non-grafted controls.

Our work is one of the few studies testing the efficacy of human pluripotent stem cell-derived GABAergic precursors in preclinical chronic models of epilepsy. When comparing our results with data published elsewhere, small differences in the TLE model, time for transplantation, and time for therapeutic readout can be found although all showing encouraging results. Cunningham and colleagues transplanted hESC-derived MGE-like precursors three weeks after SE in the pilocarpine mouse model and performed functional assessment during 5-10 days at three months PT, which revealed a 93% of reduction in the overall SRS frequency (Cunningham et al., 2014). In another study from Upadhya and colleagues, hiPSC-derived MGE

cells were transplanted seven days after KA-induced SE in a TLE rat model under a cyclosporin A immunosuppression regime, followed by functional analysis over three weeks at five months post-SE, which revealed a reduction of 72 % in SRS frequency (Upadhya et al., 2019). In the second study, they also confirmed that the anti-seizure effect was induced by the grafted cells by using chemogenetics, the inhibitory DREADD hM4Di. In addition, the timing of transplantation is also important when aiming to translate the therapeutic outcome. In this line, our study is more translational since patient candidates for intracerebral cell therapy would be those displaying refractory epilepsy usually for a long time.

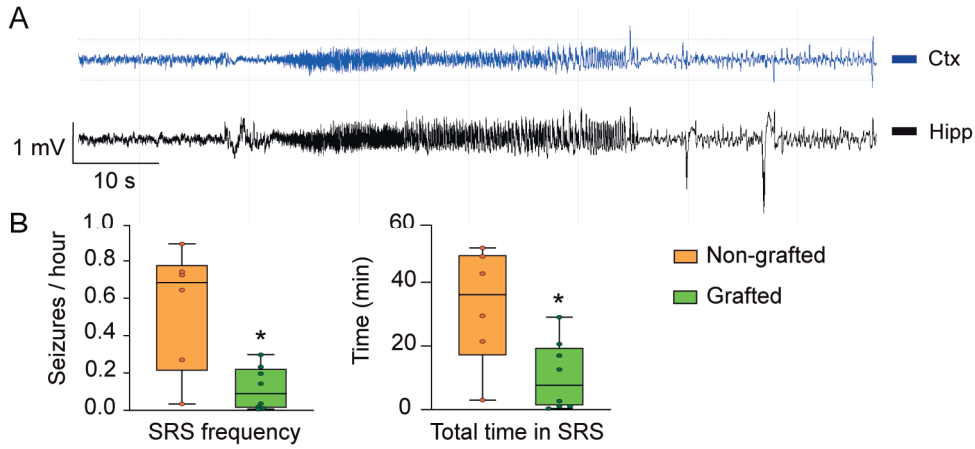

Figure 15. Spontaneous recurrent seizures in KA epileptic rats four months post SE induction and
(A) Representative EEG trace of a SRS detected by the cortical electrode (Ctx, blue) and the hippocampal electrode (black, Hipp). **(B)** Box plots for the median motor SRS frequency and the total time in motor SRS in control epileptic animals (non-grafted) and epileptic animals grafted with hdINs. SRS, spontaneous recurrent seizure; Ctx, cortex; Hipp, hippocampus. *Adapted from Paper II.*

Assessment of the therapeutic outcome of hdINs transplantation into the hippocampus of *Cntnap2* KO mice

One of the peaks of prevalence for epilepsy is during childhood (Forsgren et al., 2005), which in that case is frequently associated with other neurodevelopmental disorders such as intellectual disability and ASD (Lo-Castro & Curatolo, 2014). Nearly 7 to 20 % of children suffer from refractory epilepsy (Xue-Ping et al., 2019), thereby seizures can have a profound effect on the education, social and cognitive functioning of the child. Developmental malformation of the cortex is a common cause of refractory epilepsy in children accounting for the most severe symptomatic epilepsies (Guerrini, 2006; Lortie et al., 2002). Several genetic variants underlying cortical dysplasia associated with epilepsy have been identified.

In **Paper III**, a mouse model for the familiar recessive loss of function in the *Cntnap2* gene was used since it has been associated with CDFE syndrome both on

patients (Strauss et al., 2006) and in the animal model (Penagarikano et al., 2011). The *Cntnap2* expression in mice is already detected at E13.5 in the MGE and it is highly expressed in inhibitory neurons. Loss of function of this gene has been shown to influence neuronal migration disturbances and a reduction in the inhibitory tone. Moreover, it replicates clinical phenotypes including hyperactivity, repetitive behavior, social deficits, and epileptiform discharges leading to the development of SRS around six months of age (Penagarikano et al., 2011; Vogt et al., 2018). Previous studies have focused on underpinning functional consequences of *Cntnap2* loss on cortical and striatal circuits, and associated behaviors during juvenile and early adulthood. However, less is known about late adulthood when mice develop seizures. Recently, Paterno and colleagues have highlighted the implication of the hippocampus in ASD by showing a reduction in hippocampal PV interneuron density, and consequent reduction in inhibitory input to CA1 pyramidal cells, which lead to frequency-dependent circuit changes within the hippocampus (Paterno et al., 2021). Nonetheless, all those observations were done in mice at P90-120 and did not elucidate the period of seizure onset and their origin. Therefore, the effect of *Cntnap2* mutation at later postnatal stages and into the time of ictogenesis remains unknown.

Characterization of the epileptic phenotype in Cntnap2 KO mice

In **Paper III**, the effect of *Cntnap2* mutation at the time of ictogenesis, older than six months of age, was investigated both at behavioral and electrical brain activity levels by continuous video-EEG monitoring for two weeks. Electrodes were placed in CA1 and the contralateral cortex and later bilaterally in CA1 since studies published elsewhere and our previous results suggested the implication of the hippocampus in seizure initiation. CDFE patients display focal intractable seizures arising from the temporal region identifying seizure-onset zones distributed over the hippocampus-amygdala complex (Strauss et al., 2006). In **Paper III**, a similar EEG pattern was found in *Cntnap2* mutant mice with electrographic seizure activity detected in the hippocampi prior to its behavioral component (Figure 16A-B), which had a wide range of severity from stage 2 to 6 (Racine, 1972). In addition, clinical studies described interictal epileptiform discharges (IEDs) frequently in CDFE patients that originate in seizure onset zones (Strauss et al., 2006). Similarly, to the clinical findings, we confirmed the presence of IEDs in the hippocampi of *Cntnap2* KO mice (Figure 16C-D). IEDs presented a positive correlation with seizure occurrence, so IEDs were more frequent in animals that displayed seizures. Importantly, there was high heterogeneity in the epileptic phenotype of the mutant mice, from mice displaying an EEG comparable with WT mice with no seizures to those suffering from many IEDs and SRS (Figure 16D). Such heterogeneity is also observed in the clinical manifestation of CDFE and other neurodevelopmental disorders themselves due to the developmental environment and individual differences in resilience (Batalle et al., 2018). Nevertheless, extended periods of video-EEG recording could help to gain further understanding of the dynamics and

diversity within the genotype. In addition, *Cntnap2* mutant mice displayed impaired hippocampus-dependent long-term memory after seizure onset. Taken together, these first findings in **Paper III** highlight a hippocampal involvement in the epileptic phenotype of the *Cntanp2* KO mouse model of ASD and CDFE.

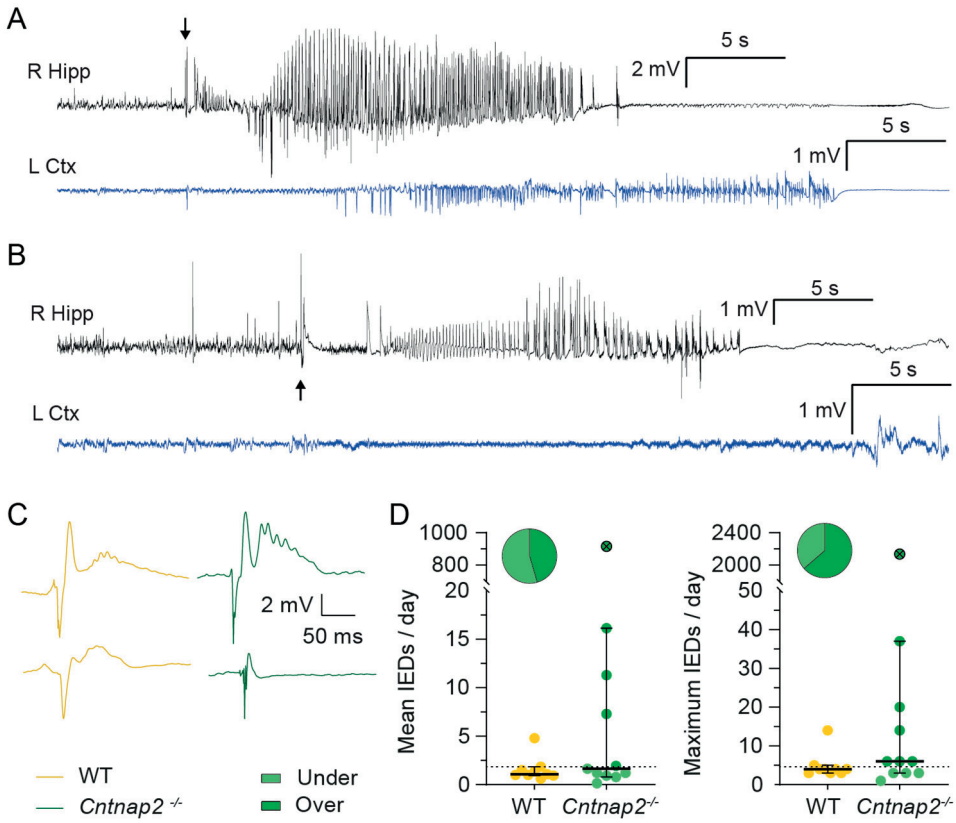

Figure 16. Spontaneous behavioral seizures and IEDs in *Cntnap2* knock-out mice older than six months of age
(A) Representative EEG trace from *Cntnap2* knock-out mice showing a stage V seizure being detected first at the right hippocampus (black) and later at the contralateral cortex (blue). **(B)** Representative EEG trace from *Cntnap2* knock-out mice showing a stage I seizure being detected at the right hippocampus (black), but not at the contralateral cortex (blue) since it did not generalize. Arrow: the beginning of the seizure. **(C)** Representative traces of IEDs observed in WT (yellow) and *Cntnap2* knock-out (green) mice, recorded in CA1. **(D)** Mean daily IEDs (left) and maximum daily IEDs (right), for a 14-days recording period. The crossed circle signifies an animal displaying behavioral seizures. The dotted line identifies the limit of 95% CI of the WT mice group. In the top left corner, pie-chart for data distributions in *Cntnap2* knock-out mice illustrating two different subpopulations over and under the 95% CI of the WT for each parameter. Median ± 95% CI. Hipp, hippocampus; Ctx, cortex; IED, interictal epileptiform discharges. *Adapted from Paper III.*

Although we cannot exclude the seizure origin in other subcortical regions i.e. the amygdala, the data demonstrate that all seizures detected in the hippocampus were always preceding the seizures in the cortex, and that some seizures were only detected focally in the hippocampus. A possible explanation for the hippocampal origin of the seizures is that the hippocampus, together with the amygdala, is a structure with the lowest after-discharge thresholds (Handforth & Ackermann,

1995; McIntyre & Gilby, 2008). In addition, previous studies have obtained positive results targeting the hippocampus with the therapy, even though the insult was induced in other regions i.e. the amygdala (Colasante et al., 2020). Therefore, our results highlighted hippocampal involvement in the epileptic phenotype in these animals and suggested that is a potential therapeutical target.

Increase of the adult epileptic phenotype of Cntnap2 KO mice after transplantation of hdINs at the early postnatal period

Our results demonstrating electrographic seizures in the hippocampus preceding cortical involvement, together with other studies demonstrating reduced inhibitory input and circuit alterations in this area (Paterno et al., 2021), suggest the hippocampus as a potential therapeutic target. Moreover, a reduction in GABAergic interneurons already at early postnatal development has been identified in *Cntnap2* mutant mice (Paterno et al., 2021; Penagarikano et al., 2011). Overall, these data justified transplantation of INs into the hippocampus as a potential therapeutic approach. The effect on pathophysiological processes of hdIN transplantation into the hippocampus of early postnatal *Cntnap2* KO mice was thereby explored in **Paper III**.

Promising results on the reduction of seizure frequency and severity have been achieved in animal models of TLE by transplanting allogeneic mouse MGE-derived GABAergic progenitor cells into the hippocampus (Casalia et al., 2017; Hattiangady et al., 2008; Henderson et al., 2014; Hunt et al., 2013). From a translational perspective, human pluripotent stem cells, both hESC and hiPSC, have been used to generate MGE-like GABAergic progenitors accomplishing attenuation of SRS after transplantation (Cunningham et al., 2014; Upadhya et al., 2019; Upadhya et al., 2016), as we also shown in **Paper II** (Waloschková et al., 2021). Importantly, seizure reduction in a genetic model of epilepsy has only been achieved by transplanting allogeneic mouse MGE progenitors into early postnatal neocortex of *Kv1.1* mutant mice (Baraban et al., 2009). On the other hand, transplantation of allogeneic mouse neuronal precursors into the medial prefrontal cortex improved behavior deficits in a maternal immune activation model of ASD (Donegan et al., 2018) and the *Pten* mutant mice (Southwell et al., 2019; Southwell et al., 2020). Therefore, little is known about the therapeutic potential of human-derived GABAergic INs in genetic epileptic models and even less in ASD models.

To this end, in **Paper III**, the effect of transplanting hdINs into the hippocampus of neonatal *Cntnap2* KO mice on epilepsy development and behavior was investigated at time points prior to and after the time of expected seizure onset. Animals transplanted with hdINs had an increased IED rate and seizure occurrence when compared to the sham group (Figure 17A). Altogether the results suggest that hdIN transplantation did not prevent epileptogenesis as hypothesized, but rather potentiated seizure occurrence. Seizures were more predominant in males than females (Figure 17B), and animals suffering seizures tended to display increased

mean IEDs per day (Figure 17C). Interestingly, a similar proportion of animals exhibiting non-pathological EEG phenotype was present in both hdIN-transplanted and sham mice (Figure 17D), implying that grafted hdINs did only aggravate the epileptic phenotype in the subset of animals already developing the pathology. Increased mean IEDs and maximum number of IEDs were observed in hdIN-transplanted mice (Figure 17E) and was consistent in both genders (Figure 17F). Nonetheless, no differences were identified between hdIN-transplanted and sham mice regarding spatial learning and memory.

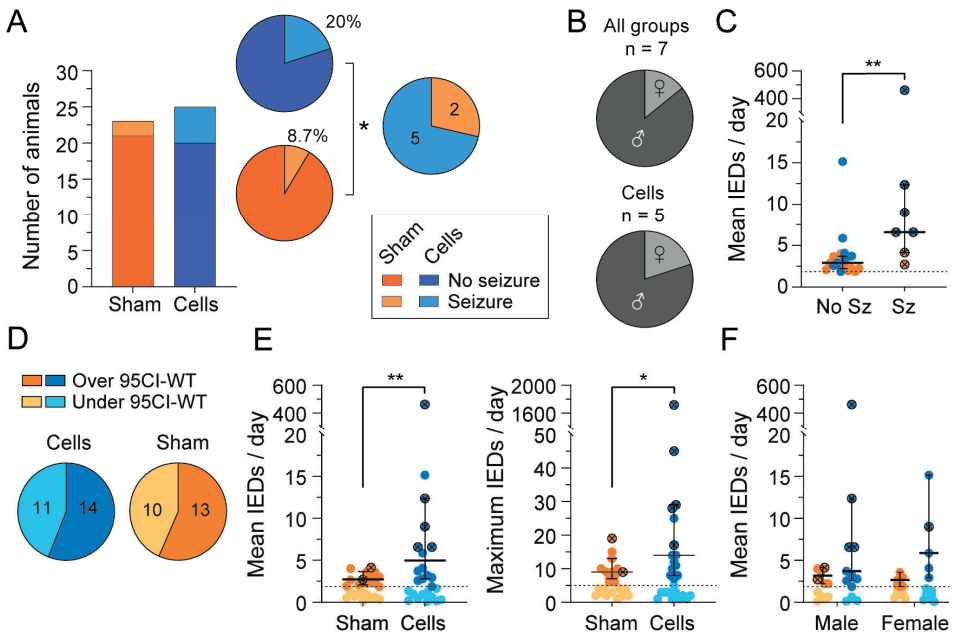

Figure 17. SRS and IEDs in *Cntnap2* KO mice transplanted with hdINs or sham
(A) Bar plot illustrating the number of animals suffering seizures or not in both treatment groups. On the right, pie charts represent the values in proportions of animals with seizures within treatment groups. **(B)** Seizures by gender overall in all groups and only in the cell-transplanted mice. **(C)** Mean daily IEDs pooled from both treatment groups and grouped by animals displaying seizures and not. **(D)** Sub-populations of mice within treatment groups with IED rate over and under the 95CI-WT (dashed lines setting the limit). **(E)** Mean daily IEDs (left) and maximum daily IEDs (right), for the 14-day recording period. **(F)** IED rate by gender and treatment group. Median ± 95% CI. Sz, seizures; IED, Interictal Epileptiform Discharges; 95CI-WT, 95% confidence interval limit value for the WT group. Crossed sphered represent animals that had suffered one or more seizures during the monitoring period. The dotted line identifies the limit of 95CI-WT. Light color spheres below the threshold (dashed line) are not included in the statistics. *Adapted from Paper III*.

These results stress the complexity and importance of better understanding the pathology of the disease, and choosing cell therapy approaches as a possible therapeutic intervention in ASD and developmental epilepsy. There are several possible explanations for the increased severity of the epileptic phenotype after hdIN transplantation (Figure 18). (1) It could be that hdINs engendered disinhibition by inhibiting host INs which would lead to increased excitability (Figure 18, 1). (2) Another explanation could be a change in GABA polarity due to disrupted chloride

homeostasis since alterations in NKCC1/KCC2 regulation has been linked to several genetic models associated with epilepsy and ASD (Chao et al., 2010; Duarte et al., 2013; Schulte et al., 2018; Tyzio et al., 2014), and patient tissue from tuberous sclerosis resections (Ruffolo et al., 2016; Talos et al., 2012) (Figure 18, 2). In support of this hypothesis, it is described that *Cntnap2* KO mice have an overall decrease in brain oxytocin levels due to a decrease in cells producing this hormone in the hypothalamus (Peñagarikano et al., 2015), and it is known that the timing of excitatory-to-inhibitory GABA switch is regulated by the oxytocin receptor via the chloride cotransporter KCC2 (Leonzino et al., 2016). Therefore, it would not be surprising if *Cntnap2* KO mice also present alterations in NKCC1/KCC2 regulation, although it requires further confirmation. This situation has been already proven for the fragile-X syndrome model, the *Fmr1* KO mouse, which has reduced oxytocin immunoreactivity in the hypothalamus (Francis et al., 2014), reduced number of GABAergic interneurons (Selby et al., 2007; Tyzio et al., 2014), and it is used as a model for epilepsy and seizures. (3) The last possible explanation is an increase in circuit synchronization due to aberrant innervation by hdIN of principle cells or other INs, so that it leads to an unstable and seizure prone network (Jiruska et al., 2013; Trevelyan et al., 2015) (Figure 18, 3). Further experiments are needed to clarify the underpinning mechanism of hdINs contributing to increased hippocampal excitability.

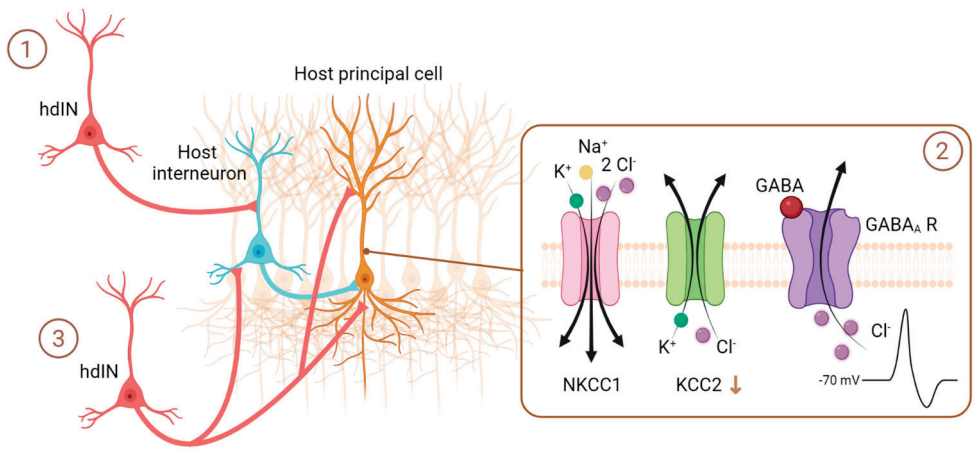

Figure 18. Hypothetical explanations for the pro-excitatory effect of grafted hdINs in *Cntnap2* KO mice
(1) Deshinhibition. (2) Excitatory GABA. (3) Unstable network.

While numerous approaches have demonstrated seizure suppressant effects in models of TLE, none of them examined the prophylactic effect of transplanting IN precursors but rather performing an intervention once the disease has already developed and SRS are present. That situation is different when approaching neurodevelopmental disorders and, in particular, the *Cntnap2* KO mice where various defects are already present at early postnatal development although seizures

do not appear until six months of age. Therefore, the present intervention by transplantation could be considered as prophylactic in terms of epilepsy treatment. Only one study has transplanted interneuron precursors in a genetic model of epilepsy, the *Kv1.1* mutant mice, although in this model seizures already begin around the second to third postnatal week (Baraban et al., 2009). Moreover, even though it is described that seizures originated from the hippocampus or amygdala in this *Kv1.1* model (Robbins & Tempel, 2012), the transplantation was performed into the cortex. Therefore, it is very difficult to compare our study with theirs. One remarkable difference between the studies is the number of cells transplanted and used for inclusion criteria of animals. Baraban and colleagues performed bilateral injections of mouse MGE precursors (4×10^5 cells per mouse) in four depots at P2 and included in the analysis only mice with a survival of 40,000 cells per cortex or more (150 days PT). First of all, their intervention was bilateral, and the cells transplanted were still in the proliferative stage since otherwise, they could not have obtained the same or higher amount of cells after grafting. In our study, cells were transplanted unilaterally and were post-mitotic to avoid any risk of teratoma formation, therefore the survival was lower than the initial amount transplanted, with a final survival of approximately 1400 cells in total per individual animal at nine months PT. While reduced viability after cell transplantation in studies using human-derived cells *in vivo* in comparison to primary mouse MGE cells has been observed before (Southwell et al., 2014), there is a remarkable difference in cell numbers that could affect the outcome observed. Unfortunately, Baraban and colleagues did not mention the results in mice with fewer cells transplanted than the cut-off value, which could have been useful for drawing possible conclusions. Similar scenario to ours where grafted cells represented only a fraction percentage of the initial numbers of transplanted ones, have been reported before by Cunningham and colleagues, averaging 19 % of the injected cells (Cunningham et al., 2014), and still achieving seizure suppressant effects. Moreover, it is being reported that effects are largely insensitive to the higher number of cells transplanted since transplant-mediated inhibition in the cerebral cortex reaches a plateau with transplant sizes of 5-10 % of the normal IN population (Southwell et al., 2010). Herein an interesting question would be if in our study we have approached the size of the graft to reach this plateau of the effect or not. Further investigation will also require investigating the formation of synaptic connections from the grafted cells to the host neurons since it may be a key factor for exerting an effect on network excitability. Anderson and colleagues pointed out that this reasoning could be a possible explanation for not detecting seizure suppressing effect after transplanting hESC-derived interneuron progenitors into pilocarpine-induced TLE mice, despite that grafted cells restored memory deficits (Anderson et al., 2018). In this last study, authors also performed longer monitoring periods of 30 days instead of 5-10 days as most of the publications elsewhere do, which seems to be more accurate due to the fact that mice display seizures in clusters (Henderson et al., 2014).

In summary, early postnatal interneuron transplantation did not seem to ameliorate the epileptic phenotype of *Cntnap2* KO mice at six months, but rather supported it. Consequently, a further investigation on chloride homeostasis, as well as elucidating disease mechanisms are highly warranted in these animals, which can, in turn, lead to refinement in treatment design. In line with this notion, discoveries in other ASD models could bring insights into alterations taking place in this model since they seem to share underlying mechanisms. Recently, whole-brain and region-specific organoids are being used for disease modeling of genetic epilepsies to investigate key brain developmental processes such as migration and connectivity. In particular, de Jong and colleagues have generated forebrain organoids from patient-derived hiPSCs with a homozygous protein-truncating mutation in *Cntnap2* and observed overgrowth of the organoids and differentially expressed genes compared to controls (de Jong et al., 2021).

PSAM^4-GlyR / uPSEM^{817} chemogenetic approach decreases excitability of principal neurons in the epileptic hippocampus

– Paper IV

Previous strategies presented in this thesis were based on the transplantation of GABAergic INs that upon light stimulation release the neurotransmitter GABA. Then, GABA activated $GABA_A$ receptors that are chloride permeable channels, leading to a hyperpolarization of the principal neuron. As mentioned above, this strategy however may result in the opposite, unexpected outcome, facilitating the excitability of principal neurons due to altered chloride homeostasis in epileptic tissue. We next asked whether this could be the case in mice epileptic tissue. To mimic $GABA_A$ receptor activation, we expressed in principal neurons of the epileptic hippocampus a chimeric chloride channel, activated by an otherwise inert selective agonist. At the same time, this approach was evaluated as potential gene therapy for epilepsy, albeit operating on the same principle, but as an alternative to cell transplantation.

In **Paper IV**, the chemogenetic tool PSAM^4-GlyR was used as gene therapy to inhibit the principal neurons of the epileptic hippocampus. The PSAM^4-GlyR is constituted by the ligand-binding domain of the α7 nicotinic acetylcholine receptor (α7-nACh) fused to the chloride-conducting pore of a glycine receptor (GlyR) (Magnus et al., 2011; Magnus et al., 2019), so that it is permeable for chloride ions when activated. AAV8 viral vectors were generated as a delivery system for introducing the *PSAM^4-GlyR* transgene, and its expression selectively targeted CamKIIα expressing pyramidal cells and GCs in the hippocampi. The PSAM^4-GlyR can be regulated externally in terms of dose and time by its specific activator

molecule uPSEM^{817}. On-demand regulation of the therapy represents an advantage towards clinical translation in comparison with more classical gene therapy strategies, since it affords to achieve an optimal degree of modulation of network excitability, and the external activator could be discontinued in case of an adverse effect on normal brain function.

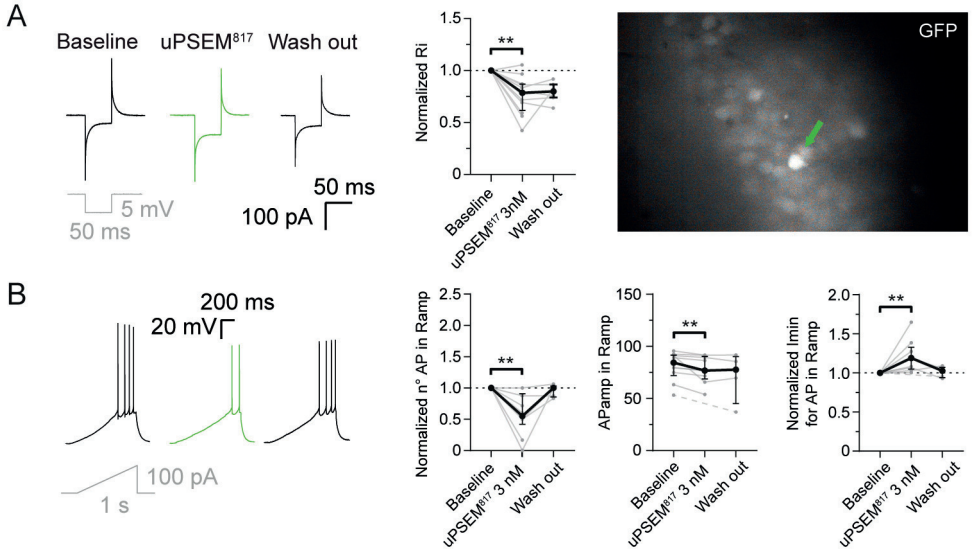

Figure 19. Effect of uPSEM^{817} on electrophysiological intrinsic properties of PSAM^4-GlyR expressing cells
(A) Test pulse response in the different treatments, green indicating during uPSEM^{817} application. On the right, normalized values for Ri in PSAM^4-GlyR-eGFP+ cells before, during, and after uPSEM^{817} application. Whole-cell patch-clamp recording of a GFP+ cell (green arrow). **(B)** Differential cell response to 0-100 pA ramps of depolarizing current in different treatments, green indicating during uPSEM^{817} application. Next, from left to right, normalized number of APs, measurements of the AP amplitude, and normalized minimum current (Imin) for the first AP in response to ramps of depolarizing current in PSAM^4-GlyR-eGFP+ cells before, during, and after uPSEM^{817}. Ri, input resistance; AP, action potential; APamp, action potential amplitude. *Adapted from Paper IV*.

Initially, the functional effect of PSAM^4-GlyR was studied at a cellular level using whole-cell electrophysiology. Since PSAM^4-GlyR is permeable for chloride ions, its effect will depend on the chloride levels at the extracellular space and inside the cell. Chloride homeostasis has been implicated in several epilepsies, as has been pointed out previously in this thesis (Bregestovski & Bernard, 2012). Hyperpolarising inhibition by chloride currents has been challenged due to some data showing a collapse in gradients of transmembrane chloride (Miles et al., 2012). Studies suggested that the activation of $GABA_A$ receptor has depolarizing effects on cells in tissue from epilepsy patients (Cohen et al., 2002), whereas others showed the hyperpolarisation (Duveau et al., 2016; Krook-Magnuson et al., 2013; Lieb et al., 2018). In acute hippocampal brain slices from chronic TLE mice, in **Paper IV**, the activation of the chloride-permeable channel PSAM^4-GlyR by uPSEM^{817} decreased the neuronal response to depolarizing currents (Figure 19B), so that it decreased the effective membrane resistance (Figure 19A) presumably leading to a

shunting inhibition of the neuron. A larger current was needed for triggering an action potential (AP) for a given depolarizing ramp current when chloride-permeable PSAM^4-GlyR channels were opened by the ligand (Figure 19B). Moreover, smaller AP amplitudes were observed during uPSEM^{817} effect compared to baseline.

To investigate whether the effect of uPSEM^{817} activation of PSAM^4-GlyR on the cellular level could be translated to an effect on seizures *in vivo*, the intrahippocampal kainic acid (IHKA) TLE mouse model was used. TLE is the most common form of focal refractory epilepsy in humans, and it is a well-characterized epileptic syndrome both in patients and animal models (Cendes et al., 2014; Engel, 2001). First, AAV8-CaMKIIα-PSAM^4-GlyR-IRES-eGFP was injected bilaterally into both hippocampi, and two weeks after, SE induction was performed by IHKA administration in the right dorsal hippocampus, and in the same surgery session, a recording electrode was placed into the same location for video-EEG monitoring (Figure 20A). After SE, a latent phase of two weeks was waited, corresponding to the average time needed for epileptogenesis to occur and leading to subsequent development of SRS. Then, continuous video-EEG recordings started (Figure 20A). Characteristic histopathological changes of TLE, such as structural network reorganization by mossy fiber sprouting in the inner molecular layer of the DG (observed by ZnT3 immunoreactivity) and GC layer dispersion, were observed in all animals (Figure 20B).

Despite the clear effect on the excitability of the principal cells *in vitro*, the *in vivo* administration of uPSEM^{817} intraperitoneally (i.p.) at dose 0.03 mg/kg did not affect electrographic seizures in mice with chronic TLE (Figure 21A-B). Although a tendency to decrease in IED rate was observed in those animals (Figure 21B), overall PSAM^4-GlyR gene therapy seemed to be insufficient to drive an anti-epileptic effect *in vivo*. On the other hand, the administration of the positive control treatment, the ASM phenobarbital (PHB), did affect both electrographic seizures and IEDs, decreasing their rates (Figure 21A-C). PHB effect was visually clear in all animals although statistically significant only for IEDs because of the low statistical power caused by the small sample size. Nonetheless, PHB belongs to the barbiturate family and acts on $GABA_A$ receptors. Therefore, the observed effect of PHB was indicative of normal chloride homeostasis. The advantage of PSAM^4-GlyR / uPSEM^{817} approach is the cell-specific effect in a very restricted area, only on the PSAM^4-GlyR expressing neurons in the epileptogenic focus, while PHB and similar drugs potentiate the action of $GABA_A$ receptors indiscriminately in cells and all brain leading to considerable side effects and therefore restricting the therapeutic value of these drugs.

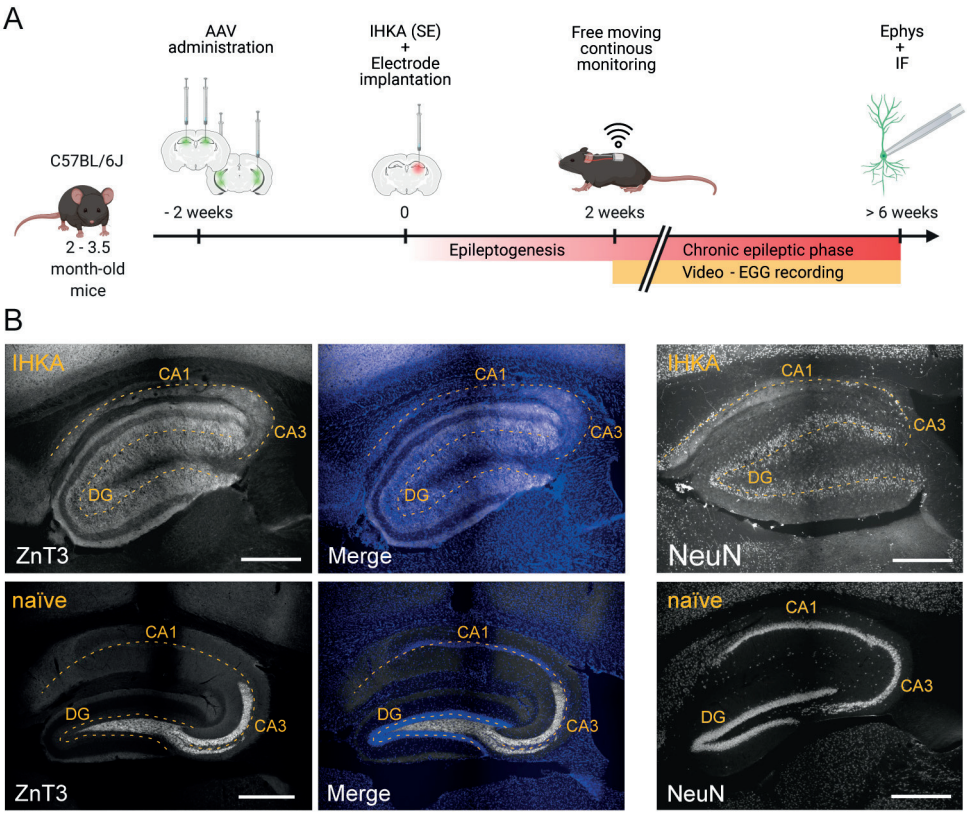

Figure 20. Experimental design, and histopathology after the intra-hippocampal KA induction
(A) Schematics of the study timeline. **(B)** On the left, immunofluorescence for mossy fiber sprouting, using antibodies against ZnT3, in naïve and IHKA mice. On the right, immunofluorescence for neuronal nuclei, NeuN, in naïve and IHKA mice. Images correspond to the surrounding area of the IHKA depot and electrode location in the right dorsal hippocampus, sagittal sections. The dashed yellow line in B represents the anatomical structure of the hippocampus. Scale bar: 500 µm. *Adapted from Paper IV*.

Previous studies using a different chemogenetic approach have demonstrated encouraging results as a valid strategy for refractory epilepsy treatment (Avaliani et al., 2016; Călin et al., 2018; Desloovere et al., 2021; Desloovere et al., 2019; Kätzel et al., 2014; Wang et al., 2018; Zhou et al., 2019). The approach is called DREADD and relies on G-protein-coupled receptors. DREADDs have the advantage that only a small fraction of the receptors needs to be activated to have a maximal downstream effect, although they can desensitize over time (Wacker et al., 2017). In contrast, $PSAM^4$ relies upon an extended gene expression to have the maximal response. At the same time, that could be an advantage since the activation of the chloride-permeable $PSAM^4$ channel is a more rapid response that shares similarities with the mechanism of action with well-known ASM acting on $GABA_A$ receptors. Unlike DREADDs, for $PSAM^4$, the nature of the channel influences the efficacy of the treatment. In other words, the activation of chloride-permeable $PSAM^4$-GlyR could

lead to a change in chloride loading in the cell over time and thereby change the chloride reversal potential, which ultimately might decrease the efficacy of $GABA_A$ receptor-mediated inhibition. Nonetheless, changes in chloride reversal potential due to extended activation of the chemogenetic receptor were not observed in a study using eGluCl, another LGIC constituted by a chloride pore although activated by glutamate (Lieb et al., 2018). Although, for eGluCl, the chloride currents are more restricted in time since the receptor is only active during seizures when the extrasynaptic glutamate is elevated. Despite the mentioned drawbacks of $PSAM^4$, it has been pointed out as a potential alternative therapy for epilepsy with a more clinical profile compared to DREADDs. The reason is that DREADDs pose a challenge for clinical translation due to the pharmacology and off-target effects of the external activator CNO (Gomez et al., 2017; Manvich et al., 2018). On the other hand, $PSAM^4$ can be activated by $uPSEM^{817}$, but also by varenicline, a drug already available in the market for smoking cessation. The fact of repurposing a molecule already used in healthcare facilitates the clinical translation by not requiring undergoing extensive safety studies before being accepted by the EMA and/or FDA (Lieb et al., 2019).

Some possible explanations for the absent or insufficient effect of $uPSEM^{817}$ activation of $PSAM^4$-Gly *in vivo* could be speculated. One explanation could be an insufficient viral coverage of the epileptogenic foci since in the present study the viral expression was mainly restricted to the dentate GCs both at dorsal and ventral hippocampi. The limited coverage could be a consequence of both coordinates that were designed to target DG accompanied by a limited spread of the particles, and/or viral particles serotype (Aschauer et al., 2013; Castle et al., 2016). In **Paper IV**, AAV8 was used, while publications elsewhere showing different expression coverage used lentiviral particles (Lieb et al., 2018) and various AAV serotypes, such as AAV1 and 2 mixture (Noè et al., 2008; Woldbye et al., 2010), AAV2/7 (Desloovere et al., 2021; Desloovere et al., 2019), or AAV8 in combination with Cre-recombinase cell-specific targeting (Călin et al., 2018). The aiming of the expression of $PSAM^4$-Gly in the dentate GCs was expected to enhance inhibition of the cells, which seems to be decreased in TLE (Williamson et al., 1999). The DG is also considered as one of the key players in seizure generation (Hsu, 2007; Kobayashi & Buckmaster, 2003; Zhang et al., 2012). Dentate GCs undergo structural and migratory abnormalities after SE leading to formation of excessive *de novo* excitatory synapses and recurrent excitatory loops (Zhou et al., 2019). Thereby, GCs that are innately resistant to activation, stop acting as a filter to the hippocampus, but rather produce, amplify, and propagate recurrent excitatory signals (Botterill et al., 2019; Hsu, 2007; Stringer et al., 1989; Zhou et al., 2019). Taking this into consideration, selective $PSAM^4$-Gly driven inhibition of the GCs was taken to be sufficient to affect seizures, but, based on the study outcome, may prove to be suboptimal. Another possible explanation could be a suboptimal experimental design since in our hands the IHKA mouse model slightly differed from what was expected from the literature (Arabadzisz et al., 2005; Lentini et al.,

2021; Löscher et al., 2020; Riban et al., 2002), i.e. not all the animals displayed electrographic seizures after SE, while the rest of the animals presented a more severe seizure phenotype. This might have affected the outcome assessment of the therapy. The reason for these differences in model could be that cells expressing the transgene were more sensitive to KA excitotoxicity, or that combination of IHKA and electrode implantation in the same coordinates triggered a larger spread of KA within the hippocampus along the electrode tract that consequently created larger damage and leakage of the BBB. In follow-up studies, one could consider inducing SE first, and then performing viral vector administration and electrode implantation.

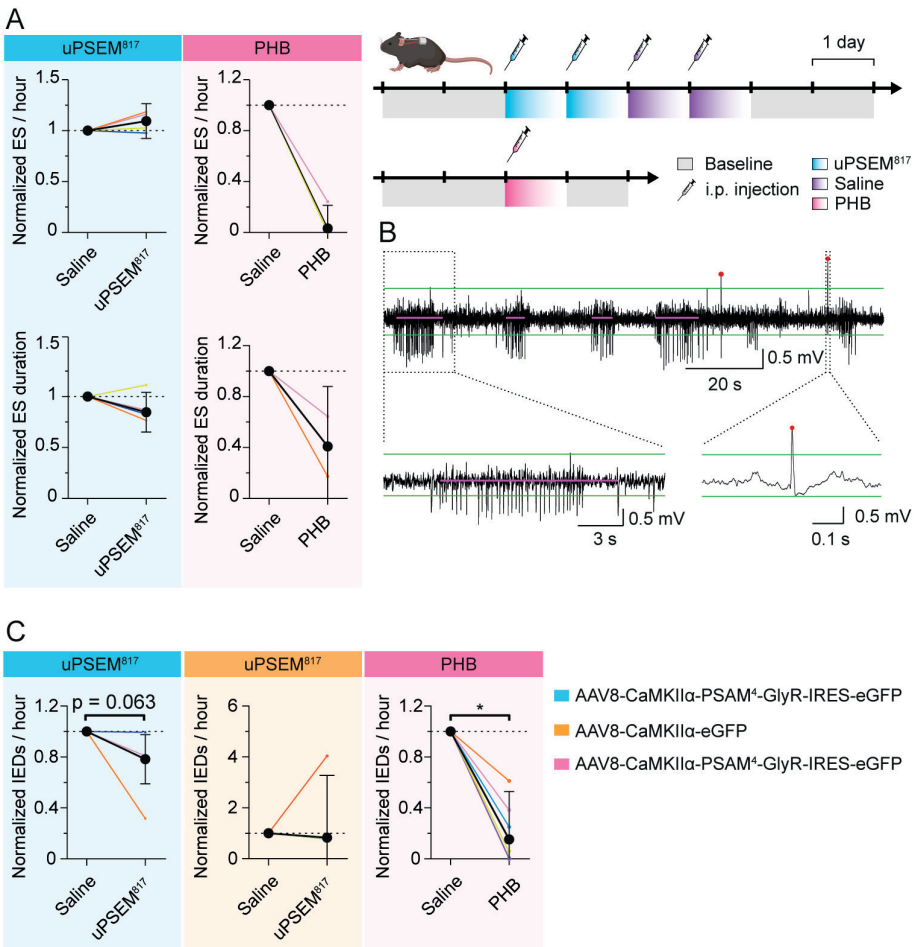

Figure 21. Effect of uPSEM^{817} on ES and IEDs in a chronic model of TLE in mice expressing PSAM^{4}-GlyR
(A) Normalized effect of uPSEM^{817} (cyan) or PHB (magenta) i.p. administration to saline ip injection in PSAM^{4}-GlyR animals on ES rate and mean ES duration. (B) Example of raw signal with electrographic seizure detections (magenta line) and IED detections (dark red points). Green lines mark the thresholds for the spike detection. Magnification of an ES on the left and of an IED on the right. (C) Normalized effect of uPSEM^{817} (cyan) or PHB (magenta) i.p. administration in PSAM^{4}-GlyR animals on IEDs rate, and effect of uPSEM^{817} (orange) in control animals. Median ± interquartile range. ES, electrographic seizures; IED, interictal epileptiform discharges; I.p., intraperitoneal; PHB, phenobarbital. *Adapted from Paper IV.*

In conclusion, these results indicate that PSAM^{4}-GlyR-based chemogenetics is a promising approach but requires further optimization to be able to achieve an anti-seizure effect. Exploring the effect of uPSEM^{817} on epileptiform activity in acute brain slices *ex vivo* or even testing the therapy in hippocampal organotypic cultures could help to clarify if our findings are due to low and/or limited expression of PSAM^{4}-GlyR, or whether there are some other unexpected factors affecting the system itself in the pathological *in vivo* milieu.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

A reality of gaps in epilepsy treatment is evident and inspired the development of this thesis. Epilepsy is a collection of diseases of different etiologies that share a common hallmark, the development of spontaneous recurrent seizures. This diverse disorder is orchestrated by various continuously ongoing underlying processes that facilitate the disease, and yet remain not fully understood. The heterogenicity among epilepsies and the lack of deep comprehension of some underpinning mechanisms has hindered the progress in the field. Nonetheless, new tools in neuroscience research, such as optogenetics and chemogenetics, may help us to understand the function and circuit dynamics of the brain and bring clues on how to better aid dysfunction.

Within this urge for improving our understanding, and thus for developing new therapeutic approaches for refractory epilepsies, this thesis evolved. Based on the evidence of a GABAergic synaptic transmission impairment in the epileptic neuronal network, the overarching strategy of this thesis was to potentiate neuronal inhibition in the epileptic foci with the goal of diminishing hyperexcitability and counteracting seizures.

The studies presented in **Paper I** and **II** provide a better *in vitro* and *in vivo* characterization of the functional intrinsic properties, maturation, and establishment of synaptic connections of the hdINs. The knowledge gained from that work helped to better understand and explain that enhanced inhibitory synaptic transmission was the mechanism of action through which grafted cells modulated the network excitability and exerted an anti-seizure effect. Moreover, analysis of the cell graft composition highlighted the fact that a heterogeneous population of INs may be advantageous for achieving such effect, results that differed from some speculations conferring this ability exclusively to fast-spiking PV-expressing INs. Nevertheless, several challenges remain to be addressed and elucidated in more detail in future preclinical studies. For instance, elucidate if by reducing network excitability, hdINs have also a disease-modifying effect i.e. in aberrant mossy fiber sprouting. However, results elsewhere did not observe any change in mossy fiber sprouting (Hattiangady et al., 2008; Hunt et al., 2013). Moreover, behavior and comorbidities associated with epilepsy should be addressed. In this line, Cunningham and colleagues demonstrated that epileptic mice receiving grafts displayed alleviation of

hyperactivity and aggressive behavior, as well as exhibited improved short-term working memory and novel object recognition memory (Cunningham et al., 2014). Assessment of learning and memory functions is important since they are not caused entirely by SRS, rather influenced by multiple alterations in the epileptic hippocampus i.e. severe decline in neurogenesis, disruption of the tri-synaptic circuit due to the loss of principal neurons in the CA3 and CA1 subfields. Importantly, it should be elucidated if the timing of cell transplantation from the SE induction is relevant to that matter since discrepancies on learning and memory improvements have been noticed when comparing early grafting after SE (Cunningham et al., 2014; Hunt et al., 2013) and protracted grafting (Waldau et al., 2010). This concern is highly relevant and requires resolution since intracerebral cell therapy candidates most likely will be refractory epilepsy patients with some months and/or years of clinical history, and enduring comorbidities and/or mood dysfunction, since patients having significant seizure control with ASM are unlikely to seek for invasive therapies at first. Last, graft survival and seizure suppressor effect must be evaluated at extended periods of time after transplantation, since some studies have reported a diminished in cell survival at a prolonged time after grafting (Zaman & Shetty, 2001).

In addition, results from **Paper I** in the human epileptic tissue further strengthen the translational value of using hdINs as treatment for TLE. The incorporation of *ex vivo* organotypic human epileptic brain slices into the preclinical roadmap represents a plausible testing platform for new therapeutic approaches, potentially saving resources, time, and effort. Indeed, to move from discovery in rodents to testing in humans has proven to be challenging, with many drugs that showed promising results in animal models failing in the early phases of clinical trials. We foresee organotypic human brain cultures, as well as the acute usage of this resected tissue, as an intermediate step in this translation. Within the endeavors for moving cell therapy into clinics, non-immunogenic hPSC lines could potentially be used without immunosuppression (Greco et al., 2015; He et al., 2017; Zheng et al., 2016).

A recent study by Lentini and colleagues have investigated the use of an endogenous cell source to generate interneurons that then would enhance inhibition in the epileptic hippocampus. The authors had induced *in vivo* reprogramming of the reactive glia toward INs, observing promising results in seizure control (Lentini et al., 2021). Direct *in vivo* reprogramming could be a valid avenue to treat epilepsy given its combinatorial nature of reducing astrogliosis and diminishing network excitability by enhancing the inhibitory tone at the same time.

It is worth noticing that most of the work discussed and cited here is done in TLE models. Despite it being one of the most common causes of refractory epilepsy in adults, as it has been mentioned at the beginning of this section, epilepsy is a collection of diseases of different etiologies. Hence, the suitability and effect of those therapies must be examined in other epilepsy models such as focal cortical dysplasia since it is the most common cause of refractory epilepsy in the pediatric

population and the second to third most common etiology in adults. Often pediatric epilepsies are associated with other neurodevelopmental disorders such as ASD, and therefore genetic models of epilepsy and ASD might present challenges when testing therapies that have worked in TLE models. To this goal, in **Paper III**, we first better characterized the epileptic phenotype of the *Cntnap2* KO mouse model of ASD and CDFE syndrome. The study presented in **Paper III** elucidated a heterogeneous phenotype regarding pathological EEG, the hippocampus preceding the cortical areas in seizure generation and propagation, and the positive correlation in terms of IEDs and seizures in this model. A deterioration in long-term memory was also observed after seizure onset in mutants compared to WT. Furthermore, hdIN transplantation in this mouse model did not prevent the development of epilepsy as it could be expected from results in the rat model of TLE, but on the contrary, potentiated seizures. Further investigation of the possible reasons for this outcome are planned in the coming future, including electrophysiological recordings in cell transplanted mice to assess potential disinhibition of principal neurons exerted by grafted cells leading to an increase in network excitability, as well as NKCC1 and KCC2 protein quantification at different ages to assess chloride homeostasis in this model. Another potential consideration is a plausible gradual dysfunction of grafted hdINs in parallel with the progression of the disease into advanced phases and epilepsy occurrence, a phenomenon that has been reported earlier in animal models of Parkinson's disease and even in patients (Brundin & Kordower, 2012). Overall, there is a clear need to better understand the underpinning mechanisms of the disease and its progression, which in turn would lead to refinement of the therapeutic interventions. In this direction, de Jong and colleagues have generated forebrain organoids from patients with a homozygous protein-truncating mutation in *Cntnap2* (de Jong et al., 2021). In the future, it could be interesting to generate dorsal and ventral forebrain spheroids and merge them into assembloids to study IN migration abnormalities (Bagley et al., 2017; Sloan et al., 2018), or investigate E/I balance and network alterations (Nieto-Estévez & Hsieh, 2020; Samarasinghe et al., 2021).

Lastly, in **Paper IV**, a gene therapy approach was used to the same aim as before, potentiate neuronal inhibition in the epileptic foci. Gene therapy presents some advantages in comparison to cell therapy, such as no risk for teratoma formation or ethical considerations. Although, it has the disadvantage of not sensing the network as a grafted neuron would do, and consequently does not have autonomous temporal regulation. To overcome this limitation, we used chemogenetics restricting the effect in time, on-demand. Even though positive results were obtained at the cellular level by affecting the depolarizing currents, further investigation needs to be done to evaluate the efficacy of $PSAM^4$-GlyR therapy on seizures. Perhaps, it would be a better strategy to take a step back and first assess the effect of the approach on epileptiform activity in acute brain slices *ex vivo* or perhaps even test the therapy in mouse hippocampal organotypic cultures. Whether $PSAM^4$-GlyR therapy shows

positive results on seizure control, it would represent a more translational approach compared to DREADDs and its activator CNO.

Finally, one can further envision the clinical translation of these preclinical studies as local administration targeting the epileptic foci planned to be resected in a patient with refractory epilepsy. In case of a positive outcome results from the therapy, the resective surgery might be unnecessary. However, whether a negative outcome or unexpected side effects arise, the resective surgery can be performed as planned and the treated tissue will be removed.

These are exciting times regarding cell and gene therapy for refractory epilepsy, in particular TLE, with a couple of clinical trials recently started or on the verge of being initiated. For the other epilepsies, the future holds many interesting avenues to pursue and investigate to finally improve the lives of those affected.

KEY EXPERIMENTAL PROCEDURES

In this section, only selected methods that have been important in the studies included in the thesis and carried out by the author are described. For additional details about other methods used in the different projects, including the derivation of human primary fetal neurons, SE induction in rats, and bioinformatic analysis of the video-EEG recordings, I kindly refer the reader to the method section of the respective papers in the *Appendix*.

Molecular tools for neuronal activity modulation

In the past decades, two new powerful techniques were developed and had been crucial in gaining understanding and untangling the complexity of specific classes of neural cells and neuronal circuits' mechanism and function in freely moving animals. Those techniques are known as optogenetics and chemogenetics. Both methods require a genetic introduction of the effector protein in the specific neuronal population, so that other cell populations will remain unaltered. They differ from the actuator. Optogenetics depends on light activation that affords a fast effect relevant to the physiological processes in the brain. Chemogenetics relies on a drug actuator being simple to use and resulting in a long effect window. Both techniques had been used in this thesis for different purposes.

Optogenetics

Optogenetics emerged from the discovery of light-activated ion channels and pumps in other organisms such as algae (Nagel et al., 2002; Nagel et al., 2003; F. Zhang et al., 2007). In 2005, Karl Deisseroth's lab demonstrated for the first time that these microbial opsin genes could be used safely to confer neurons the capability to respond to light stimulation (Boyden et al., 2005). Currently, optogenetics is broadly used in neuroscience due to the enormous advantages that offer: the possibility to target a certain cellular population by using specific promoters preceding the opsin

gene, and a high temporal resolution since the ion-channel proteins are activated by light (Deisseroth, 2015).

The first light-activated channel developed and proven in mammalian cells was ChR2 (Boyden et al., 2005). ChR2 is a cationic channel activated by blue light (activation peak at 470 nm) and was first observed in *Chlamydomonas reinhardtii* since this unicellular green algae use this opsin to control phototaxis. The activation of ChR2 in mammalian neurons results in the passive diffusion of positively charged ions across the channel leading to a depolarization of the cell membrane potential. Since then, several engineered versions of natural opsins have been developed, as well as opsin mutants with improved properties, and it is possible to both excite and inhibit specific cell populations by using various ion channels and wavelengths of light (Gong et al., 2020; Lin, 2011; Feng Zhang et al., 2007).

In this thesis, an engineered version of ChR2 with improved expression levels and photocurrent amplitude, ChR2(H134R), is used in Paper I and II to offer a fast and specific control of the activity of genetically modified neurons, for testing integration of grafted cells to host tissue and therefore their capacity to modulate the neuronal network excitability.

Chemogenetics

Chemogenetics permits the reversible remote control of neuronal function and neuronal circuitry by systemic drug administration. Importantly, chemogenetics skips the difficulties in translating optogenetic approaches to the clinic since it does not have problems with light diffusion and penetration. For a successful chemogenetic tool, the modified receptor must not be receptive to any endogenous ligand and should not have endogenous activity in the absence of ligand binding, as well as the actuator must not have pharmacological activity at other endogenous receptors (Urban & Roth, 2015).

The most extensively used chemogenetic strategies are G-protein coupled-receptors. The earliest evidence of a specifically engineered G-protein coupled-receptor was in 1991, when Strader and colleagues mutated the β-adrenoceptor to be activated by catechol-containing esters and ketones (Strader et al., 1991). Since then, various attempts were performed on developing engineered receptors activated by synthetic compounds such as the receptor activated solely by synthetic ligand, abbreviated as RASSL (Conklin et al., 2008; Pei et al., 2008), until the development of DREADDs in 2010 (Dong et al., 2010). The prototype DREADD is a variant of the human M3 muscarinic receptor that is no longer activated by acetylcholine, its endogenous ligand, but by CNO, an inert metabolite of the antipsychotic drug clozapine (Armbruster et al., 2007). Various muscarinic-based DREADDs have been developed that either excite (Alexander et al., 2009) or inhibit specific cell populations (Armbruster et al., 2007).

In 2011, Magnus and colleagues developed a similar method LGIC that permit the control over ion conductance and therefore activation or inhibition of neurons (Magnus et al., 2011). LGIC combines ligand-binding domains and ion pore domains of different channels. An example of these LGIC is the pharmacology selective actuator molecule (PSAM^4) that combines the ligand-binding domain of the a7 nicotinic acetylcholine receptor with the ion pore domain of the glycine receptor (used in Paper IV) (Magnus et al., 2019).

Viral vectors

Two different viral vectors were used in this thesis depending on the specific finality for each project (Figure 22). For Paper I, II, and III, the Tet-On system with the transcription factors *Ascl1* and *Dlx2*, as well as ChR2 were introduced into the hESCs using LVs. The genetic information encoded in the construct of the LV is integrated into the genome of the cell and it will remain in the progeny after cell division. On the other side, for Paper IV, AAVs were used to deliver the chemogenetic system, PSAM^4-GlyR. In this case, we were targeting principal neurons in the adult brain and AAVs are non-integrative vectors with a small size that allows higher spreading through the tissue.

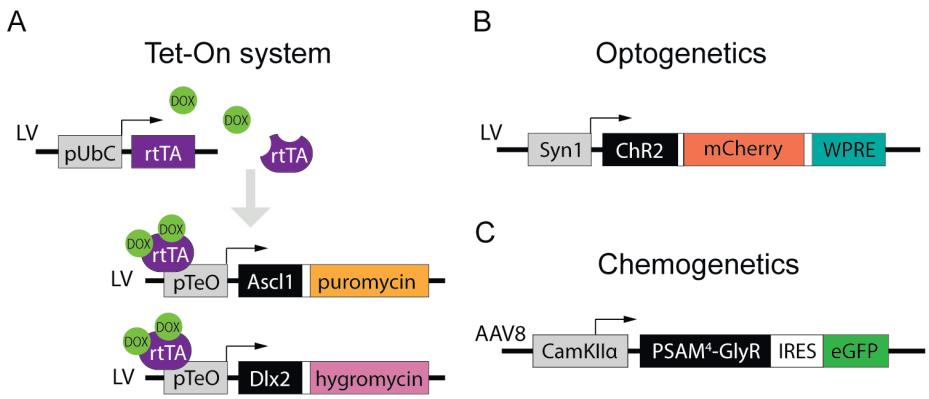

Figure 22. Construct maps of the different viral particles used in this thesis
(A) Construct maps of the LVs constituting the Tet-On system, FUW-rtTA (Addgene #20342), FUW-TetO-Ascl1T2A-puromycin (Addgene #97329), and FUW-TetO-Dlx2-IRES-hygromycin (Addgene #97330), together with an illustration of how doxycycline (Dox) would contribute to the system mechanism. **(B)** Construct map of the LV used for optogenetics, hSyn1-ChR2(H134R)-mCherry-WPRE (obtained by cloning at the lab, from Addgene #20945). **(C)** Construct map of the AAV used for chemogenetics in Paper IV, CaMKIIα-PSAM^4-GlyR-IRES-eGFP (Addgene # 119744).

Lentiviral particles production and titration

For both LV and AAV viral production, we used human embryonic kidney (HEK) 293T cells as packaging cells. For LV, approximately 12,5 x 10^6 HEK293T were seeded in a T175 flask the day before the transfection using DMEM with GlutaMAX supplemented with 10 % fetal bovine serum (FBS) and Penicillin (100 U/ml) / Streptomycin (100 µg/ml) (P/S) (all from Gibco), to achieve confluency of 75-90 % at the time of transfection. Two T175 were used for the generation of 1 batch of LVs.

Before transfection, the medium was replaced by fresh one. Then, the transfection solution was prepared by combining a total of 28 µg DNA and 84 µg PEI (1:3 ratio) for each T175. DNA was composed of the transgene plasmid, the packaging plasmids (pMDLg/pRRE and pRSV-Rev), and the envelope plasmid (pMDG2) in a ratio of 5:4:1. The transfection solution was diluted in DMEM with P/S so that it became 10 % of the total final volume in the flask. Before the addition of the transfection solution to the culture, it was mixed by vortexing for a few seconds and consequently incubated at room temperature (RT) for 15 min. After the addition of the transfection solution, flasks were placed in the incubator, carefully shaken to evenly distribute the solution, and left at 37 °C and 5 % CO_2 until harvesting of the medium.

Cell supernatant was collected 45 hours after transfection and centrifuged at 800 x g for 10 minutes at 4 °C. Then, the supernatant was filtered through a 0,45 µm pore size filter, and the filtrate was transferred to a Beckman ultracentrifugation tube for further centrifugation at 25000 x g for 1,5 hours at 4 °C. The supernatant was discarded, and the pellet containing the viral particles was resuspended in DPBS. The pellet was left to dissolve overnight (O/N) at 4 °C, and then aliquoted and stored at – 80 °C until use.

LVs were titered by quantitative polymerase chain reaction (qPCR) once frozen. First, 1 x 10^5 HEK293T cells were seeded in each well of a 6-well plate in the same media as mentioned above. After 2h from seeding, cells were transduced with 3, 1, or 0.3 µl of the virus diluted using DPBS, to one well each concentration. In addition, three wells were kept without any addition as a mock sample, and three others were transduced with a reference virus at the same concentrations as described. The reference virus expressed GFP and its titer was previously determined by two different methods: manual quantification of the number of cells transduced, and GFP+ cells quantified by a flow cytometer. Once all the conditions were set, cells were left at 37 °C and 5 % CO_2 for 72 hours.

Cells were rinsed in DPBS and the genomic DNA was extracted. The final number of LVs was determined using qPCR, and primers targeting the WPRE sequence and the human albumin gene were used (Table 1). The values detected from the albumin primers were used to normalize the values from the WPRE primers, then the normalized value was compared to the reference virus to calculate the predicted functional titer.

Adeno-associated viral particles production and titration

AAVs were produced as previously described (Negrini et al., 2020). Similar to the LVs, 11 x 10^6 HEK293T were seeded in a T175 flask using the same culturing media as indicated before the day before transfection, so that confluency was 65 % at the time of transfection. In this case, also two T175 were used for the generation of 1 batch of AAVs.

Before transfection, the medium was replaced with fresh one. Then, the transfection solution was prepared by combining a total of 28 µg DNA and 84 µg PEI (1:3 ratio) for each T175. DNA was composed of the transgene plasmid, and the capsid plasmid (rep and cap genes together with adenoviral helper function; pDP8.ape, PlasmidFactory) in a ratio of 1:1. The transfection solution was diluted in DPBS so that it became 10 % of the total final volume in the flask. Before the addition of the transfection solution to the culture, it was mixed by vortexing for a few seconds and consequently incubated at RT for 15 min. After the addition of the transfection solution, flasks were placed in the incubator, carefully shaken to evenly distribute the solution, and left at 37 °C and 5 % CO_2. After 24 hours, media was replaced by Opti-Pro medium (Gibco) with P/S.

Virus harvesting was performed 72 hours after media change. First, cells were detached from the flask by using a cell-scraper, and collected together with the supernatant onto a falcon tube (total volume of 30 ml, from one T175). Then, 3 ml of chloroform was added to the solution and vortex continuously for 5 min. Next, 7,65 ml of 5M NaCl were added, quickly vortex for 10 s, and centrifuge at 3000 x g for 5 minutes at 4 °C. The aqueous phase (upper phase) was transferred to a new falcon tube, where 9,4 ml of 50 % PEG 8000 solution were added. The solution was mixed by vortexing and incubated on ice for 1 hour.

After the incubation, the tubes were centrifuged at 3000 x g for 30 minutes at 4 °C. The supernatant was discarded, and the pellet was resuspended by pipetting in 1,4 ml 50 mM HEPES. Next, a mixture of 3,52 µl 1M $MgCl_2$, 14 µl DNase I, and 1,4 µl RNase A was added to each tube, and those were incubated at 37 °C for 20 min. Then, the solution was placed in two Eppendorf tubes with approximately 700 µl each. Chloroform was added to each tube at a 1:1 ratio, vortexed for 1 min, and centrifuged at 3000 x g for 5 minutes at 4 °C. The aqueous phase was transferred to a new Eppendorf tube, and the process from the addition of chloroform was repeated three more times. After the last transfer of the aqueous phase, tubes were left with the lid open for 30 minutes for evaporation of the chloroform.

The aqueous phases were loaded onto 100 KDa molecular weight cut-off ultrafiltration columns (Amicon Ultra Centrifugal Filter, Millipore), one column for each AAV batch. Columns were then centrifuged at 14000 x g for 5 minutes at RT, and the flowthrough was further discarded. This step was repeated until all the aqueous phases of each sample had been through the column. Next, the solution remaining in the column was diluted with 400 µl DPBS, mixed by pipetting, and

centrifuged at 14000 x g for 5 minutes at RT. The steps including the addition of DPBS and further centrifugation were repeated 4 times. The column was then removed from the collection tube, and placed upside down into a new clean collection tube, which was centrifuged at 1000 x g for 2 minutes at RT. Finally, the AAV solution was immediately transferred to a glass vial, properly sealed, and stored at 4 °C until use.

AAV solution was titered using qPCR. First, 1 µl of AAV solution was diluted in 89 µl of DPBS and 10 µl of 10X DNase I reaction buffer. In parallel, a negative control sample was also generated by using 1 µl of DPBS instead of the AAV solution. Then, 2 units of DNase I were added (1 µl), followed by incubation for 10 minutes at 37 °C. DNase I was then inactivated by adding 1 µl of 0.5M EDTA and heated for 10 minutes at 65 °C. Next, AAVs were lysed by adding 1 µl of Proteinase K (20 µg) and thus incubated for 60 minutes at 50 °C, followed by its inactivation for 20 minutes at 95 °C.

For AAV particle quantification, first, a standard curve was prepared by linearizing an AAV transfer plasmid, and then prepared 10-fold dilution series from 1 x 10^9 to 1 x 10^5 copy number / µl. Next, the final number of AAVs was determined using qPCR, and primers targeting the ITRs (Table 1), followed by interpolation of the values to the standard curve results.

Tabel 1. Titration primers
Forward and Reverse primer sequences for both LV, *ALB*, and WPRE, and AAV, ITR, titration by qPCR. ALB, albumin.

Target sequence	Forward	Reverse
ALB	TGAAACATACGTTCCCAAAGAGTTT	CTCTCCTTCTCAGAAAGTGTGCATAT
WPRE	GGCACTGACAATTCCGTGGT	AGGGACGTAGCAGAAGGACG
ITR	GGAACCCCTAGTGATGGAGTT	CGGCCTCAGTGAGCGA

Cells and Tissue

The majority of work included in my thesis has been conducted using the hESC-line H1 (WA01, WiCell), in particular Papers I, II, and III. Nevertheless, other primary cells, as well as tissue have been used for specific purposes primarily in Paper I and therefore are also described in this thesis.

Culturing of hESCs

H1 (WA01) ESC were obtained from WiCell Research Resources (WiCell, WI) and used for Paper I, II, and III. Human ESCs were maintained as feeder-free cultures on Matrigel-coated (Corning) 6-well plates using Essential 8 Flex medium (E8F; Gibco) and/or mTeSR1 (STEMCELL Technologies), and passaged as colonies

using ReLeSR (STEMCELL Technologies) and/or Accutase Cell Detachment Solution (STEMCELL Technologies). To enhance the survival of cells seeded at low-density, 10 μM ROCK inhibitor Y-27632 (Y; Stem Cell Technologies) was added to the medium for the first 24 hours after passage.

Mouse primary glial cells

As support for the hdIN differentiation and maturation, mouse primary glial cells were used as co-culture. Before the co-culture, those glial cells were harvested from the cerebral cortex of newborn C57BL/6J mice at P3-5, and then kept in culture for a minimum of one to a maximum of five passages. The harvesting process started with the extraction and dissection of the cortex, followed by cutting it into small pieces. The tissue was all the time kept in ice-cold Hank´s Balanced Salt Solution (HBSS, Gibco). Once at the cell culture lab, the tissue was digested using trypsin 1 % for 30 minutes at 37 °C, and manually inverting the tube every 10 minutes. Then, the suspension was centrifuged at 300 x g for 5 minutes at RT, and consequently, the supernatant was removed by decantation. Glial media based on MEM medium (Gibco) and supplemented with 5% FBS, 0.4% D-Glucose (w/v; Sigma), 2% B-27 (Gibco), 1% GlutaMAX (Gibco), and 1% P/S; was added onto the pellet and used for dissociating the tissue to a single-cell suspension by pipetting. The cell suspension was loaded onto a 45 μM cell-strainer and collected into a new tube so that big aggregates of cells were removed. Finally, primary glial cells were plated onto T75 flasks coated with poly-D-lysine (Sigma), maintained at 37 °C and 5 % CO_2, and passage at confluency using trypsin.

Organotypic cultures of adult human brain tissue sections

There is approximately 30 % of epileptic patients that do not respond to ASM, as it has been explained before, and the majority of these refractory patients suffer from focal epilepsy. For a small number of these refractory patients, fewer than 5 %, surgical resection of the epileptogenic focus could be an effective treatment. In those cases, prior to the hippocampal or cortical resection, patients undergo extensive pre-surgical evaluations including the identification of the epileptic focus by extracranial (or even intracranial) EEG recording and neuroimaging using magnetic resonance imaging, as well as neurological and neuropsychological assessment, among others. The goal of these tests is to evaluate if by removing the tissue, the benefits will outweigh the risks of the surgery *per se*.

This surgically resected tissue from drug-resistant epileptic patients is an invaluable platform for gaining understanding of the pathology, as well as testing potential therapeutic approaches. To that goal, written informed consent is required from the patients to use the tissue for research purposes, and all the procedures have been approved by the local Ethical Committee in Lund (#212/2017).

As illustrated in Figure 23, brain tissue resections were obtained as one big piece from the surgery team in the same surgery room. Immediately after the tissue was extracted from the patient, it was submerged in an ice-cold sucrose-based human cutting solution, containing (in mM): 200 sucrose, 21 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 NaH_2PO_4, 1.6 $CaCl_2$, 2 MgCl (all from Sigma-Aldrich, Sweden), adjusted to 300–310 mOsm, 7.4 pH. Within the cutting solution, the tissue was transported to the laboratory and transferred into a fresh cutting solution, continuously bubbled with 95% O_2 and 5% CO_2. If needed, meninges and blood vessels sticking to the pial surface of the brain were carefully removed, and then, the tissue was transferred to the slicing chamber of a Vibratome (Leica VT1200S) connected to a mini chiller (Huber) at 3 °C. The tissue was cut in 300 µm sections, at vibration amplitude of 1.7 mm and forward speed through the tissue 0.05 mm/s. Next, slices were transferred to a cold rinsing media, containing HBSS, HEPES (4.76 mg/ml; Sigma), D-Glucose (2 mg/ml), P/S (50 µl/ml), bubbled with 95% O_2 and 5% CO_2. After 15 minutes in the rinsing media, slices were transferred to the cell culture lab and placed onto membrane inserts (Millipore, PIHP03050) in 6-well plates submerged with BrainPhys culture medium (STEMCELL Technologies) supplemented with 2 % B27, GlutaMAX (1:200), and P/S (10 µl/ml), and incubated at 37 °C and 5 % CO_2. The medium was changed the day after and two-three times per week thereafter.

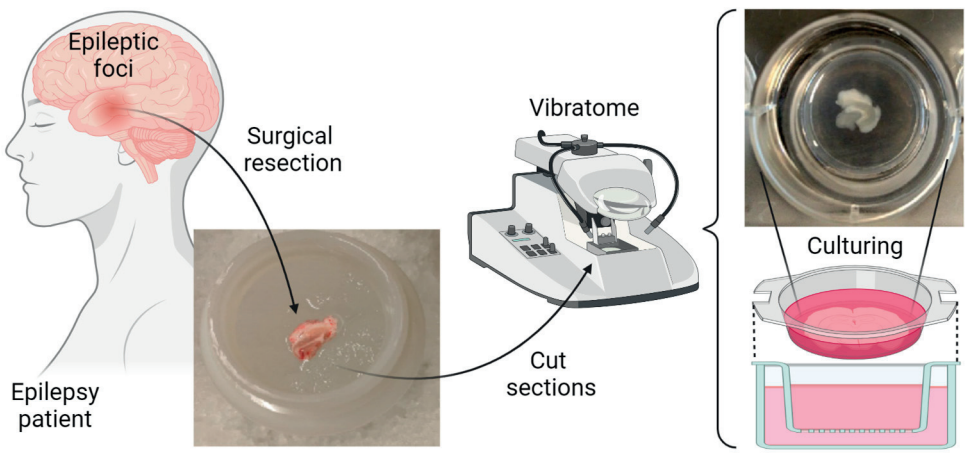

Figure 23. Preparation of organotypic cultures of adult brain surgical resections
From left to right, brain tissue from surgical resections of the epileptic foci of refractory epilepsy patients is transported from the surgery room to the laboratory where it is sectioned in the vibratome. After rinsing, slices are cultured in kept in the incubator.

Differentiation of hESCs into GABAergic neurons *in vitro*

To initiate the differentiation, hESCs were transduced with LVs carrying the Tet-On system for the TFs AD at MOI 5:2.5:2.5 and ChR2 at MOI 2 (Figure 24), in fresh E8F medium containing 10 µM Y.

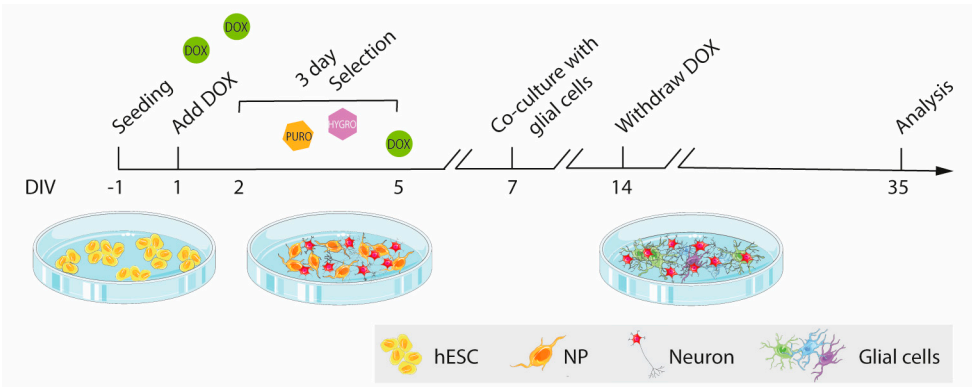

Figure 24. Differentiation protocol for the generation of GABAergic interneurons
Schematics illustrating the timeline protocol used for the generation of hESC-derived GABAergic interneurons by overexpression of the two transgenes *Ascl1* and *Dlx2* using the doxycycline-inducible Tet-On system. DIV, days *in vitro*; DOX, doxycycline; PURO, puromycin; HYGRO, hygromycin; NP, neuronal precursor.

The differentiation was carried out as described in Yang *et al*. (2017), with the addition of some modifications (Gonzalez-Ramos et al., 2021; Yang et al., 2017). Briefly as summarized in Figure 24, hESCs were plated on a Matrigel-coated 6-well plate at a density of 3 x 10^5 cells/well in E8F containing Y on -1 DIV. At 1 DIV, the medium was replaced with N2 medium containing DMEM/F12 (Gibco) supplemented with N2 Supplement (1:100; Gibco) and containing doxycycline (Dox; 2 g/l; Sigma-Aldrich) to induce the transcription of the Tet-On system genes AD and induced the conversion towards the GABAergic lineage. Dox was added to the medium for 14 days. At 2 DIV, an antibiotic-resistance selection period for the expression of both TF was started by the addition of puromycin (0,5 µg/ml; Gibco) and hygromycin (750 µg/ml; Invitrogen) to the fresh medium. At 5 DIV, the antibiotic-resistance selection was finalized, and instead, a cytotoxic compound was added to the medium, cytosine β-D-arabinofuranoside (Ara-C; 4 µM, Sigma), so that proliferative cells died and, consequently, cells initiating the differentiation process were selected. After a week, 7 DIV, cells were detached into a single-cell suspension using Accutase and plated together with mouse primary glial cells on Matrigel-coated 24-well plates at a density of 3-5 x 10^5 and 5 x 10^4 cells/well respectively. Moreover, at this time point the medium was replaced with a Growth medium, consisting of Neurobasal medium supplemented with 2% B27, 1% GlutaMAX, 5% FBS, and P/S. Then, medium changes were done every 2-3 days, until analysis, and supplemented with cytosine β-D-arabinofuranoside (Ara-C)

again from 10 DIV onwards to avoid glial cells overgrowth, and from 15 DIV with brain-derived neurotrophic factor (BDNF, 14 ng/ml, R&D Systems). Additionally, Dox was withdrawn from the medium at 14 DIV.

Cell grafting *in vitro*

In Paper I, the survival and integration of hdINs were studied in two different *in vitro* systems: a 2D culture of human fetal primary cortical neurons, and in organotypic cultures of adult human brain tissue. For both cases, the preparation of the hdINs was similar.

At 7 DIV, hdIN precursors were detached into a single-cell suspension using Accutase, centrifuged at 300 x g for 5 minutes at RT, and resuspended to a desired concentration. Then, for the co-culture with human fetal primary cortical neurons, 15 x 10^4 hdIN precursors were resuspended in Growth medium supplemented with Dox and 10 µM Y and plated in each well of a 24-well plate onto the human primary neuronal cultures. Then, both cell types were cultured together following the differentiation protocol described above for 4 weeks (reaching 35 DIV for hdINs), when co-cultures were analyzed.

For the transplantation onto the organotypic cultures, hdIN precursors were resuspended to a concentration of 10000 cells/µl in HBSS (Gibco) containing 2 µg/µl Dox, 10 µM Y, and DNase I (1 µg/ml, STEMCELL Technologies), and kept on ice until transplantation. This procedure was exactly the same for transplantation into the animal models performed on Papers II and III (explained below). Coming back to the organotypic cultures, those were kept for at least 1 day in culture before hdINs precursors were seeded onto the tissue. For the seeding, organotypic cultures were kept in an air-liquid interface and 3 µl of the hdIN suspension was applied on top of the tissue, followed by 30 minutes of incubation at 37 °C and 5 % CO_2 so that cells had time to penetrate the tissue before media was added to cover the tissue surface. Thereafter, human adult organotypic cultures were cultured in the same media as before with the addition of Dox for seven more days (from 7 DIV to 14 DIV), so that the hdIN differentiation continued within the tissue.

Animal work

Experimental animals were kept at the local animal facility in individually ventilated cages, 12 hours light / 12 hours dark cycle with access to food and water *ad libitum*. All experimental procedures performed were approved by the Malmö/Lund Animal Research Ethics Board, ethical permit number indicated in each specific project (Table 2), and conducted in agreement with the Swedish Animal Welfare Agency regulations and the EU Directive 2010/63/EU for animal experiments. For more

information regarding numbers, cohorts, and treatment groups, I kindly refer the reader to the respective papers in the appendix

Tabel 2. Summary of the main practicalities regarding experimental animals of each paper
Information regarding the specie, strain, gender, age of therapeutic intervention, company, and ethical permit. M, males; F, females; w, week; m, month; P, postnatal day.

Paper	Specie	Strain	Gender	Age	Company	Ethical permit
I	Mouse	C57BL/6J	M + F	P3-5	Janvier Labs *	M49-15
II	Rat	NIH-Foxn1^{rnu} (RNU)	M	7-8 w	Charles River	M47-15, M49-15
III	Mouse	C57BL/6J	M + F	P2	Janvier Labs *	02998/2020
III	Mouse	B6.129(Cg)-Cntnap2^{tm1Pele}/J	M + F	P2	Jackson Lab *	12548-19
IV	Mouse	C57BL/6J	M	2-3.5 m	Janvier Labs	02998/2020

(*) Animals were bred in-house, only founders were ordered from the company.

General stereotaxic surgery considerations

All surgical procedures, except the early neonatal transplantation (Paper III), were performed under general anesthesia by isoflurane gas inhalation (Baxter), 1.2-1.8 % (4 % for induction) in air. Experimental animals were placed in a stereotaxic frame (Kopf Instruments) and local analgesic bupivacaine (< 0.5 ml, Marcaine, AstraZeneca) was injected sub-cutaneous to the skull surface after shaving the area. Viscotears were used to maintain the eyes hydrated, and physiological saline (~0.5 ml) was supplemented sub-cutaneous to maintain hydration in all the body. Before and after surgery, the incision area surface was cleaned with Chlorhexidine (Fresenius Kabi) and after the surgery, the wound was closed with resorbing thread (Ethicon). During the surgery, body temperature was maintained using an electric heat pad.

Induction of status epilepticus in mice

Epilepsy is a collection of diseases with different etiologies that share certain hallmarks. In epilepsy, several pathological factors contribute to hyperexcitability and seizure generation (Löscher, 2002). Different models have been used to study the underlying mechanisms and can be classified in many ways, for instance, they can be easily divided in acute seizure models, such as pentylenetetrazol (PTZ) seizure test and the electrical kindling model, or chronic seizure models, also known as post-SE models (Löscher, 2017; Simonato et al., 2014). These second ones are generated by an insult (either electrical or chemoconvulsive) that gives rise to SE, followed by an epileptogenic process, and further development of SRS, as well as pathological hallmarks of the disease (Kandratavicius et al., 2014). Therefore, chronic models do better resemble features of human patients, have higher predictive value for new treatments for refractory epilepsy, and thereby are the ones chosen on Paper II and IV. Importantly, in some cases, models are chosen because

they are relevant etiologically, such as the traumatic brain injury model (Thompson et al., 2005), or models of genetic epilepsies like CDFE, the *Cntnap2* KO used on Paper III (Paterno et al., 2021; Penagarikano et al., 2011).

In Paper IV, we use a well-established mouse model of chronic TLE with hippocampal sclerosis, the IHKA mouse model, used by the NIH/NINDS Epilepsy Therapy Screening Program to test the efficacy of new antiepileptic treatments since it mimics drug-resistant seizures as described in TLE patients (Wolfgang Löscher et al., 2020; Wilcox et al., 2020). SE was induced by unilateral IHKA injections into the right dorsal hippocampus of adult mice, using the stereotaxic setup explained above. A single administration of 45 nl of 20 mM KA solution (total dose 0.9 nM KA; Abcam ab120100) was injected at a rate of 25 nl/min, followed by 3 minutes of waiting time before retraction of the glass capillary. The coordinates used are indicated in Table 3. After KA administration, animals were video-EEG monitored for 24 hours and evaluated in a modified six-level Racine scale (Racine, 1972). KA is a glutamate analog that interacts with kainate receptors, which happen to be highly expressed in the hippocampus (Bloss & Hunter, 2010). Because of massive KA-inducing robust neuronal depolarization, the hippocampus becomes susceptible to excitotoxicity and triggers acute sustained seizures or SE (Vincent & Mulle, 2009). This epileptogenic insult leads to the development of SRS after a few days or weeks without any ictal activity (seizure-free period).

Cell transplantation

On 7 DIV, hdINs precursors were dissociated into a single-cell suspension using Accutase, washed, counted and resuspended in HBSS containing 2 µg/µl Dox, 10 µM Y, and 1 µg/ml DNase I to a concentration of 100.000 cells/µl. Cells were kept on ice during the entire duration of the surgical procedure and transplanted in a 4-5 hours window from the time the cells were prepared. Moreover, to ensure a homogeneous density of the cell preparation, cells were gently mixed with a sterile pipette prior to loading into the glass capillary or Hamilton syringe. This part of the procedure was common for the cell treatment used in Paper II and III, although the main difference relies on the stereotaxic injection *per se*.

In addition, in both cases, cells remaining after transplantation were re-plated on Matrigel-coated coverslips in 24-well plates and cultured in N2 medium for 24 hours. Then, cells were fixed with 4 % paraformaldehyde (PFA) containing 0.25 % glutaraldehyde, and used for immunofluorescence.

Cell transplantation in adult rats

In Paper II, a total of 600.000 cells were transplanted bilaterally into both hippocampi of epileptic RNU rats under isoflurane anesthesia (see coordinates in Table 3). Rats were given Dox in drinking water (1 mg/ml in 0.5 % sucrose) for two

days before and four weeks PT to continue with the Tet-On system induction for the cell differentiation *in vivo*.

Cell transplantation in newborn mice pups at P2

In Paper III, transplantations were performed in newborn mice at P2, still depending on the mother for survival and feeding. Therefore, pups were taken from the breeding cage one by one, diminishing the stress to the mother, then briefly anesthetized by isoflurane and kept anesthetized on ice during the procedure (surgeries lasted less than 15 minutes in total for each animal). Mice pups were placed on the stereotaxic frame using a homemade Play-Doh-like stage (see Figure 25) and flipped ear bars so that flat edges were touching the head of the mice. The skin surface was clean with ethanol and air-dried prior to the injection. A pre-hole in the skull cartilage using a 30G insulin needle was done at the desired coordinates (Table 3) prior to cell injection. A total of 100.000 cells were transplanted unilaterally into the dorsal right hippocampus in a final volume of 1 µl at a rate of 0.2 µl/min. using a Hamilton syringe with a 33G needle. After cell delivery, three minutes were waited before retracting the needle, and then the pup was warmed up using the heat from the experimenter's fingers for a few seconds until it woke up, so that it could be placed back in the breeding cage. Mice were given Dox in the drinking water following the same pattern as described previously for the adult rats.

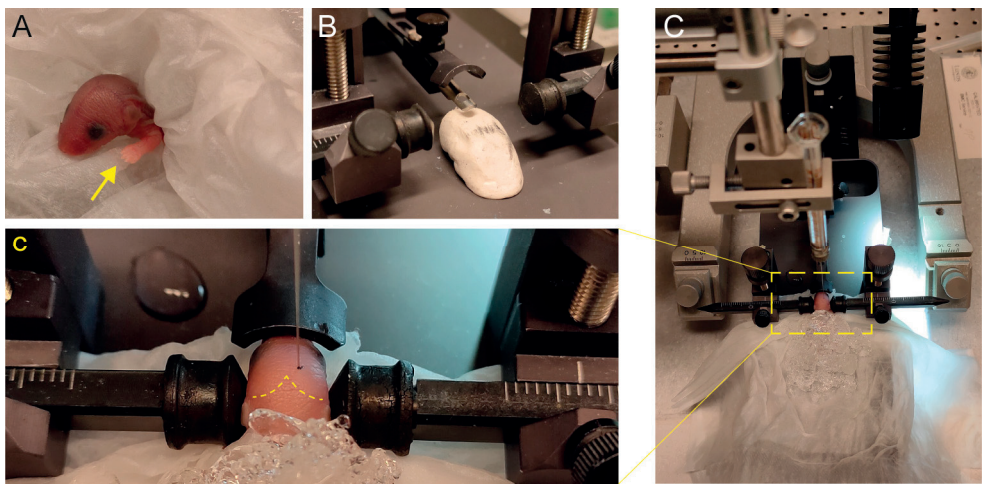

Figure 25. Stereotaxic transplantation in newborn mice pups at P2
(A) White front paw (yellow arrow) indicative of the reduced blood flow in that area, so that the pup is experiencing anesthesia by hypothermia. **(B)** Play-Doh-like stage for holding the pup's body in position and inverted ear bars. **(C)** Overview of the setup with the pup already covered by ice over the soft tissue paper. **(c)** Closed-zoom of the head of the pup, with the Hamilton needle already inserted into the brain (yellow dashed line indicating lambda and lambdoid sutures).

Viral vector delivery

The delivery of AAVs into the hippocampus of adult mice was performed via stereotaxic surgery as described above. A volume of 0.4 µl was injected at a rate of 0.1 µl/min in each deposit (Table 3) using a glass capillary and injection pump (Nanoliter 2010, World Precision Instruments). Hence, a total of 1.2 µl were injected in 3 different deposits in each hemisphere, with an additional 3 minutes after administration allowing for diffusion of the vectors before retraction of the needle and moving to the next coordinate.

Video-EEG monitoring

Two different EEG monitoring systems were used in this thesis. In Paper III, electrodes connected via cables were used, while in Paper IV a telemetry system was used allowing free-moving of the animals which was important for the more frequent behavioral seizures occurring in the IHKA mice model compared to the genetic *Cntnap2* KO mouse model used in the previous paper (see Figure 26). In Paper II, rats were monitored also using a telemetry system, although for specifics I kindly refer the reader to this paper in the *Appendix*.

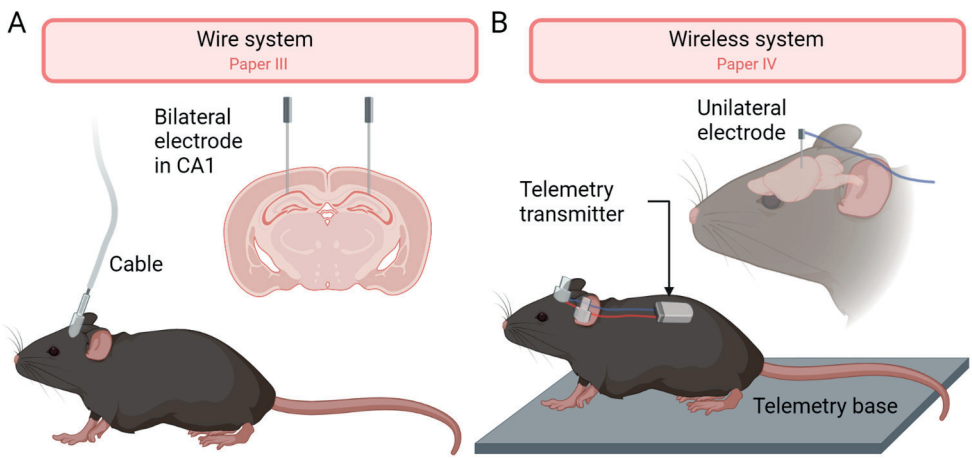

Figure 26. EEG recording systems
(A) Wire system used for EEG monitoring in Paper III, where two recording electrodes were placed bilaterally in CA1. **(B)** Wireless system, also called telemetry system, for EEG recording in Paper IV, where a single recording electrode was placed in the right dorsal hippocampus.

Going into detail, in Paper III two insulated stainless-steel wire twisted pair electrodes (127 µm diameter) were implanted to the dorsal CA1 bilaterally (see coordinates in Table 3), secured by cyanoacrylate glue (Loctite), dental cement (AgnTho's) and two skull screws (AgnTho's), placed in frontal and occipital bones. The ground electrode was placed into the neck musculature. All electrodes, the two twisted pairs and the ground one, were attached to six-channel plastic pedestals

(E363/3 & MS363, P1 Technologies). After four days of recovery, being the last day also acclimatization to the recording cage, mice were connected via cable and swivel (P1 Technologies) to amplifiers providing common-mode rejection and digitized at 2 kHz (BioAmp & PowerLab 8/35, AD Instruments) and recorded to a Windows PC using LabChart 8. Video was registered by two HD cameras (Logitech), integrated into Open Broadcaster Software (OBS Studio), and recorded via the video-plugin in LabChart (720p/25fps). Mice were monitored 24/7 for 13-14 days, and red-spectrum restricted light sources were used during the dark cycle.

Tabel 3. Coordinates used for the different stereotaxic interventions

Summary of the coordinates (in mm) used for cell transplantation (Paper II and III), viral delivery (Paper IV), KA administration (Paper IV), and electrode implantation (Paper II, III, and IV). For all the interventions coordinates are given in relation to Bregma for AP and ML, and the *dura mater* for DV. Cell transplantation in Paper III used Lambda as reference for AP and ML coordinates. AP, antero-posterior axis; ML, medio-lateral axis; DV, dorso-ventral axis; Loc, location; D, dorsal; V, ventral.

Paper	Injection	Intervention	Loc	AP	ML	DV	Volume
II	Bilateral	Cell suspension	D-V	- 6.2	± 5.2	- 6.0; - 4.8; - 3.6	1 µl / deposit
II	Unilateral	Electrode impl.	V	- 6.2	+ 5.2	- 6.0	-
			Ipsilateral *dura mater*, above motor area M2/M1				-
III	Unilateral	Cell suspension	D	+ 0.85 *	+ 1.35 *	- 1.1	1 µl
III	Bilateral	Electrode impl.	D	- 2.0	± 1.75	- 1.3	-
IV	Bilateral	AAV delivery	D	- 2.2	± 1.7	- 1.9	0.4 µl / deposit
			V	- 3.3	± 3.0	- 3.7; - 2.7	0.4 µl / deposit
IV	Unilateral	KA injection	D	- 2.0	+ 1.6	- 1.9	45 nl
IV	Unilateral	Electrode impl.	D	- 2.0	+ 1.6	- 1.9	-

(*) Coordinates based on Lambda as reference.

In Paper IV, electrodes were implanted on the same surgery that IHKA was administered. First, a telemetry transmitter (MT10B, KAHA Sciences) was implanted subcutaneously in a previously created skin pocket over the right dorsal thoracic region. Then, the negative (reference signal) lead of the transmitter was fixed to a skull screw (AgnTho's) in the occipital or parietal bone, and sealed with conductive paint (Bare Conductive). Next, the positive lead was connected before insertion by clamping to the electrode connector, an insulated stainless-steel electrode (203 µm diameter) (E363/2, P1 Technologies) that was placed on the dorsal CA1 of the right hemisphere (see Table 3), same coordinates than the KA deposit. The clamp was also sealed with conductive paint. Finally, connections were fixed on the skull and insulated with first cyanoacrylate glue (Super Glue, Loctite) followed by dental acrylic cement (AgnTho's), and the opening of the skin pocket containing the transmitter was sutured with resorbing thread. The signal from the telemetry transmitter was collected by the telemetry system (MT110 tBase, Kaha Sciences) when the cage with the animal was placed above. Then, the telemetry system was connected to an ADC (PowerLab, AD Instruments) with LabChart Pro software (AD Instruments) on a Windows PC. In this case, the sampling rate was 1 kHz and video was registered using two HD ethernet cameras (Axis Communications), combined in the open-source Open Broadcaster Software (OBS

Studio), and synchronized and recorded using LabChart. After the surgery, the animals were video-EEG monitored for SE during the next 24 hours, followed by being returned to the stables. Two weeks later, during the chronic phase, continuous video-EEG monitored commenced until the different treatment conditions were tested.

Behavioral tests

A battery of behavioral tests was performed in Paper III, to assess locomotion, stereotypies, and perseveration, as well as memory capacity before and after the predicted seizure onset at six months of age. *CNTNAP2* gene is strongly associated with ASD and CDFE (Peñagarikano & Geschwind, 2012; Rodenas-Cuadrado et al., 2014; Strauss et al., 2006), and several behavioral deficits previously reported in human patients has also been described in juvenile *Cntnap2* KO mice (Penagarikano et al., 2011). Together with deficits in the three core ASD behavioral domains, mice also showed hyperactivity and epileptic seizures, also as reported in humans.

A period of acclimatization was performed for three days before starting the behavioral tests, consisting of daily handling of the mice for 5 minutes and habituation to the testing room at least for 1 hour before the start of the test. Importantly, tests were performed in dimmed light, during the early morning around 8-9 a.m. Three different tests were performed, open field, memory test, and spontaneous alternation T maze, at two different ages of the animals, 4 and 7 months. Moreover, nest building was assessed on the video-EEG recording cages at 7 months of age.

For the open field test, mice were placed inside a black Plexiglas arena (40 cm x 40 cm) for 30 minutes and their general activity was video recorded. There were detectors on both sides of the box at 6.5 cm of height to assess rearing, which is an indicator of exploratory behavior. Analysis was done using data collected with ANY-maze (Stoelting Europe), and only data between 10-30 minutes was used. In addition, motor stereotypies were studied by analyzing self-grooming in the last 10 minutes of the test.

The hippocampal formation is responsible for processes underlying memory formation and spatial navigation (Bird & Burgess, 2008; Burgess, 2008). So, we considered that hippocampal functions needed to be assessed in Paper III since we identified the hippocampus as the focus of epileptic seizures in the *Cntnap2* KO model, and it was the target area for the cell transplantation. The open field was used as habituation to the arena the day before starting the memory tests. Short-term spatial memory (STM) was investigated by an object location task (OLT) with 20 minutes retention time. In this test, there were four objects placed in the arena and mice had 15 minutes to explore them before being placed back to the home cage. After 20 minutes, the location of two of them was exchanged and mice could explore

them again for 15 minutes. Long-term memory (LTM) was also studied using a novel object recognition test (NOR) with 24 hours retention time. In this case, one object out of four was changed for a different one, although maintaining the location, and the time spent in the novel object was compared to the mean of time spent in the other three ones.

To study perseveration, the spontaneous alternation T maze was performed (Deacon & Rawlins, 2006). Mice were placed on the base of the T maze facing the wall at the end of the corridor, and given the choice to explore both arms of the maze (either right or left), for ten consecutive trials. When the animal was released from the hand of the experimenter, it run along the corridor deciding for one of the arms, meaning that at least stepped with the four paws into the arm, and thus the gate was closed behind the animal allowing it to explore for 5 seconds before the next trial started. Then the number of alternations were counted.

Last, nesting behavior was observed during the video-EEG monitoring. Three different materials were used, and the average was the final nest-building score. It represents the amount of nest material used after 24 hour period and the shape of the nest (1, poor; 5, good) (Deacon, 2006).

In vitro Electrophysiology

A substantial portion of the results of this thesis rely on *in vitro* electrophysiological experiments, especially Paper I, II, and IV. Mainly, electrical recordings from individual neurons, known as whole-cell patch-clamp (Figure 27B), were performed, although in some cases also electrical recordings from an area of the tissue called extracellular field recordings (Figure 27A) were also included (Paper II).

Whole-cell patch-clamp recording

One way of obtaining functional information of a cell and the surrounding population network is by performing whole-cell patch-clamp recordings. In this type of recording, the solution surrounding the recording electrode is exchanged by diffusion with the interior of the cell enabling a low resistance electrical access to the cell functional properties. For instance, by interpreting the electric parameters (current, voltage, and resistance), it is possible to characterize the maturation of neuronal cultures, both at individual cell properties and afferent synapses level.

The principle of the whole-cell patch-clamp techniques is that the recording electrode is surrounded by a glass micropipette containing a solution that mimics the intracellular content of the cell. It is possible to carefully adjust the composition

of the intracellular solution to better visualize certain properties of the cell. The glass micropipette is placed pressing against the cell surface and by applying gentle suction through the tip it forms a tight seal with the cell membrane, called "giga-ohm seal" (GΩ) (Figure 27C, steps 1-2). Once the pipette is so closed to the cell membrane that the resistance is at the giga-ohm range means that the cell is securely attached to the pipette. Thus, the next step is rupturing the piece of membrane in between by a short suction, gaining access to the inner cell (cytoplasm) although leaving the seal intact (Figure 27C, step 3). Now, electrical information can be detected, further amplified, and recorded. At this step, it is important to assess the resistance through the pipette to the ground, known as series resistance (Rs) or access resistance (Ra), which gives information if the access to the cell from the recording electrode is fine. Then, the recording can be done in two different modes: voltage clamp and current clamp. In the first case, the membrane potential is controlled by the experimenter, usually set to -70 mV, and the recording trace illustrates changes in current in the interior of the cell (in the range of pA). In the second case, the current clamp, current inside the cell is controlled by the experimenter and the recording trace indicates the membrane potential. For instance, if just after rapturing the cell membrane, the experimenter sets the recording in current-clamp mode with 0 pA holding current, the trace will show the resting membrane potential of the cell, which is characteristic of the different cell types. This is only an example, there are more specifications in the *intrinsic properties* section below.

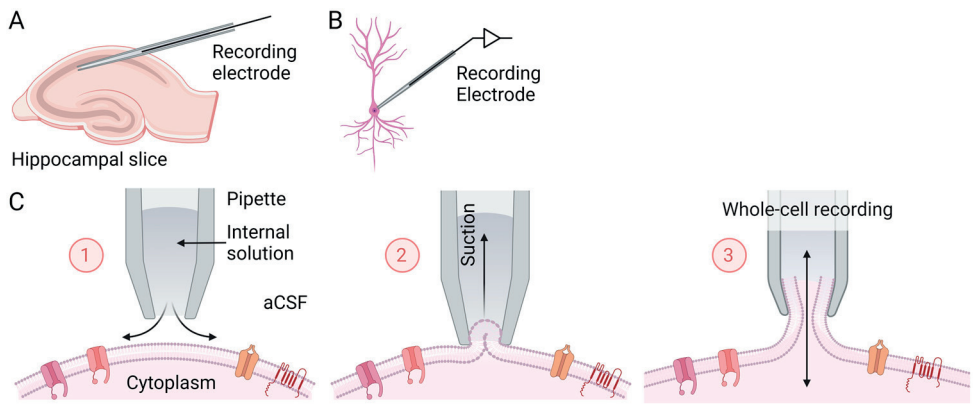

Figure 27. Principle of field and whole-cell patch-clamp electrophysiology.
(A) Pipette placed in the extracellular space in CA1 of a hippocampal slice for field recording of the activity from several cells. **(B)** For whole-cell recording, the neuron is approached with the glass pipette containing the recording electrode. **(C)** Principle of whole-cell recording and the different steps: 1, the glass pipette approaches the cell membrane applying pressure; 2, the glass pipette contacts the cell membrane, and a tight seal is formed by gentle suction; 3, a higher suction causes the membrane to rupture establishing the whole-cell configuration.

Extracellular field recording

The extracellular field recordings, unlike whole-cell patch-clamp recordings, measure responses from a population of neurons by detecting local field potentials. In this case, the glass micropipette is filled with aCSF since we want to mimic the extracellular composition. Moreover, the pipette is placed in the extracellular environment of a specific area of interest, although not attached to any cell. With this technique, the experimenter will observe changes in the field potential as a result of several cells being active at the same time.

Setup

To perform the electrophysiological experiments, the recording setup was composed as follows. Glass pipettes were made from borosilicate glass capillaries and pulled with a Flaming-Brown horizontal puller (P-97, Sutter Instruments). Pipette resistance for whole-cell patch-clamp recordings was between 2.5 - 6.5 MΩ, while for field recording was 1-3 MΩ. Importantly, pipette capacitance was corrected online before giga-seal formation, while fast capacitive currents were compensated for during cell-attached configuration. Then, pipettes were backfilled with the appropriate solution and a silver electrode was placed inside as a recording electrode. The recording electrode was connected to an EPC-9 amplifier (HEKA Electronik), which was then connected to a computer with the Patchmaster software. Data were acquired with a sampling frequency of 10 kHz with a 3 kHz Bessel anti-aliasing filter.

For visual guidance when approaching the pipette to the cell, an infrared differential interference contrast microscope (Olympus) with a 40x water immersion objective was used. The microscope and movement of the electrode were controlled by a micromanipulator (SM-5 9 Luigi and Neumann). In the case of fluorescent cells (Paper I, II, and IV), those were identified first by differential interference contrast microscope and then by external activation of a LED light source and setting of the specific wavelength filter (see *Optogenetics* section below for more information). For a retrospective confirmation of the recorded cell using immunofluorescence, during the recording, the intracellular solution within the pipette contained biocytin (0.5-1 mg/ml, Biotium).

Recordings were performed with a continuous flow of aCSF, bubbled with carbogen (95% O_2 and 5% CO_2), to afford a constant nutrient and oxygen supply to the sample. The aCSF was continuously pumped via masterflex BPT tubing (ColePArmer instrument) through a dual-channel inline solution heater (Supertech) passing by a drop chamber, to ensure a constant flow before entering the stage where the sample was. Exit from the stage was also through a drop chamber. The stage or recording chamber was also heated from the holder, maintaining a stable temperature usually set at 32 °C.

Optogenetics

For the experiments involving optogenetics in Paper I and II, blue light of 460 nm wavelength was generated by a LED light source (Prizmatrix, Modiin Ilite) connected to the microscope via a waveguide. Then it was illuminating the sample through the water immersion 40x microscope objective. In addition, orange-red light (595 nm) was used as a negative control of ChR2 stimulation. Specific light protocols with different frequencies and duration of light pulses were programmed and controlled through the Patchmaster software. In particular, blue light was delivered as a square pulse for a duration of 500 milliseconds, or by 5 pulses of 3 milliseconds repeated at 10 Hz.

Acute hippocampal slice preparation

Mice (Paper IV) or rats (Paper II), were removed from the video-EEG monitoring setup and transported to the electrophysiology lab. Once there, animals were briefly anesthetized with isoflurane, decapitated and brain was extracted from the skull. The brain was then transferred to an ice-cold sucrose-based cutting solution containing (in mM): 75 sucrose, 67 NaCl, 26 $NaHCO_3$, 25 D-glucose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$ (all from Sigma Aldrich), equilibrated with carbogen (95% O_2 and 5% CO_2), with pH 7.4 and osmolarity ~305-310 mOsm. Cerebellum was removed and hemispheres of the brain were separated and cut on a vibratome (VT1200S, Leica Microsystems) into 300 µm (Paper II) or 400 µm (Paper IV) thick slices. Slice orientation differed in the different studies, in Paper II slices were cut quasi-horizontal by performing the "magic-cut" before attach the tissue to the vibratome. On the other side, in Paper IV right hemisphere slices were cut sagittal to better preserve the tri-synaptic circuit of the dorsal hippocampus where the KA was administered and the electrode was implanted, and the left hemisphere was cut quasi-horizontal as in Paper II. Slices were incubated in the cutting solution for 30 minutes at 34 °C, and subsequently transferred to aCSF containing (in mM): 119 NaCl, 26.2 $NaHCO_3$, 11 D-glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$ and 1.3 $MgCl_2$ (pH 7.4, osmolarity 305-310 mOsm). Slices were kept in aCSF at RT until recordings were performed.

Intrinsic properties of the neurons

Cell's intrinsic electrical and morphological properties determine the neuronal firing pattern and are regulated by synaptic interactions with the surrounding cells.

One of the intrinsic electrical properties of the cells is the resting membrane potential (RMP). The phospholipid bilayer, cell membrane, is an effective barrier for the movement of charged particles driven by the concentration gradients between intra- and extracellular compartments. In resting conditions, charges on

either side of the membrane are not in balance, so that the inner side of the cell is more negatively charged than the outside. This difference in charged particles generates an electrical potential over the membrane known as RMP, and is characteristic of different neuronal populations. When performing whole-cell patch-clamp, a way to measure this parameter is right after the opening of the cell, before any diffusion of solutions can occur, and measuring the voltage for a few seconds in current-clamp mode at 0 pA of holding current.

It is possible to measure the resistance of the membrane for the movement of charged particles, called input resistance (Ri). The membrane is not a closed barrier, but the flow of charged particles or ions across the membrane is facilitated by specialized proteins such as ion channels, transporters, and pumps. The current of ions is limited by the resistance of the membrane, and it is proportional to the driving force (in volts) of the ions following Ohm's law [voltage (V) = current (I) x resistance (R)]. For instance, if we wish to quantify the Ri of a cell, we can induce a voltage change of 5 mV (test pulse), and measure the current that travels across the membrane in response to the stimulation. Ri provides information about the excitability of the neuron, so if the Ri is high the neuron is more excitable than if it is low. When Ri is low means that most of the ion channels in the membrane are in an open configuration, increasing the leaking current and doing more difficult to excite this neuron since to get a certain voltage deflection, we will need a higher injected current ($Ri\downarrow \times I\uparrow = V$).

As mentioned, the intracellular compartment is more negatively charged than the extracellular compartment creating a certain membrane potential, that can be calculated using the Nernst equation and which exerts an electromagnetic field across the membrane from the inner to the outer part of the membrane. That field attracts charged particles that accumulate near the membrane, anions in the inner part, and cations in the outer part. This capacity of the membrane to store charge at a given potential is the cell membrane capacitance, and it is proportional to the surface area. Membrane capacitance is calculated from the charge integration of the transient current response to a change in the membrane potential (the test pulse). This is because membrane capacitance imparts a delay since any change in the membrane potential must first overcome a change in stored charge.

In conclusion, RMP, series resistance, Ri, and membrane capacitance were calculated from a series of 5 mV current pulses of 100 milliseconds duration (test pulse), applied through the patch pipette immediately after the rapturing of the membrane and opening of the cell. Then, recordings were analyzed subsequently with Igor Pro (Wavemetrics).

Next, to determine the ability of the cell to fire APs and to study the properties of those APs, 500 milliseconds current steps ranging from -40 pA to 200 pA in 10 pA steps were applied to the cells at a holding potential of -70 mV. Moreover, 1 second linear ramps currents were injected to determine the AP threshold. Other properties

of the APs were also determined, for instance AP amplitude was measured from the threshold to peak, AP duration was measured as the width at the threshold, and afterhyperpolarization amplitude was measured as the difference between the afterhyperpolarization peak and the AP threshold. Two important ion channels involved in intrinsic firing and AP generation are voltage-gated sodium and potassium channels. Therefore, observation of sodium and potassium currents is also indicative of the existence of those channels in the membrane and thereby the ability of the cell to generate APs. Tetrodotoxin (TTX)-sensitive voltage-dependent sodium currents and tetraethylammonium (TEA)-sensitive voltage-dependent potassium currents were evoked by a series of 100 milliseconds long voltage steps ranging from -90 mV to +40 mV in 10 mV steps.

Spontaneous postsynaptic currents

Spontaneous postsynaptic currents (sPSCs) and potentials (sPSPs), depending on the mode of recording, are reflections of spontaneous synaptic transmission from other cells in the network to the one that is being recorded indicating that the targeted cell is receiving synapses from the surrounding cells. Recordings in Paper I were done in human aCSF contained (in mM): 129 NaCl, 21 $NaHCO_3$, 10 glucose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, and 1.6 $CaCl_2$. For detection of sPSCs, cells were recorded in voltage-clamp mode at -70 mV, and for sPSPs in current-clamp mode with the holding current need it for reaching a membrane potential of -70 mV. Then, currents were analyzed offline with Igor Pro (Wavemetrics) and Python using a customized Python script (https://github.com/AMikroulis/xPSC-detection). For the detection algorithm used, a postsynaptic current or potential template was generated from the cells recorded, which were low-pass filtered at 400 Hz. Events with a correlation coefficient to the template lower than 0.6 were excluded from the analysis, and next the following excluding criteria were applied: (1) events with smaller amplitude than 5 pA were excluded (due to the amplifier's intrinsic noise floor at 4 pA p-p), (2) events with larger rise-time larger than 3 miliseconds were excluded; (3) events with longer decay-time than 20 miliseconds were excluded; and (4) events with decay-time shorter than 1.5 times the rise-time of the event were also excluded.

Importantly, using different intracellular and extracellular solutions is possible to better visualize and even discriminate between different types of synaptic transmission since they alter the equilibrium potential (V_{Eq}) for the different ions (see Table 4). To this goal, three different internal solutions have been used in the different publications: solution 2 and 10 in Papers I and II, and solution 13 in Paper IV. The composition of the different internal solutions is:

- Solution 2 containing in mM: 122.5 K-gluconate, 17.5 KCl, 10 KOH-HEPES, 0.2 KOH-EGTA, 2 Mg-ATP, 0.3 Na_3GTP, and 8 NaCl.

- Solution 10 containing in mM: 140 KCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, 0.4 Na_3GTP, and 10 NaCl.
- Solution 13 containing (in mM): 140 K-Gluconate, 4 NaCl, 10 KOH-HEPES, 0.2 KOH-EGTA, 2 Mg-ATP, and 0.3 Na_3GTP.

The reasoning is that we used solution 2 for hdINs *in vitro* and *in vivo* to be able to compare the intrinsic properties among them. Then, we used solution 10 for the host cells both *in vitro* and *in vivo* to facilitate the visualization of the inhibitory events mediated by $GABA_A$ receptor that is a chloride channel, since this solution inverts the polarity of chloride currents inward while increasing their amplitude compared to solution 2 (larger difference between holding potential, -70 mV, and chloride V_{Eq}, see Table 4). Finally, solution 13 was used in Paper IV because it allows the discrimination between excitatory and inhibitory currents without the need of adding any antagonist to the aCSF. Excitatory signals are mediated by the interaction between glutamate with AMPA and NMDA receptors, which are permeable for cations like sodium and calcium, so that sodium ions flow inwards depolarizing the membrane potential. On the other hand, inhibitory signals are mediated by the interaction of GABA with $GABA_A$ receptors, which are permeable for chloride, so that when using solution 13 chloride ions flow inwards (outward current) hyperpolarizing the cell, whether when using solution 2 and 10 chloride ions flow outwards (inward current) depolarizing the cell.

Tabel 4. Equilibrium potentials for the different ions

Overview of the different combinations of external solution (aCSF), rodent or human, and internal solution (2, 10, or 13) that have been used in the different Papers, so that equilibrium potential (V_{Eq}) for the different ions also differed. Human aCSF composition is detailed above, and rodent aCSF composition is detailed in the previous section *Acute hippocampal slice preparation*. Internal solutions are detailed above as well. V_{Eq} is indicated in mV.

Paper	aCSF	Internal sol.	$V_{Eq}\,Na^+$	$V_{Eq}\,K^+$	$V_{Eq}\,Cl^-$
I	Human	2	+77.29	-102.9	-43.25
I	Human	10	+70.39	-101.05	+3.36
II	Rodent	2	+75.48	-107.69	-41.86
II	Rodent	10	+69.54	-105.84	+4.73
IV	Rodent	13	+92.77	-107.69	-90.56

Drugs

The addition of certain molecules during specific recording protocols helps to gain a better understanding of the properties of the phenomena that we are observing. Most of them are added to the aCSF and pumped into the recording chamber. For instance, the addition of synaptic antagonists to the aCSF can aid to discriminate different types of synaptic transmission (used in Paper I and II).

To discriminate the excitatory or inhibitory nature of the spontaneous synaptic currents observed in the experiments, as well as the cellular phenotype originating

light responses when using optogenetics; in the thesis, two glutamate-receptor blockers were used: the NMDA receptor blocker AP5 (50 µM, Abcam), and the AMPA receptor blocker NBQX (5 µM, Abcam Biochemicals). Similarly, the $GABA_A$-receptor blocker PTX (100 µM, Tocris) was also used to the same goals.

During the assessment of intrinsic properties, two molecules were used to aid to confirm the channels involved in certain measured currents. TTX (1 µM, Abcam) was used to block voltage-gated sodium channels; and TEA (10 mM, Abcam) was added to block voltage-gated potassium channels.

Cell and Tissue Analysis

The main cellular and molecular analysis used in this thesis have been immunofluorescence and reverse-transcription quantitative PCR (RT-qPCR), and are therefore explained below.

Cells and Tissue fixation

All different cell cultures intended for electrophysiology and histology were plated on glass coverslips placed within the 24-well plate (7 DIV). For fixation, the medium was removed, and cells were rinsed with PBS with calcium and magnesium, to avoid any cell detachment and maintain cell adhesion to the coverslip. Next, cells were fixed in 4 % PFA for 20 minutes at RT, followed by three washes in KPBS. For GABA detection, cells were fixed with 0.25 % glutaraldehyde in 4 % PFA instead.

For histology, brain tissue was extracted after perfusion of the animals so that blood vessels were empty of blood and to ensure better processing of the tissue. Mice and rats were briefly anesthetized using isoflurane to disconnect from the EEG recording cables or extract the wireless transmitter system, and then sacrificed by i.p. injection of sodium pentobarbital. Following the loss of pain reflex, before the heart stopped beating, animals were transcardially perfused with RT 0.9 % saline, and then with ice-cold 4 % PFA, pH (7.4 ± 0.2). After perfusion, the brains were extracted from the skull and post-fixed for 24 hours in 4 % PFA at 4 °C. Then, brains were transferred to 20 % sucrose in 0.1 M PBS and kept at least for 24 hours at 4°C or until the brains have sunk to the bottom of the vials, indicating complete sucrose penetration. Brains were cut on a microtome into 30 µm thick sections, collected in a 1 in 8 series (every 8^{th} section belonged to the same series), and stored in a glycerol-based antifreeze solution at -20 °C until stained.

Some of the animals were not perfused, but used for functional analysis using electrophysiology (see *Acute hippocampal slice preparation* above). In those cases,

brain slices were transferred to 4 % PFA solution directly from the electrophysiology rig, kept for 24 hours at 4 °C, and transferred then to KPBS and kept at least for 24 hours at 4 °C.

Histology

Immunofluorescence was performed on either coverslips within 24-well plates in the case of cell cultures or free-floating brain sections in the case of tissue from animals.

For staining, samples were washed thoroughly three times with KPBS, and then pre-incubated for 1h in blocking solution containing 10% normal serum (of the species-specific to the secondary antibody) in KPBS containing 0.25 % Triton-X-100, for 1h at RT. Primary antibodies diluted in the blocking solution were incubated overnight at 4°C (Table 5). Following primary antibody incubation, samples were washed three times with KPBS and incubated again in blocking solution for 1 hour at RT. Then, samples were incubated with secondary antibodies for 2 hours at RT. This secondary antibody was either fluorophore-conjugated (AlexaFluor Plus 488/555/647, 1:1000, ThermoFisher) or biotinylated (in the case of STEM121) for further streptavidin amplification (1:200, Vector Laboratories). In the case of biotinylated secondaries, a consequent incubation with streptavidin-conjugated fluorophores was done (1:2000, Jackson Immunoresearch), for 2 hours at RT. Next, samples were washed three times with KPBS, and nuclei were counterstained with Hoechst 33342 (1:1000) diluted in the last rinsing with PBS before mounting with PVA-DABCO mounting media. Images were acquired either by confocal microscopy (Nikon Confocal A1RHD microscope) or by epifluorescence microscopy (Olympus BX61 and Leica DMi8).

For staining human organotypic culture slices or acute brain slices from electrophysiology (300 µm thickness), slices were washed thoroughly three times with KPBS, incubated for 1 hour at RT in permeabilization solution (0.02 % BSA + 1 % Triton X-100 in PBS) and 2 hours at RT in blocking solution (5 % normal serum + 1 % BSA + 0.2 % Triton X-100 in PBS). Primary antibodies were diluted in blocking solution and incubated for 48 hours at 4 °C. Next, slices were washed three times with KPBS, incubated again in blocking solution for 2 hours at RT, and then secondary antibodies were applied in blocking solution for 48 hours at 4°C. Next, samples were washed three times with KPBS, and nuclei were counterstained with Hoechst 33342 (1:1000) diluted in the last rinsing with PBS for 20 minutes before mounting with PVA-DABCO mounting media. Images were acquired by confocal microscopy (Nikon Confocal A1RHD microscope).

Tabel 5. Summary of primary antibodies and dilutions used for immunofluorescence
Antibodies used for immunofluorescence detection of different markers for cellular characterization. *, streptavidin amplification was used for the immunofluorescence.

Antibody	Host specie	Dilution	Company
OCT4	Rabbit	1:500	Abcam ab19857
Sox2	Rabbit	1:1000	Abcam ab97959
Nestin	Mouse	1:500	Abcam ab6142
β-III-tubulin	Rabbit	1:1000	Abcam ab18207
MAP2	Mouse	1:500	Sigma-Aldrich M2320
MAP2	Chicken	1:2000	Abcam ab5392
NeuN	Mouse	1:400	Millipore MAB377
mCherry	Chicken	1:2000	Abcam ab205402
GFP	Chicken	1:400	Abcam ab13970
Ki67	Rabbit	1:250	Novocastra NCL-Ki67p
STEM121 *	Mouse	1:400	Takara Bio Y40410
GABA	Rabbit	1:2000	Sigma Aldrich A2052
GAD65/67	Rabbit	1:500	Sigma Aldrich G5163
Calretinin (CR)	Rabbit	1:2000	Swant CR7697
Calbindin (CB)	Rabbit	1:1000	Swant CB-38a
Parvalbumin (PV)	Rabbit	1:5000	Swant
Parvalbumin (PV)	Mouse	1:1000	Swant PV235
SST	Rat	1:150	Millipore MAB354
NPY	Rabbit	1:5000	Sigma Aldrich N9528
CCK	Rabbit	1:1000	Sigma Aldrich C2581
TH	Mouse	1:200	Millipore MAB318
VGLUT1	Mouse	1:200	Synaptic Systems 135511
KGA	Rabbit	1:1000	Abcam ab93434
GFAP	Guinea Pig	1:500	Synaptic systems 173004
GFAP	Mouse	1:150	Sigma Aldrich G3793
Iba1	Rabbit	1:500	WAKO 01919741
CD68 (ED1)	Rat	1:200	Bio-Rad MCA1957
Galectin 3	Goat	1:500	R&D systems AF1197
CD8	Rabbit	1:200	Abcam 203035
IL1	Goat	1:400	Santa Cruz Biotech. SC-106
ZnT3	Rabbit	1:500	Synaptic systems 197003

RT-qPCR

In Paper I, gene expression analysis of the hdINs was performed at specific time points to better characterize the different subpopulations generated along with the differentiation. Three different biological replicates from different batches of differentiation were used for each time point. RNA was extracted from hdINs using RNeasy mini kit (Qiagen) and then reversed to cDNA using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher). For qPCR, cDNA was prepared with PowerUp SYBR Green Master Mix (Thermo Fisher). Candidate genes related to different stages of neurodevelopment (such as *POU5F1*, *SOX2,* and *MAP2*), and

neuronal subtypes (*GAD1* and *SLC17A7*) were selected for gene expression analysis. A complete list of the primers used is shown in Paper I, Table S3. All the samples were run in technical triplicates, and the average Ct-values were used for calculations. Data was represented using the ΔCt method, in which all gene expression values are calculated as the average change based on two different housekeeping genes (*ACTB* and *GAPDH*).

Statistical analysis

For analysis of the immunofluorescent images of cell cultures and brain tissue (Paper I, II, and IV), Fiji/ImageJ was used. Although in Paper III, quantification of the STEM121+ cells, as well as the different interneuron subtypes was done directly at the epifluorescence microscope at 20x (Leica DMi8).

Analysis of the electrophysiological data was performed offline with Igor Pro (Wavemetrics) and Python. Usually, individual values are plotted as dots and population data is presented as mean ± SEM, if it is normally distributed, and as median [interquartile range], if it is non-normally distributed.

Different statistical methods were used to assess significant differences between groups. Unpaired Student t-test was used when comparing the mean of two normally distributed populations. When data were non-normally distributed, the Mann-Whitney test was used for comparison of medians, and Wilcoxon rank test for comparison of the median when data were paired. For normally distributed data, one-way or repeated-measures ANOVA was used, followed by Tukey's posthoc test for multiple comparisons or Bonferroni correction when comparing three groups. Fisher's exact test was used for comparing proportions, or the binomial test was used when there was an expected distribution of observations. All statistical analyses were performed with Prism (GraphPad) with the significance level set to $p < 0.05$. In addition, the Kolmogorov-Smirnov test was used when analyzing differences in distribution of amplitudes, inter-event internal and other parameters of spontaneous postsynaptic currents. For an equal statistical representation of the different neurons analyzed, an equal number of events were analyzed for all neurons. When performing these comparisons of distributions, the level of significance was set to $p < 0.01$.

For more specific and accurate details about data and statistical analysis, I kindly refer the reader to the method section of the respective papers (see *Appendix*).

Some of the graphics used to illustrate this thesis have been created with BioRender.com

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ACKNOWLEDGMENTS

Along this PhD journey, I have shared adventures with many people that have contributed directly or indirectly and scientifically or personally to it. People without I could not have accomplished this. There are so many people I would like to thank for that and for contributing to make my time in Lund special and enjoyable. I will try to put in words those feelings.

First, I want to express my gratitude to **Merab Kokaia**, my main supervisor, for giving me the opportunity to join the lab and guiding me on the process despite the ups and downs. During these four years and a half under your supervision, I have learned a lot and I had the opportunity to grow as an independent researcher, gaining critical thinking and planning thanks to the freedom and trust you have given me.

I am very grateful to have counted on you, **My Andersson**, as co-supervisor on this journey. I am extremely glad to have shared the Cntnap2 adventure with you, so we have experienced many meetings, students, ideas, and theories together. I love your enthusiasm and curiosity about science in general. Thanks for caring about me and being always available to listen and give me advice.

Last but not least from the three wise kings (MMM), thanks to **Marco Ledri** for the molecular perspective in all the meetings and also for taking care of me on my first trip to the US for the SfN.

Eliška Waloschková, thank you for sharing with me this PhD journey full of experiences and research schools. Traveling with you has been a lot of fun, even we reached the optimized morning schedules. Sharing problems and laughs with you have made things easier.

During my last two years, I had the pleasure to share two projects with you, **Fredrik Berglind**. I appreciate your perfectionism and structure when working, as well as your feedback on the manuscripts. You make things look so simple. Working with you has been highly inspiring.

Jenny Wickham, the first time I sat in the rig, it was while you were patching some of your last PhD experiments. It seemed like driving a spaceship, extremely complicated. Like then I learned from you about electrophysiology, later you have given me many times good advice. I am glad you are back. Thank you for helping me to get a view from a more experienced perspective.

I would like to thank **Apostolos Mikroulis**, for sharing his expertise in electrophysiology and making my life much easier by using his Python script to analyze spontaneous synaptic currents.

Thank you, **Esbjörn Melin** and **Jan Kudláček**. I have learned from working with you both at different scenarios and have truly enjoyed all the interesting chats about Swedish history, science, physics, and just life in general.

Susanne Geres, your help has been invaluable. Thank you for always being willing to help with a smile, for all the conversations and time during the behavior test in the early mornings, for helping me with virus injections when I felt and I had my hand full of wounds, for bringing me a banana to eat on the long surgery days,… – I would sum up this countless list by thanking you for always being there.

Besides you have joined at the end of my PhD, you have also been remarkably helpful. **Ling Cao**, I really appreciate your willingness to learn and help, always combining numerous experiments to be able to aid everybody. Thank you also for sharing your experience with life during breakfast, lunch, and fika.

Despite not being at the lab anymore, I would like to express my gratitude to former members for their excellent technical and administrative help: **Nora Perna**, **Susanne Jonsson**, and **Ann-Katrin Häger**.

In this line, I was extremely lucky to share my first years in the lab with **Valentina Di Maria**. You were like an older sister to me, taking care of me, helping with the experiments, and even saving me when I locked up myself outside of the office on weekends. Thank you. During your time here, you had two amazing students that I had the pleasure to meet personally and that I am happy to still be in touch with, **Marine** and **Bianca**. I had so much fun sharing the "gossip office" with all of you!

I am thankful to my first students, **Mohanad** and **Liza**. From you I have learned how to teach and guide students, and that each one is unique and different from the rest. I also had the pleasure to supervise **Diana Hatamian** in her master thesis at the lab. Thanks for being such a kind person, and a fountain of energy and positive attitude. After all what we went through together during that year, I can consider you as a friend. The best coincidence was to share this time also with **Kerstin Laurin**, at that time bachelor student of Marco. I loved our short breaks on the bench in front of A10 the three of us eating the white chocolate and strawberry ice cream! It was also a lot of fun our little afterworks and celebrations at the office, with the signed cork of the bottles as a memory. All of that happened during the year of my halftime seminar, and now the year of my PhD defence, you are back in the lab for your master thesis Kerstin. Thank you for being in two very important moments of my PhD, always by my side supporting, cheering me up, and celebrating. I wish you well in the coming adventures, wherever you go they will be infinitely lucky to count on you.

One of the good sides of a close relationship between three different PIs is that you get to know and spend time with many students. Some have been mentioned, but I would like to also thank **Antonino**, **Daniela**, **Adrien**, **Anja**, **Arianna** and **Karuna** for the time we have shared in the lab, but also the laughs and joy outside those hours.

To the best neighbor group we could have, with even a sofa at the office, thank you **Matilda Ahl**, **Una Avdic**, **Marie Taylor** and **Filip Bäckström** for fikas, epilepsy retreats, and celebrations. Thanks, **Christine Ekdahl**, for the critical and valuable feedback I could get from you at the epilepsy retreats and Neuroscience Day.

I am highly indebted to **Johan Bengzon** for allowing me to be present in the surgical resections for refractory epilepsy patients and for being so kind to explain practicalities during those. The possibility to observe those surgeries is always a learning experience that boosts the motivation for pursuing the projects.

Thanks to LBIC, especially **Sebastian** and **Lina**, for the help in confocal and electron microscopy matters.

As mentioned before in the *Context of the thesis* section, during my PhD I was part of a European consortium named **Training4CRM**. As an early-stage career member, I had the chance to learn about a wide variety of topics during the research schools, present my data in oral presentations, and get to do an internship where I got crucial knowledge for my thesis projects. I am truly grateful to meet all the people involved in the consortium, both senior and early-stage researchers. I have learned a lot from you and had fun during our research schools. Thank you: **Shashank**, (partner in crime for the optical fibers and sharing representatives' duties), **Cristina** (always so kind and easy to talk with), **Robin**, **Theresa**, **Camille**, **Alireza**, **Christin**, **André**, **Sandrina**, **Afia**, **Hakan**, **Salman**, and **Mozhdeh**. As well as **Jenny** and **Mette** for leading and coordinating everything, and all the senior members. I also want to thank the people in **Josep M Canals'** group who kindly welcomed me to the lab and make my time during the internship enjoyable and full of laughs, and to Josep himself for your closeness and caring about people.

A special thanks to all the people at **A11** (now and on the previous four years) for the nice company during lunch breaks and fika. I will miss the warm and open atmosphere and the nice interaction between people in different groups. - **Janitha**, **Marcella**, **Tiago C**, **Shelby**, **Jessica**, **Fredrik N**, **Janko**, **Edo S**, **Raquel**, **Daniela G**, **Elin**, **Luis**, **Per**, **Sara N**, **Andy**, **Dee**, **Yu**, **Ale**, **Marie PV**, **Sol**, **Ulla**, **Jenny**, **Paulina**, **Cilla**, **Johan** and **Malin**. - *I apologize for the ones that might not be mentioned in this text, I can assure you are not forgotten.* I would like to emphasize **Marcus Locko** for your support and nice conversations. Despite you not being the most expressive person, I have felt that you care about me, and so do I for you.

One of the beauties of BMC is the interconnection between floors and people, so thanks to everyone at **A10**, **B10** and **B11** (and the ones spread on other floors) for

the help in scientific matters but also the nice moments together. First, thanks to **Zaza** for giving me the opportunity back in 2015 to come for the first time to Lund and discover the amazing world of stem cells and regenerative medicine at your lab. It was back then when I decided I wanted to do a PhD, and where I made friends for life. Besides being considered the "cookie monster" together with Sara P, your thoroughness on the projects set the foundations of my way of doing science. When I came back for my PhD some of the lab members were still here, and have been of great help uncountable times, especially **Emmanuela Monni**. Many other great people on this and different floors have contributed remarkably to my time in Lund – **Giedre**, **Linda**, **Natalia**, **Jonas**, **Francesco**, **Sabine**, **Edo**, **Andreas E**, **Sertan**, **Laura Andreoli**, **Andy H**, **Marcus D**, **Niko**, **Hani** – and my halftime seminar opponents **Henrik Alhenius** and **Daniella Ottosson** which gave me plenty of good tips and advice. A special mention to **Sophie**, **Shayini**, **Raquel**, **Ella**, and **Andrea** who I had nice chats about both science and non-science stuff, and shared dinners, beers, parties, and much more!

Andreas Bruzelius, I am fortunate to have met you because you are one of the persons that have conditioned my PhD journey to a great extent. This is not only because we both are electrophysiologists, which sometimes helps. You are one of the most loyal and fair persons that I have ever met, and most importantly, *you have been one of the stars that had shine the most during the darkness*.

As much as science and a great work environment, life needs color and happiness. It has been plenty of fun gathering and going out with **Be Creative** and later **Be Festive**. From little gatherings in the sauna at B15 and attending ESN board game nights to a big bbq in the park and night outs in apartments and Malmö. Thank you to the originals **Fabio D**, **Marine**, **Eliška**, **Mo, Jessica** and **Martino**, and to all the ones that joined afterward – **Fabio F**, **Marija**, **Roberta**, **Bianca**, **Isak**, **Tiago B**, **Tiago C**, **Julie**, **Chang**, **Adrien**, **Carletto**, **Kritika**, **Albert**, **Nadja**, **Nic**, **Scott**, **Mert** and **Carolina**.

Claudio, my first corridor mate here in Lund and my friend. Thank you for all the dinners together cooking amazing Italian food, and having conversations about any and everything until very late. **David**, gràcies per la multitud de riures i confidències. Pels sopars quan vivíem junts i el tornar junts de festa.

During the last two years, another source of joy has been climbing, and for that, I count on wonderful people. Thanks **Virginia**, **Alejandro** and **Barbara** for the time in the klätter hanging like monkeys from the problems, but also the hikes and dinners.

When living abroad, you find people that can be considered family. ¡Gracias **Daniel Tornero** por todo! Gracias por cuidar de mí en mi primera experiencia en el extranjero e introducirme a tu círculo de amigos, por enseñarme la magia de las células madre y los enigmas del cerebro, por conseguir que participase en una publicación sólo siendo estudiante de grado (la cual me abrió muchas puertas), por

siempre estar ahí y darme consejos, gracias por ser mi "padre científico" y un gran amigo. Siempre al lado de un gran hombre hay una gran mujer (y viceversa), gracias **Talía** por haber sido participe de muchas de las cosas aquí mencionadas. Gracias por ser tan sensible y cariñosa siempre. Me alegra que estéis en Barcelona y os pueda seguir viendo a menudo.

Gracias a mi hermanita, **Sara P**, por lo que sigue a todo heheh Juntamente con Dani hicisteis mi primera experiencia en el extranjero y contacto real con la ciencia increíble. Gracias por nuestros café a media tarde con +2 galletas (con razón nos ganamos el apodo de "cookie monsters"), viaje a Helsingør y a Stockholm (durmiendo en el aeropuerto antes de volver). Por invitarme a tu defensa de tesis y celebración. Siempre recordaré al volver de la fiesta y ver que te habían contratado "por Facebook" y empezar a saltar de alegría porque volvíamos a Lund juntas. Gracias por haber estado siempre a mi lado durante estos años de tesis, así como cenas, bbq y "fin de año" - eres una gran amiga.

Con vosotros también conocí a los miembros de **Luñd**, con los que he disfrutado de fantásticas cenas, cafés y cervezas - **Antonio**, **Tania**, **Sarito**, **Alba**, **Irene** (y también **Martin**), **Agi**, **Rodrigo** y **Maca**. Gracias en especial también a ti **Isa**, por este tiempo, charlas, risas y consejos. Me gustaría resaltar a **Laura TG**, la "wonder woman". Siempre me sorprendes para bien. Eres luchadora y leal, y me encanta tu locura.

Isaac quina casualitat que dos martorellencs es trobessin a Suècia. Tot i voler dissimular i amagar els sentiments, ets un tros de pa qui cuida als amics. Estic molt contenta d'haver-te conegut i de què m'hagis fet partícip d'un moment tan especial de la teva vida com el desembre de l'any passat. M'alegro de veure't feliç amb la **Leal** i la **Laia**.

Sometimes, stars arrange as constellations. Thank you to **Comida de mi vida**, my wonderful friends and network support here in Lund. This journey would have not been the same without you. I will start with my dear girls because they are always there, for bad news, bad days, random and casual coffees or dinners, laughs and craziness, gossip nights, pole-dance classes, spa trips, nights out, and even adventures in Iceland and Malta. **Laura**, per mil xerrades, consells, i paranoies juntes. Gràcies per ser algú amb qui sempre puc comptar. Tot i això, m'alegro d'haver superat la nostra obsessió de visitar la tenda de segona mà cada dissabte al matí quan vivíem juntes. **Marta**, el pequeño terremoto! Gracias por esa energía y predisposición a intentar todo. Me encantan las excursiones e ir a buscar setas contigo. **María**, por tu inigualable dulzura y detallismo. Reflejas a la perfección el dicho, en los pequeños detalles está la diferencia. **Myriam**, divertida, activa y atenta. Tus dotes teatrales son un diamante en bruto, pero uno que siempre nos hace reír. Gracias por ese paseo la tarde antes del halftime, y por muchos otros momentos de pánico. **Kat**, for your effectiveness in fixing issues and getting presents, and deep thoughts during conversations. **Martina**, for all the amazing meals that you have

prepared for us, and because I love your mindfulness attitude. **Lavanya**, for arranging an amazing trip to your country, and for your selfless caring. **Matilde** and **Martino**, for the boosts of motivation when I find you at the corridor, and your love for food and nature. **Alex**, for always passing by the office or lab when I needed it the most, your amazing dinners and cakes, and overall generosity and kindness. **Mo**, because I feel we have grown and changed so much together since the first time we met. **Oscar**, a beautiful voice from a kind heart. Thank you for getting us into the Swedish culture, Melodifestivalen, and also Eurovision. **Sid**, for feeding us with herring and snaps on Midsummer and other celebrations. **Tabor**, for the joy and fun stories that you always bring

To my other star constellations back home. Gràcies a les **CHAIE** i als **Montserratins** per la vostre amistat des de sempre i durant els diferents camins que hem anat seguint. – **Cristina**, **Helena**, **Irene**, **Esther**, **David**, **Albert**, **Angel**, **Josep**, **Martí**, i **Carlos** - I gràcies a les meves **Biomeds** per seguir compartint experiències un cop acabat el grau. – **Aina**, **Anna**, **Clàudia**, **Marta** i **Núria** -

Jordi, no hi ha res que pugui escriure que no sàpigues ja, tot i això ho intentaré. *De totes les estrelles tu ets el meu sol.* Primer de tot, gràcies per creure en mi i encoratjar-me a complir els meus somnis, tot i les dificultats. Gràcies per sempre estar allà dia rere dia, quan hi havia distància i ara vivint junts. Per fer possible el impossible. No tinc paraules per agrair que decidissis unir-te a la meva aventura a Suècia, només puc dir-te que sóc immensament feliç al teu costat. Gràcies per tota l'ajuda i els tocs de realitat durant aquesta recta final, no ho hagués aconseguit sense tu. Gràcies per fer-me millor persona i donar llum a la meva vida. T'estimo.

Para finalizar, quiero agradecer a mis padres, **Elena** y **Cecilio**, por haberme ayudado a ser la persona que soy y a perseguir mis sueños. El cariño, sencillez, y espíritu luchador que he vivido en casa des de pequeña han ayudado a modelar mi persona. Sé que siempre he podido y puedo contar con vuestro apoyo y amor para todo. Gracias por alentarme cuando decidí irme a vivir a otro país para hacer lo que me gusta, y gracias por haber hecho que esa distancia física nunca fuese real en los sentimientos. Nunca hubiese conseguido llegar hasta aquí sin vosotros y vuestra ayuda. Os quiero. Gracias a ti también **Miquel**, por haber crecido juntos compartiendo tantos momentos, por saber que siempre nos tenemos el uno al otro, y por hacerme participe de una parte tan importante de tu vida – **Ani** y **Bruno** -. Por último, agradecer a mis abuelos **Miguela** y **Juan José**, todos los momentos de amor y cariño que me habéis dado. Soys muy importantes para mí.

— Gracias. Os quiero mucho.

APPENDIX

About the author

ANA GONZALEZ RAMOS graduated in Biomedical Sciences from the Autonomous University of Barcelona and then performed a seven-month Erasmus Program internship at the Stem Cells and Restorative Neurology group at the Lund Stem Cell Center. There, she developed her curiosity and passion for regenerative medicine and neuroscience, which brought her to complete her Master's in Regenerative Medicine: Clinical and Industrial delivery at the University of Edinburgh. In the fall of 2017, she started her doctoral studies in the Experimental Epilepsy Group, under the supervision of Merab Kokaia, as a member of the Marie Skłodowska-Curie Innovative Training Network – Project Training4CRM. During her Ph.D., Ana has investigated novel therapeutic strategies targeting refractory epilepsy, by developing and testing cell and gene therapies with the goal to increase neuronal inhibition. Moreover, Ana combines her Ph.D. studies with her participation in the Doctoral student council (MDR) at the faculty of medicine, and also organizing outreach activities such as the Pint of Science.

Printed by Media-Tryck, Lund 2022 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences, Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2022:44
ISBN 978-91-8021-205-2
ISSN 1652-8220