DOCTORAL THESIS DISSERTATION

Nrg1 signaling in the development of cortical circuits: molecular basis of schizophrenia

Universitat Politècnica de València

PhD in Biotechnology

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Valencia, June 2024

VALENCIA BIOMEDICAL RESEARCH FOUNDATION
CENTRO DE INVESTIGACIÓN PRÍNCIPE FELIPE

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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CERTIFY that:

The work titled "**Nrg1 signaling in the development of cortical circuits: molecular basis of schizophrenia**" has been developed by Ángela Rodríguez Prieto in Centro de Investigación Príncipe Felipe (CIPF), under the supervision of Dr Pietro Fazzari and the academic tutorship of Dr Máximo Ibo Galindo Orozco, as a Thesis Project to achieve the PhD degree in Biotechnology at the Universitat Politècnica de València.

Valencia, June 2024

Dr. Pietro Fazzari

Dr. Máximo Ibo Galindo Orozco

“The feeling of awed wonder that science can give us is one of the highest experiences of which the human psyche is capable. It is a deep aesthetic passion to rank with the finest that music and poetry can deliver. It is truly one of the things that make life worth living and it does so, if anything, more effectively if it convinces us that the time we have for living is quite finite.”

Richard Dawkins

Unweaving the Rainbow

AGRADECIMIENTOS

En primer lugar, quiero expresar mi más sincero agradecimiento a Pietro Fazzari. Hace seis años en Madrid confiaste en mí para hacer el doctorado contigo y desde entonces has sabido dedicarme todo tu tiempo, sabiduría y paciencia.

Este proyecto hubiera sido inviable sin la ayuda de mis compañeras de laboratorio. Gracias a Carmen, por ser el mejor referente como investigadora, persona y amiga. A Yaiza, por haber estado para mí en los momentos más duros, tanto experimentales (ese *Western blot* con forma de arbolito lo acredita), como personales. A Ana, mi compañera y amiga desde el máster, gracias por tu asertividad y presencia constante. Os admiro profundamente a todas y siempre os voy a estar agradecida por haber sido un refugio para mí.

También agradecer a todos los servicios y personas que hacen del CIPF lo que es, especialmente a Ally, Estefanía, Alberto, Mara, Majo, Xavi, Gemma y al laboratorio del I-44.

Gracias también a todas esas personas con las que he tenido el placer de compartir camino estos últimos años. Gracias al grupo de Jóvenes CIPF y a sus quedadas semanales. Gracias a Anita, Sonia, Mili e Isa, por escucharme, aconsejarme y acompañarnos mutuamente. Sois todas especiales, os llevaré siempre conmigo.

A mis amigos de Sevilla, por hacer que la vuelta a casa siempre sea mejor. A Marta, por enseñarme tanto y quererme incondicionalmente. Qué suerte la mía por seguir creciendo contigo. A Valentina, por tu presencia y cariño este último año. Gracias por sostenerme en una época tan compleja.

Por último, a mis abuelos y a mis padres. Habéis hecho posible todo esto, no solo por dedicarme cada recurso con el que contabais, sino por inculcarme desde pequeña que la vida es un juego que se disfruta explorando y descubriendo. Aunque no entendáis mucho de esta tesis, que sepáis que mi amor por la ciencia existe gracias a vosotros.

ABSTRACT

Schizophrenia (SZ) is a neurodevelopmental disorder that affects cognitive processes and social behavior, impacts approximately 1% of the population but presents a major socio-economic impact. Unlike other neuropathologies, the brains of SZ patients do not display obvious histological hallmarks and their molecular mechanisms remain unknown, making it very difficult to prevent and treat effectively. The most consistent endophenotypes in SZ include reduced neuropil, impaired functional connectivity between cortical areas and specific changes in synaptic connections. Therefore, SZ is a pathology based on abnormal cortical neuronal connectivity. The corpus callosum (CC), connecting the brain's hemispheres, is the largest bundle of cortico-cortical nerve fibers and its development is a complex process crucial for proper cortical circuitry formation. Converging evidence supports the hypothesis that the CC is hypoconnected in SZ patients.

While the developmental etiology of SZ remains largely unresolved, it is well established that SZ has a strong genetic component. A key gene implicated in SZ risk is *neuregulin 1* (*NRG1*), which controls several aspects of neuronal development. Prior research has primarily focused on Nrg1 signaling in inhibitory interneurons, neglecting its role in excitatory neurons. To understand the function of Nrg1 signaling in the development of cortical circuits, we studied the Nrg1's role in the development of callosal axons and specifically, the cell autonomous function in the excitatory neurons. To this aim, we sought to 1) evaluate the Nrg1's involvement in the development of callosal projecting neurons; 2) investigate the Nrg1's cell autonomous effect on neurites outgrowth; 3) study the molecular mechanisms underlying Nrg1's role in neuron development; 4) explore the Nrg1's potential in reprogramming astrocytes to neurons, as a novel therapeutic approach following brain injury.

First, we developed an *in vivo* loss-of-function model to determine the role of Nrg1 in the development of callosal projections. We employed newborn Nrg1 knockout mice and performed neuronal tracing of the callosal projections, as well as we traced those projections in wild type mice by *in utero* electroporation. We found that the deletion of Nrg1 in a conditional mouse model impaired the development of callosal axons *in vivo*.

On a mechanistic level, we found that the intracellular signaling of Nrg1 was sufficient to promote axonal development in cortical neurons *in vivo.*

Second, to specifically determine the role of Nrg1 in axonal development of excitatory neurons, we employed a suitable *in vitro* model with a more reductionist approach. We performed primary cultures of cortical neurons. Using transfection by electroporation, we achieved sparse labeling and obtained internal controls. In this model, we carried out gain- and loss-of-function approaches to investigate specifically the Nrg1's cell autonomous effect on dendrites and axonal development. Our single-cell experiments in primary cultures showed that Nrg1 is cell-autonomously required and sufficient to promote axonal outgrowth *in vitro*.

Third, we studied the molecular mechanisms underlying Nrg1's role in neuron development. We found by Western blot and immunofluorescence that the GAP43 protein expression is impaired in Nrg1 knockout neurons. Additionally, we observed that decreased axonal development in Nrg1 knockout neurons was rescued by overexpressing GAP43 protein. These results suggest that signaling through GAP43 may be one of the mechanisms involved in the role of Nrg1 in axonal growth.

Finally, we tested the potential role of Nrg1 in astrocyte-to-neuron reprogramming, as a novel therapeutic approach following brain injury. We failed to see consistent results indicating increased reprogramming or development of the new neurons.

In conclusion, our study indicates a crucial role for Nrg1 intracellular signaling in the development of long-range cortico-cortical connections between brain hemispheres. It indicates that Nrg1 dysfunction in excitatory neurons may contribute to SZ-associated hypoconnectivity and neurodevelopmental alterations, providing new insights into the role of Nrg1 in the etiology of SZ.

RESUMEN

La esquizofrenia (SC) es un trastorno del neurodesarrollo que afecta los procesos cognitivos y el comportamiento social. Si bien afecta aproximadamente al 1% de la población, tiene un gran impacto socioeconómico. A diferencia de otras neuropatologías, los cerebros de los pacientes con SC no muestran características histológicas evidentes y los mecanismos moleculares subyacentes a la enfermedad siguen siendo desconocidos, lo que dificulta mucho su prevención y tratamiento eficaz. Los endofenotipos más consistentes en la SC incluyen la reducción del neuropilo, la conectividad funcional deteriorada entre las áreas corticales y cambios específicos en las conexiones sinápticas. En consecuencia, la SC es una patología basada en una conectividad neuronal cortical anormal, lo que afecta a la comunicación entre las diferentes áreas cerebrales. El cuerpo calloso (CC) es el haz más grande de fibras nerviosas cortico-corticales, conectando ambos hemisferios cerebrales, y su desarrollo es un proceso complejo crucial para la formación adecuada de los circuitos corticales. Numerosa evidencia convergente apoya la hipótesis de que el CC se encuentra hipoconectado en pacientes con SC.

Si bien la etiología del desarrollo de la SC permanece en gran parte sin resolver, está bien establecido que la SC tiene un fuerte componente genético. Un gen clave implicado en el riesgo de SC es *neuregulina 1* (*NRG1*), controlando varios aspectos del desarrollo neuronal. Estudios previos se han centrado principalmente en la señalización de Nrg1 en las interneuronas inhibitorias, descuidando su papel en las neuronas excitatorias. Para comprender el papel de Nrg1 en los circuitos corticales, en este trabajo estudiamos el papel de Nrg1 en el desarrollo de los axones del cuerpo calloso y específicamente, su función celular autónoma en las neuronas excitatorias. Para este objetivo quisimos, 1) evaluar la participación de la Nrg1 en el desarrollo de las neuronas de proyección callosa; 2) investigar el efecto autónomo de Nrg1 en el desarrollo de las neuritas; 3) estudiar los mecanismos moleculares subyacentes al papel de Nrg1 en el desarrollo neuronal; 4) explorar el potencial de Nrg1 en la reprogramación de astrocitos a neuronas, como un nuevo posible enfoque terapéutico después de una lesión cerebral.

Para ello, primero desarrollamos un modelo *in vivo* de pérdida de función para determinar el papel de Nrg1 en el desarrollo de las proyecciones callosas. Empleamos ratones *knockout* recién nacidos para Nrg1 y realizamos un rastreo neuronal de las

proyecciones callosas, así como también rastreamos dichas proyecciones en ratones *wild-type* mediante electroporación *in utero*. Descubrimos que la eliminación de Nrg1 en el modelo de ratón condicional impedía el desarrollo de axones callosos *in vivo*. A nivel mecanístico, encontramos que la señalización intracelular de Nrg1 es tanto necesaria como suficiente para promover el desarrollo axonal en las neuronas corticales *in vivo*.

En segundo lugar, para determinar específicamente el papel de Nrg1 en el desarrollo axonal de las neuronas excitatorias, empleamos un modelo *in vitro* con un enfoque más reduccionista. Realizamos cultivos primarios de neuronas corticales, obteniendo un marcado neuronal disperso con controles internos, gracias a la transfección celular por electroporación. En este modelo, llevamos a cabo experimentos de ganancia y pérdida de función para investigar específicamente el efecto autónomo celular de Nrg1 sobre el desarrollo de dendritas y axones. Nuestros experimentos con cultivos primarios de neuronas confirmaron que la señalización intracelular de Nrg1 es necesaria y suficiente para promover el crecimiento axonal *in vitro*.

En tercer lugar, estudiamos los mecanismos moleculares subyacentes al papel de Nrg1 en el desarrollo neuronal. Descubrimos mediante *Western blot* e inmunofluorescencia que la expresión de la proteína GAP43 está altamente disminuida en neuronas *knockout* para Nrg1. Además, observamos que disminución del desarrollo axonal en neuronas *knockout* para Nrg1 es parcialmente rescatado al sobreexpresar la proteína GAP43. Estos resultados sugieren que la señalización a través de GAP43 podría ser uno de los mecanismos involucrados en el papel de Nrg1 en el crecimiento axonal.

Por último, probamos el papel potencial de Nrg1 en la reprogramación de astrocitos a neuronas, como un nuevo enfoque terapéutico tras una lesión cerebral. Dada una gran variabilidad experimental, no pudimos observar resultados consistentes que indicaran un aumento en la reprogramación o el desarrollo de nuevas neuronas.

En conjunto, nuestro estudio indica un papel crucial para la señalización intracelular de Nrg1 en el desarrollo de las conexiones cortico-corticales que conectan ambos hemisferios cerebrales. Nuestros resultados sugieren que la disfunción de Nrg1 en las neuronas excitatorias puede contribuir a la hipoconectividad asociada a la SC y las alteraciones del desarrollo neurológico, proporcionando nuevos conocimientos sobre el papel de Nrg1 en la etiología de la SC.

RESUM

L'esquizofrènia (SC) és un trastorn del neurodesenvolupament que afecta els processos cognitius i el comportament social. Afecta al voltant de l'1% de la població i té un gran impacte socioeconòmic. A diferència d'altres neuropatologies, els cervells dels pacients amb SC no mostren característiques histològiques evidents i els mecanismes moleculars subjacents a la malaltia continuen sent desconeguts, la qual cosa dificulta molt la seua prevenció i tractament eficaç. Els endofenotipus més consistents en la SC inclouen la reducció del neuropil, la connectivitat funcional deteriorada entre les àrees corticals i els canvis específics en les connexions sinàptiques. En conseqüència, la SC és una patologia basada en una connectivitat neuronal cortical anormal. El cos callós (CC) és el feix més gran de fibres nervioses cortico-corticals, connecta tots dos hemisferis cerebrals, i el seu desenvolupament és un procés complex crucial per a la formació adequada dels circuits corticals. Nombrosa evidència convergent dona suport a la hipòtesi que el CC es troba hipoconnectat en pacients amb SC.

Si bé l'etiologia del desenvolupament de la SC roman en gran part sense resoldre, està ben establit que la SC té un fort component genètic. Un gen clau implicat en el risc de SC és *neuregulina 1* (*NRG1*) que controla diversos aspectes del desenvolupament neuronal. Estudis previs s'han centrat principalment en la senyalització de Nrg1 en les interneurones inhibitòries, descurant el seu paper en les neurones excitatòries. Per a comprendre el paper de Nrg1 en els circuits corticals, en este treball estudiem el paper de Nrg1 en el desenvolupament dels axons del cos callós i específicament, la seua funció cel·lular autònoma en les neurones excitatòries. Per a este objectiu, 1) avaluem la participació de Nrg1 en el desenvolupament de les neurones de projecció callosa; 2) investiguem l'efecte autònom de Nrg1 en el desenvolupament de les neurites; 3) estudiem els mecanismes moleculars subjacents al paper de Nrg1 en el desenvolupament neuronal; 4) explorem el potencial de Nrg1 en la reprogramació d'astròcits a neurones, com un nou possible enfocament terapèutic després d'una lesió cerebral.

Per això, primer desenvolupem un model *in vivo* de pèrdua de funció per a determinar el paper de Nrg1 en el desenvolupament de les projeccions calloses. Emprem ratolins *knockout* nounats per a Nrg1 i realitzem un rastreig neuronal de les projeccions calloses, així com també rastregem estes projeccions en ratolins *wild-type* mitjançant la

tècnica d'electroporació *in utero*. Descobrim que l'eliminació de Nrg1 en el model de ratolí condicional impedia el desenvolupament d'axons callosos *in vivo*. A nivell mecanístic, trobem que la senyalització intracel·lular de Nrg1 era suficient per a promoure el desenvolupament axonal en les neurones corticals *in vivo*.

En segon lloc, per a determinar específicament el paper de Nrg1 en el desenvolupament axonal de les neurones excitatòries, emprem un model *in vitro* amb un enfocament més reduccionista. Realitzem cultius primaris de neurones corticals, de manera que obtenim un marcat neuronal dispers amb controls interns, gràcies a la transfeció cellular per electroporació. En este model, duem a terme experiments de guany i pèrdua de funció per a investigar específicament l'efecte autònom cel·lular de Nrg1 sobre el desenvolupament de dendrites i axons. Els nostres experiments amb cultius primaris de neurones van mostrar que la senyalització intracel·lular de Nrg1 és necessària i suficient per a promoure el creixement axonal *in vitro*.

En tercer lloc, estudiem els mecanismes moleculars subjacents al paper de Nrg1 en el desenvolupament neuronal. Descobrim mitjançant *Western blot* i immunofluorescència que l'expressió de la proteïna GAP43 està altament disminuïda en neurones *knockout* per a Nrg1. A més, observem que la disminució del desenvolupament axonal en neurones *knockout* per a Nrg1 és parcialment rescatat al sobreexpresar la proteïna GAP43. Estos resultats suggerixen que la senyalització a través de GAP43 podria ser un dels mecanismes involucrats en el paper de Nrg1 en el creixement axonal.

Finalment, provem el paper potencial de Nrg1 en la reprogramació d'astròcits a neurones, com un nou enfocament terapèutic després d'una lesió cerebral. Donada una alta variabilitat experimental, no vam poder observar resultats consistents que indicaren un augment en la reprogramació o el desenvolupament de noves neurones.

En conjunt, el nostre estudi indica un paper crucial per a la senyalització intracellular de Nrg1 en el desenvolupament de les connexions cortico-corticals que connecten tots dos hemisferis cerebrals. Els nostres resultats assenyalen que la disfunció de Nrg1 en les neurones excitatòries pot contribuir a la hipoconnectivitat associada a la SC i a les alteracions del desenvolupament neurològic, proporcionant nous coneixements sobre el paper de Nrg1 en l'etiologia de la SC.

PUBLICATIONS

Part of the results obtained in this PhD dissertation have been published in the following scientific articles:

1. **Rodríguez-Prieto, Á.,** González-Manteiga, A., Domínguez-Canterla, Y., Navarro-González, C., & Fazzari, P. (2021). A Scalable Method to Study Neuronal Survival in Primary Neuronal Culture with Single-cell and Real-Time Resolution. Journal of visualized experiments: JoVE, (173), 10.3791/62759. https://doi.org/10.3791/62759

2. Navarro-Gonzalez, C., Carceller, H., Benito Vicente, M., Serra, I., Navarrete, M., Domínguez-Canterla, Y., **Rodríguez-Prieto, Á.,** González-Manteiga, A., & Fazzari, P. (2021). Nrg1 haploinsufficiency alters inhibitory cortical circuits. Neurobiology of disease, 157, 105442. https://doi.org/10.1016/j.nbd.2021.105442

3. Martínez-Rubio, D., **Rodríguez-Prieto, Á.,** Sancho, P., Navarro-González, C., Gorría-Redondo, N., Miquel-Leal, J., Marco-Marín, C., Jenkins, A., Soriano-Navarro, M., Hernández, A., Pérez-Dueñas, B., Fazzari, P., Aguilera-Albesa, S., & Espinós, C. (2022). Protein misfolding and clearance in the pathogenesis of a new infantile onset ataxia caused by mutations in PRDX3. Human molecular genetics, 31(22), 3897–3913. https://doi.org/10.1093/hmg/ddac146

4. **Rodríguez-Prieto, Á.,** Mateos-White, I., Aníbal-Martínez, M., Navarro-González, C., Gil-Sanz, C., Domínguez-Canterla, Y., González-Manteiga, A., Del Buey Furió, V., López-Bendito, G., Fazzari, P. (2024). Nrg1 intracellular signaling regulates the development of interhemispheric callosal axons in mice. Life Science Alliance. https://doi.org/10.26508/lsa.202302250

ABBREVIATIONS AND ACRONYMS

AAVs: adeno-associated viral vectors

AD: Alzheimer's disease

AKT: protein kinase B

APP: amyloid precursor protein

Ascl1: achaete-scute family BHLH transcription factor 1

BACE: beta-site APP cleaving enzyme 1

BSA: bovine serum albumin

Ca^{2+}: calcium

CC: corpus callosum

CNS: central nervous system

COMT: catechol-O-methyl transferase

CRD-Nrg1: cysteine-rich domain Nrg1, Nrg1 type III

CRD: cysteine-rich domain

CRISPR: clustered regularly interspaced short palindromic repeats

DAPI: 4′,6-diamidino-2-phenylindole

DiD: 1,1'-dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine

DiI: 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate

DISC1: disrupted-in-schizophrenia 1

DIV: days *in vitro*

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DsRed: discosoma red fluorescent protein

DTNBP1: dysbindin

E15: embryonic day 15

EDTA: ethylenediamine tetraacetic acid

EGF: epidermal growth factor

ERK: extracellular signal-regulated kinase

FBS: fetal bovine serum

GABA: gamma-Aminobutyric Acid

GAP43: growth associated protein 43

GFAP: glial fibrillary acidic protein

GFP: green fluorescence protein

GOF: gain-of function

GRM3: metabotropic glutamate receptor 3

GSH: gamma-glutamylcysteinylglycine, glutathione

GTPases: small or monomeric guanine nucleotide-binding regulatory proteins

H_2O_2: hydrogen peroxide

HBSS: hank's Balanced Salt Solution

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HRP: horseradish peroxidase

IUE: *in utero* electroporation

JNK: c-Jun N-terminal kinase

KO: knockout

LOF: loss-of function

MAPK: mitogen-activated protein kinase

MEM: minimum Essential Medium

MGE: medial ganglionic eminence

mTOR: mammalian target of rapamycin

NAA: N-acetylaspartate

NCAM: neural cell adhesion molecule

Nes-Cre: Nrg1flox/flox mice (Nrg1 KO)

Neurog 2: neurogenin 2

NLS: nuclear localization signal

NMDA: N-methyl-D-aspartate

NPC: neural progenitor cell

Nrg1-FL: CRD-Nrg1, Nrg1 full-length, type III Nrg1

Nrg1-ICD: Nrg1 intracellular domain

Nrg1: Neuregulin 1

NRG1: Neuregulin 1 gene

OGD: oxygen glucose deprivation

OPC: oligodendrocyte precursor cell

P2: postnatal day 2

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFA: paraformaldehyde

PI3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

PNS: peripheral nervous system

PRDX: peroxiredoxin

PS: penicillin /streptomycin

PSD95: postsynaptic density protein 95

PV interneurons: parvalbumin interneurons

RGS4: regulator of G-protein signaling 4

ROS: reactive oxygen species

RT-qPCR: real time quantitative PCR

SDS: sodium dodecyl

Sema3A: semaphorin 3A

SZ: schizophrenia

TBI: traumatic brain injury

TM: transmembrane

TXN: thioredoxin

WM: white matter

WT: wild type

FIGURES AND TABLES LIST

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Supplementary material

Supplementary Figure S6. Low magnification of the representative images Nrg1-FL, Nrg1-ICD and their respective control littermates presented in the Figure 18 (Chapter 2).

Supplementary Figure S7. GAP43 protein expression is highly reduced in Nrg1 KO mice.

INDEX

GENERAL INTRODUCTION

Schizophrenia: a connectivity disorder

Schizophrenia (SZ) is a neurodevelopmental disorder that impacts around 1% of the global population and it is caused by both genetic and environmental factors. SZ affects cognitive processes and social behavior, presenting various indicators including altered perception, paranoid thoughts and motor and cognitive abnormalities (Harrison et al., 2005; Lewis et al., 2009). The SZ disorder is difficult to diagnose because, unlike other neuropathologies, the brains of SZ patients do not display obvious histological hallmarks. Additionally, there are still no effective treatments, mainly because the molecular mechanisms underlying the disorder still remain unknown. Current treatments are antipsychotic medication, being not specific for this disorder, requiring lifelong administration and being often accompanied by hospitalization. Even though all efforts, many patients still fail to cope in society.

Historically, two major theories, which are not mutually exclusive, have been proposed to explain the biological component of schizophrenia, the dopaminergic and the glutamatergic hypothesis. The dopaminergic hypothesis arose from the discovery of how conventional antipsychotics blocked dopamine receptors but its underlying molecular mechanisms remain unknown. Hyperactivity of the dopaminergic system can be related with some, but not all, symptoms of the disease. Conversely, the glutamatergic hypothesis proposes that at the core of the pathogenesis of schizophrenia is an impaired activity of glutamatergic pathways. Several studies have shown decreased expression of glutamate receptor subunits in schizophrenic patients (Stefansson et al., 2004).

More recently, studies indicate that schizophrenia is a neurodevelopmental disorder, still involving genetic factors and therefore, suggesting that defects in the neurotransmitter systems are secondary (Stefansson et al., 2004). Some of the most consistent endophenotypes in SZ involve cerebral asymmetry, reduced brain volume and neuropil, white matter (WM) abnormalities and impaired functional connectivity between cortical areas (Arat et al., 2015; Fenlon et al., 2015; Hennen et al., 2013; Hoptman et al., 2012; Innocenti et al., 2003; Kubicki et al., 2005; Mohr et al., 2000). Alterations in the morphology and molecular composition of specific neuronal and glial population, as well

as specific changes in synaptic connections, have been also observed (Bjarnadottir et al., 2007; Harrison et al., 2005; Lewis et al., 2009; B. Li et al., 2007; Lisman et al., 2008).

In vivo findings in patients provide new insights into the nature of the SZ endophenotype. In this sense, the observed decrease in size and complexity of neurons could explain the decreased N-acetylaspartate (NAA) signal, an indicator of neuronal integrity, observed in this disorder. Along with the reduction in certain neuronal and glial populations, these morphological alterations can also be contributing to the reduction in neuropil (Harrison et al., 2005). Therefore, SZ is now considered a neurodevelopmental disorder based on the abnormal circuitry of cortical neurons, with alterations ranging from abnormalities in the neurites to the cell body and the synaptic terminal, including associated glial elements. The alterations observed in SZ can be observed either as local alterations (Bjarnadottir et al., 2007; Harrison et al., 2005; Lewis et al., 2009; B. Li et al., 2007; Lisman et al., 2008) or as long-range deficits involving connections between distant brain regions (Arat et al., 2015; Fenlon et al., 2015; Hoptman et al., 2012; Innocenti et al., 2003; Mohr et al., 2000).

The corpus callosum (CC) connects the brain's hemispheres and conforms the major bundle of cortico-cortical nerve fibers. Converging evidence supports the hypothesis that the CC is hypoconnected in SZ patients (Arat et al., 2015; David, 1994; Fenlon et al., 2015; Kubicki et al., 2005). Specifically, morphological studies indicate a reduction of the CC and functional approaches, such as fMRI, show that interhemispheric coordination is impaired in SZ patients (Hoptman et al., 2012; Innocenti et al., 2003). Thus, alterations in the region-specific contralateral targeting produces an unbalance of interhemispheric activity, contributing to some of the common features of the disorder.

The CC development is a complex process crucial for the proper cortical circuitry formation. The formation of CC projections involves a timely regulated sequence of stepwise events (Fenlon et al., 2015). It begins at embryonic stages with the fusion of the brain hemispheres, forming a pathway for interhemispheric axons. Subsequently, cortical neurons of the cingulate cortex extend their axons along this route, guided by specific cues. These initial axons act as pioneers, directing the following axons. The process continues in postnatal stages with the precise targeting of the contralateral side. In the mouse, CC axons reach their contralateral target and arborize around 7 to 10 postnatal days (P7-P10) (Mizuno et al., 2007; Wang et al., 2007), a very critical period for cortical wiring that involves a peak in synaptogenesis and the concomitant activity-dependent

apoptosis of cortical inhibitory interneurons (Favuzzi et al., 2019; F. K. Wong et al., 2018). Finally, a significant amount of connections are pruned after weaning until P30 (N. S. De León Reyes et al., 2019; Noelia S. De León Reyes et al., 2020).

The development of precise contralateral connections is critical for most aspects of cortical function, including basic brain activities and higher cortical functions. Callosal wiring controls the coordination of motor actions, the three-dimensional representation of visual and auditory stimuli, the emotional processing and the verbal responses (Noelia S. De León Reyes et al., 2020; Fenlon et al., 2015). In humans, high-order cortical regions such as the Broca's and Wernicke's areas are lateralized asymmetrically. These regions control written and spoken comprehension and surgical corpus callosotomy impairs their function (Mohr et al., 2000; Riès et al., 2016). Given its major functional relevance and complex development, it is not surprising that CC deficits were associated with neurodevelopmental disorders such as SZ, since a reduced development of CC directly impairs interhemispheric communication and information processing (David, 1994; Fenlon et al., 2015; Hoptman et al., 2012; Innocenti et al., 2003; Kubicki et al., 2005).

The strong genetic component in the schizophrenia disorder: The *NRG1* risk gene

While the developmental etiology of SZ remains largely unresolved, it is well established that SZ has a strong genetic component. Twin studies unequivocally demonstrate that SZ is primarily a genetic disorder, with a risk heritability estimated around 80% (Harrison et al., 2005; Stefansson et al., 2002).

Identification of the genes responsible for this high heritability is fundamental to get to understand the mechanisms that underlie the pathophysiology of SZ. Genetic studies show that SZ is a complex genetic disorder, which is not characterized by a single causative gene and does not show simple patterns of inheritance. In fact, each of its gene's accounts for only a small fraction of the risk, which depends on its interaction with other genes and the environment. The specific set of genes and mutations involved in the etiology of SZ may vary in different populations and individuals (Harrison et al., 2005; Stefansson et al., 2002).

However, several susceptibility genes have been identified, such as *neuregulin 1* (*NRG1*), *catechol-O-methyl transferase* (*COMT*), *dysbindin* (*DTNBP1*), *disrupted-in-*

schizophrenia 1 (*DISC1*), *regulator of G-protein signalling 4* (*RGS4*), *metabotropic glutamate receptor 3* (*GRM3*), and *G72*. These genes may functionally converge on SZ risk through an influence on the development and maturation of cortical circuits (Harrison et al., 2005; Stefansson et al., 2002).

One of the most schizophrenia-risk related genes is *NRG1*. Several publications carried out in different human populations provide evidence that *NRG1* is a major susceptibility gene (Harrison et al., 2005; Stefansson et al., 2002, 2003; Tang et al., 2004; Williams et al., 2003; Yang et al., 2003). Results of a genomewide scan of schizophrenia families in Iceland shows that SZ disorder is related to the chromosome 8p, in specific to the region 8p12-8p21. *NRG1* was pointed out for the first time as a candidate for this disorder by performing a microsatellite-based whole genome scan. Using high-resolution genetic and physical mapping techniques, two risk haplotypes were found, located in the 5' domain of the *NRG1* gene (Stefansson et al., 2002). Following the initial report, evidence emerged of an association between *NRG1* and schizophrenia across multiple populations (Stefansson et al., 2003; Williams et al., 2003; Yang et al., 2003).

NRG1 hypotheses of schizophrenia

Several studies have presented evidence of the involvement of the Nrg1–ErbB4 signaling pathway in the development of SZ. *NRG1* is expressed in the synapses and has a clear role in neuronal development and synaptic plasticity, supporting the hypothesis that mutations in *NRG1* have a role in the etiology of this disorder.

The loss-of-function of either *NRG1* or its receptor, *ERBB4*, lead to impairments in pyramidal and γ-aminobutyric (GABA) expressing neuron migration, neurite outgrowth, axon projection, axon myelination and synapse formation (Mei et al., 2008). The consequent anatomical abnormalities may be the basis for the altered neurotransmission and cortical dysfunction, ultimately resulting in the psychotic symptoms and cognitive deficits associated with SZ. Heterozygous mutant mice for *NRG1* or *ERBB4*, exhibit a behavioral phenotype reminiscent of mouse models for this disorder. Also, they show a reduction in the N-methyl-D-aspartate (NMDA) receptors, which hypofunction plays a key role in SZ (Nakazawa et al., 2020; Stefansson et al., 2002). The behavioral SZ-related phenotype can be partially reversed with a treatment based on clozapine, an atypical antipsychotic drug (Stefansson et al., 2002). More recent studies also link ErbB4 to SZ

(Y.-J. Chen et al., 2010; del Pino et al., 2013). Conditional *ERBB4* mutant mice shows a subtle but consistent synaptic defect. Those wiring impairments boost cortical excitability and disrupt synchrony across cortical regions, causing a phenotype concordant with SZ symptoms (del Pino et al., 2013).

Conversely, other studies support a gain-of-function hypothesis of Nrg1–ErbB4 signaling as a potential mechanism in the pathogenesis of SZ. They are based on the evidence of the increased Nrg1 signaling found in the prefrontal cortex of patients with schizophrenia, inducing a suppression of the NMDA receptor activation (Hahn et al., 2006). Transgenic mice overexpressing Nrg1 exhibit behavioral deficits (Olaya et al., 2018) and hypofunction of glutamatergic and GABAergic pathways (Yin et al., 2013) , consistent with the reduced glutamatergic transmission and plasticity that is found in the brains of patients with SZ (Tsai et al., 2002). These results suggest that an optimal level of Nrg1 is required to maintain homeostasis of excitatory/inhibitory circuits in the cortex (Olaya et al., 2018; Yin et al., 2013).

These findings provide a strong support for the plausibility of *NRG1* and its receptor, *ERBB4*, as major schizophrenia risk genes.

NRG1 and *ERBB* genes and proteins

Neuregulins (NRGs) conform a large family of epidermal growth factor (EGF)-like proteins. NRGs are encoded by six individual genes (*NRG1–6*). Among all NRGs, *NRG1* is the best characterized. *NRG1* generates six types of proteins (I–VI) that produce at least 31 different isoforms. All six types of Nrg1 isoforms present an EGF-like domain and a distinct amino-terminal region (N-terminal), both located extracellularly. Most of them also present highly conserved transmembrane (TM) and intracellular (ICD) domains. Additionally, types I, II, IV and V have an immunoglobulin (Ig)-like domain between the N-terminal sequence and the EGF domain (**Figure 1**) (Falls, 2003; Mei et al., 2008; Navarro-González et al., 2019).

Most Nrg1 isoforms are synthesized as transmembrane precursors, called pro-Nrg1s, with the EGF domain located in the membrane-proximal region of the extracellular domain. Pro-Nrg1s undergo a process of enzymatic cleavage into the C-terminal region of the EGF domain by ADAM17, BACE and ADAM19 proteases. This

results in the release of diffusible, mature Nrg1, except in the case of type III Nrg1 (**Figure 1**) (Falls, 2003; Mei et al., 2014, 2008).

The Nrg1 type III, also referred as CRD-Nrg1 or Nrg1-FL, contains a cysteine-rich domain (CRD) in the N-terminal. The CRD domain contains a hydrophobic stretch which acts as an additional transmembrane domain (**Figure 1**). Therefore, whereas the soluble forms of Nrg1 execute their distinct functions in a paracrine manner, Nrg1 type III adopts a membrane-bound state, operating in a juxtracrine way (Falls, 2003; Mei et al., 2014, 2008).

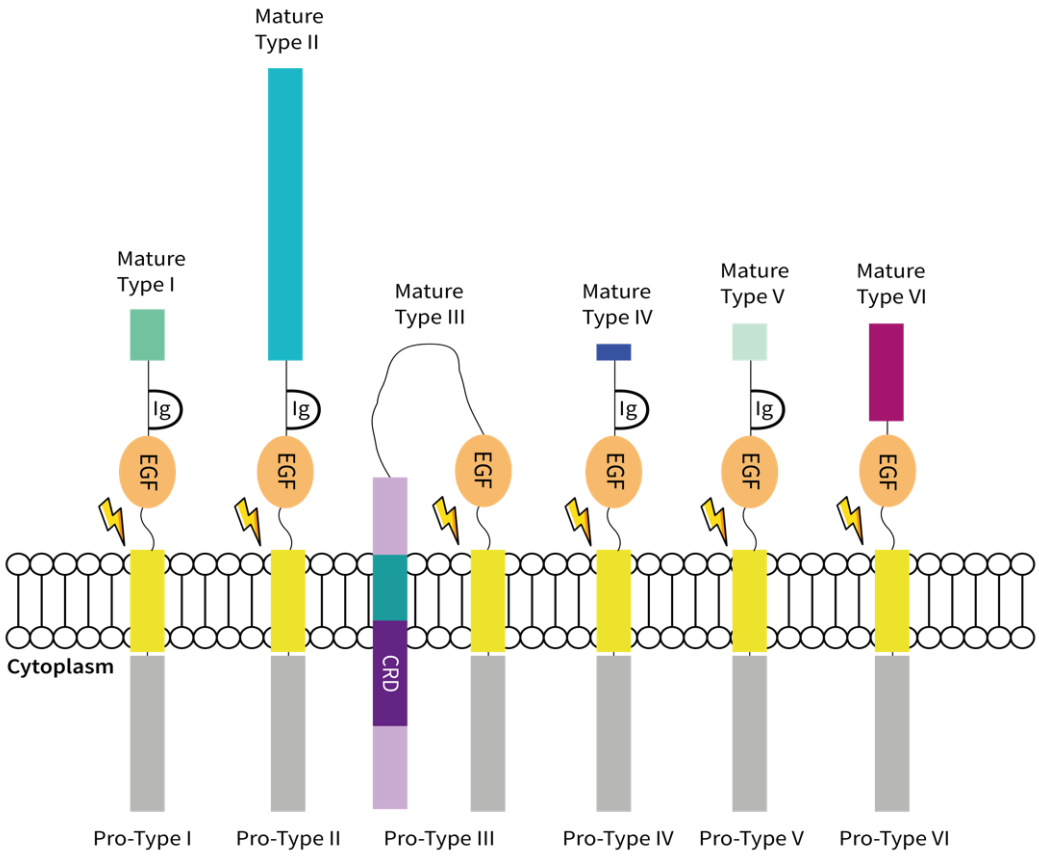


Figure 1. **Nrg1 types.** The Nrg1 isoforms are categorized into six distinct isoforms based on their distinct amino-terminal sequences. Most Nrg1 isoforms are synthesized as transmembrane precursor polypeptides (pro-Nrg1s) with the epidermal growth factor (EGF)-like domain located in the extracellular region. Types I, II, IV and V have an immunoglobulin (Ig)-like domain between the N-terminal sequence and the EGF domain. In the type III isoform, the N-terminal contains a cysteine-rich domain (CRD) with a second transmembrane domain. Following enzymatic cleavage (pointed by the arrow), every variety of Nrg1 releases a soluble form, except for the transmembrane type III Nrg1 which, due the CRD, retains both C- and N-amino terminals inside the cell. Thus, type III Nrg1 is thought to function trough cell contact (Adapted from Mei et al., 2008).

Nrg1 isoforms exhibit variations in both levels and expression patterns across different tissues, including the brain (Liu et al., 2011). Mutant mice with specific isoform inactivation display alterations in neural development, implying diverse functions for the different isoforms. Nrg1 isoforms are predominantly expressed in excitatory pyramidal neurons (C.-Y. Ding et al., 2023). The Nrg1 type III in specific is the most expressed isoform in adult neurons, and its abnormal expression was observed in schizophrenia patients (Liu et al., 2011; Mei et al., 2014, 2008).

Nrg1 signals trough the EGF-like domain, interacting with the ErbB receptors. The ErbB proteins constitutes a family of single transmembrane tyrosine kinases, formed by four members: ErbB1 (also named EGFR), ErbB2, ErbB3 and ErbB4. Structurally, ErbB receptors consist of an extracellular domain with two CRDs, a TM domain and an ICD containing the tyrosine kinase. Upon ligand binding, ErbBs undergo dimerization and activation, leading to the phosphorylation of their ICDs and the formation of docking sites for adaptor proteins. ErbB1 does not bind to Nrg1, but it can form heterodimers with ErbB4. ErbB2, by contrast, functions as a co-receptor by forming heterodimers with other ErbBs. ErbB3 kinase can interact with Nrg1 but its kinase function is impaired. ErbB4 is the only form that can both interact with Nrg1 and become activated by it as a tyrosine kinase (**Figure 2**) (Bublil et al., 2007; Mei et al., 2014).

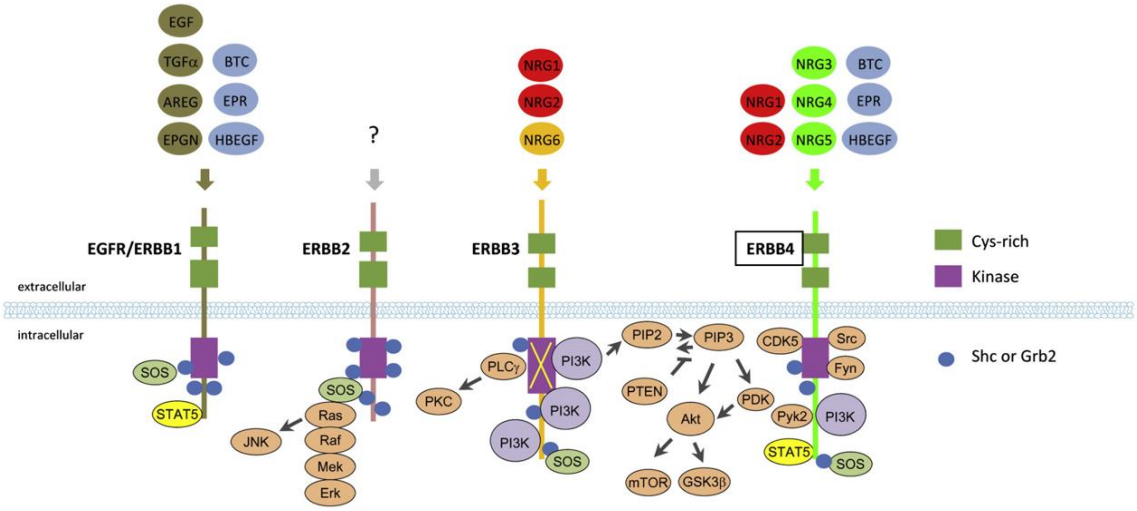


Figure 2. **Nrg/ErbB interactions.** All Nrg1s present an extracellular EGF-like domain, which is required for binding to the ErbB receptor. ErbB tyrosine kinases conform a family of four members: EGFR/ErbB1, ErbB2, ErbB3, and ErbB4. All of them has a specific group of ligands except ErbB2, whose ligand remains unknown. ErbB3 kinase activity is impaired (pointed by a cross). Ligand binding causes receptor dimerization and phosphorylation of downstream effectors (Adapted from Mei et al., 2014).

Among the ErbB proteins, ErbB4 is the best-characterized for its function in the central nervous system (CNS). Additionally, the ErbB4 receptor is the most abundant in the cortex, being mainly present in parvalbumin-expressing (PV^{+}) inhibitory interneurons (Bublil et al., 2007; Esper et al., 2006; Fazzari et al., 2010; Mei et al., 2014). No evidence suggests that ErbB2 and ErbB3 are susceptibility genes for SZ, whereas ErbB4-mutant mice exhibits behaviors characteristic of this disorder (Y.-J. Chen et al., 2010; del Pino et al., 2013; Gerlai et al., 2000). Thus, this thesis will review Nrg1 signaling and function with the main focus on ErbB4.

Nrg1 and ErbB4 signaling pathways

We and others have previously shown that the SZ risk gene *NRG1* is expressed in excitatory pyramidal neurons, whereas its specific receptor *ERBB4* is mainly found in inhibitory interneurons. Nrg1 signaling through ErbB4 is complex, involving three different pathways.

Nrg1 backward signaling

The Nrg1 backward signaling, also named intracellular signaling, operates through the highly conserved ICD present in most of Nrg1 isoforms (**Figure 3**) (Navarro-González et al., 2019).

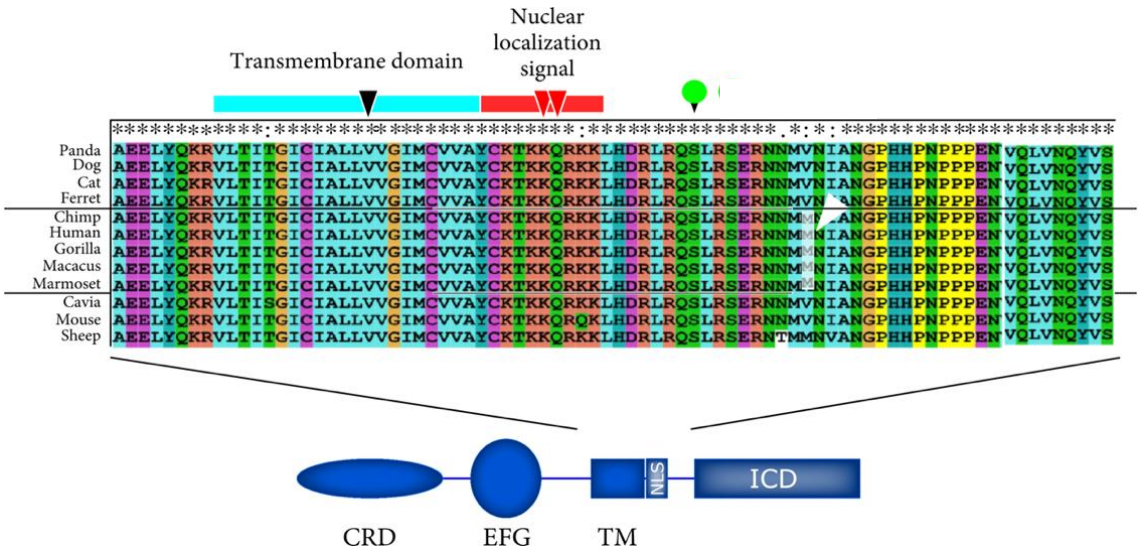


Figure 3. **Nrg1 phylogenetic conservation.** The transmembrane (TM) domain, the nuclear localization signal (NLS) and the intracellular domain (ICD) of Nrg1 are highly preserved among different species. The black arrowhead indicates the location of the Val-to-Leu schizophrenia-

linked mutation that affects γ-secretase processing. The red arrowheads indicate the amino acids in the nuclear localization signal required for the nuclear targeting of Nrg1-ICD. CRD: cysteine-rich domain; EGF: epithelial growth factor domain (Adapted from Navarro-González et al., 2019).

The physiological mechanisms leading to the activation of Nrg1 intracellular signaling are unclear but, *in vitro* studies have shown that it can be triggered by multiple stimuli, including binding to the ErbB4 receptor, neuronal depolarization and hypoxia (Bao et al., 2003; Mei et al., 2008; Navarro-González et al., 2019). Here, the type III pro-Nrg1, previously generated by extracellular proteolytic cleavage by BACE, is later processed in the transmembrane region by a γ-secretase, generating a Nrg1 intracellular domain (Nrg1-ICD). Nrg1-ICD is released to the cytosol, being able to translocate to the nucleus and regulate gene transcription (**Figures 4 and 5**) (Bao et al., 2003; Y. Chen et al., 2010; Mei et al., 2008; Pedrique et al., 2010).

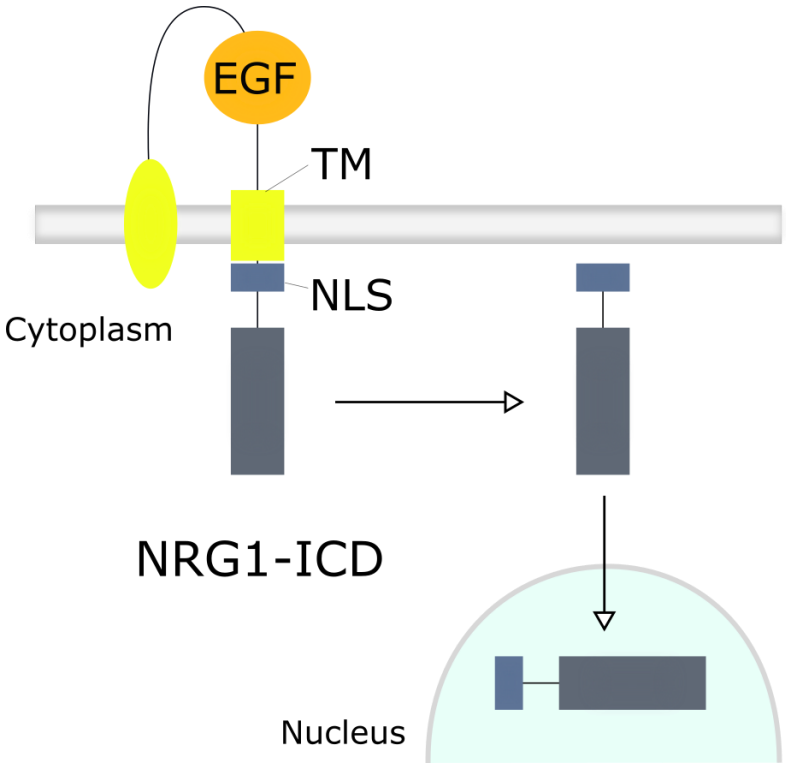


Figure 4. **Nrg1 intracellular signaling.** The Nrg1 type III intracellular domain (Nrg1-ICD) is cleaved by γ-secretase, being release into the cytosol. It can translocate to the nucleus and regulate gene expression. EGF: epithelial growth factor domain; TM: transmembrane domain; NLS: nuclear localization signal.

Nrg1/ErbB canonical forward signaling

In the canonical forward signaling, Nrg1 interacts with ErbB trough the EGF domain, promoting its dimerization in hetero- (ErbB2–ErbB3, ErbB2–ErbB4, ErbB3–ErbB4) and homodimer (ErbB4–ErbB4) structures (Bublil et al., 2007). These conformations activate the ErbB kinase domain, resulting in the auto- and trans-phosphorylation of the intracellular domains (ErbB-ICDs). As consequence, downstream phosphorylation cascades are triggered, like the MAPK/Erk and PI3K/Akt pathways. Ultimately, specific transcriptional and translational programs are activated, producing numerous long-term effects (**Figure 5, A**) (Falls, 2003; Mei et al., 2014, 2008).

Nrg1/ErbB non-canonical forward signaling

In the non-canonical forward signaling, the extracellular domain of ErbB4 first undergoes a proteolytic cleavage by TACE, releasing a soluble form (ecto-ErbB4) capable of binding to Nrg1. The remaining membrane portion is subsequently cleaved by γ-secretase, releasing the ErbB4 intracellular domain (ErbB4-ICD). ErbB4-ICD can translocate to the nucleus and regulate transcription (**Figure 5, B**) (Falls, 2003; Mei et al., 2014, 2008).

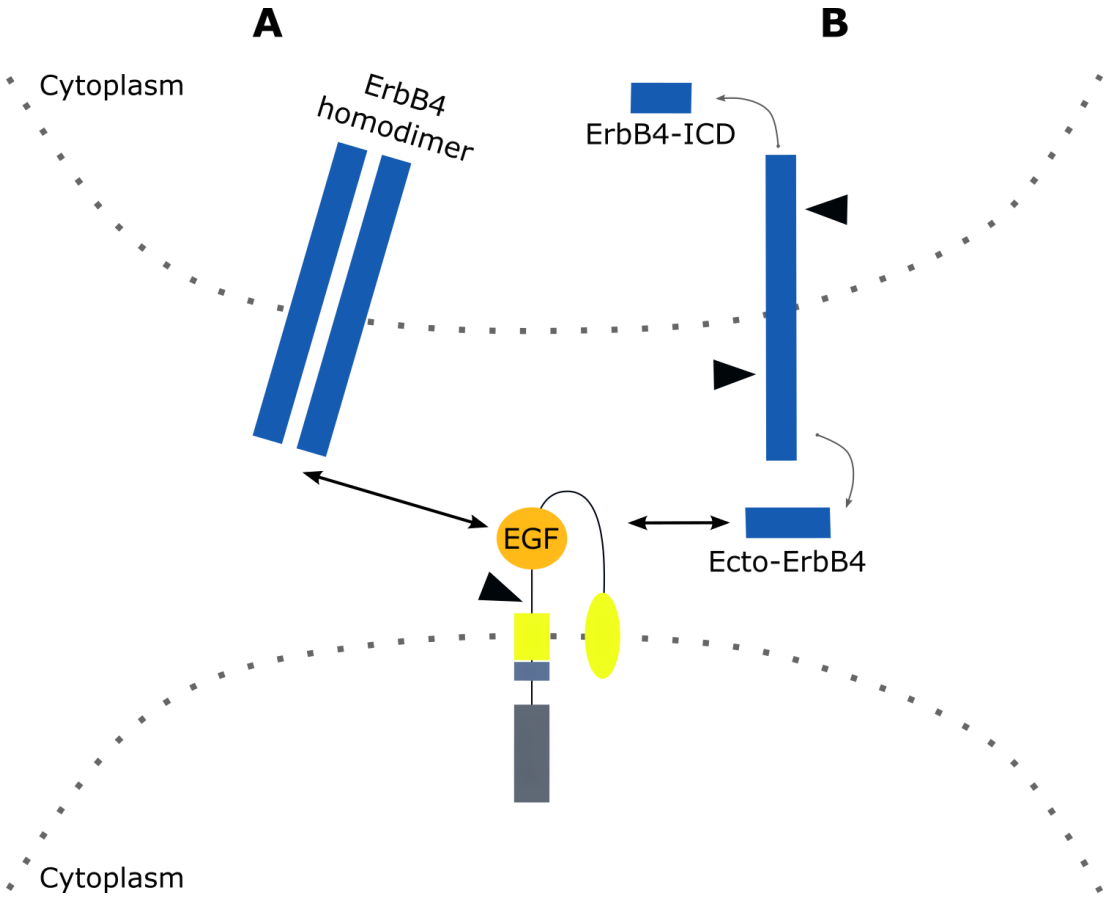


Figure 5. **Nrg1/ErbB4 forward signaling pathways.** The scheme represents the different interactions of Nrg1 and ErbB4 trough the canonical and non-canonical forward pathways. (A) In the canonical forward signaling, the EGF domain of soluble form of Nrg1 or transmembrane Nrg1 type III promotes ErbB4 dimerization, inducing the phosphorylation of downstream effectors. (B) In the non-canonical forward signaling, ErbB4 intracellular domain (ErbB4-ICD) is cleaved and translocated to the nucleus for transcriptional regulation. Simultaneously, the ErbB4 extracellular domain (ecto-ErbB4) is released to the cytoplasm, interacting with Nrg1 (Adapted from Mei et al., 2008).

Nrg1 functions in the Central Nervous System

Since Nrg1 was identified as a major SZ risk-gene (Hennen et al., 2013; Stefansson et al., 2002), most studies attempted to understand the role of Nrg1 in brain wiring and in cortical inhibition. Altogether, several studies showed that Nrg1 has multiple functions in the CNS, playing a pivotal role in various steps of cortical development and circuitry wiring.

Neuronal migration

In the developing cortex, most cortical interneurons originate in the subcortical telencephalon, but the molecules that control their migration have not yet been entirely identified. Nrg1 signaling is relevant for the formation of radial glial cells, which are required to support neuronal migration (Anton et al., 1997; Flames et al., 2004). Most tangentially migrating interneurons derive from the medial ganglionic eminence (MGE). GABAergic interneurons tangential migration from the MGE to the cortex requires the simultaneous activity of chemo-repulsive and -attractive factors (Flames et al., 2004).

It was shown that Nrg1 controls the tangential migration of interneurons precursors from the MGE to the cortex, as ErbB4 is expressed in the GABAergic interneurons that migrate through a permissive corridor that expresses type III Nrg1. Consequently, loss of Nrg1/ErbB4 signaling causes a reduction of the GABAergic interneurons in the postnatal cortex (Flames et al., 2004).

Axon guidance

Axon guidance is a critical process in brain development, wherein axons navigate precise paths under the guidance of extracellular molecules. Despite its recognized importance, the role of Nrg1 in this function remains largely unknown. Previous studies

have demonstrated that tangential migration contributes to the establishment of a permissive corridor that is essential for thalamocortical axon pathfinding, being the axon guidance mediated by Nrg1 signaling (López-Bendito et al., 2006). Additionally, another study revealed that mice lacking CRD-Nrg1 displayed insensitivity to the chemorepellent semaphorin 3A (Sema3A), resulting in aberrant axon navigation within the spinal cord and peripheral projections. Furthermore, it was shown that the *in vitro* activation of Nrg1 intracellular signaling prompts the expression of neuropilin 1, the receptor for Sema3A (Hancock et al., 2011). Altogether, these results support that type III Nrg1 is required for axon pathfinding.

Axon myelination

Several psychiatric disorders, including SZ, are associated with defects in the white matter, the regions primarily composed of nerve fibers (axons). Axon myelination is a crucial process in the nervous system where axons are wrapped in myelin sheaths. These layers are synthesized by specialized cells, known as Schwann cells in the peripheral nervous system (PNS) and oligodendrocytes in the CNS. This facilitates a more efficient transmission of electrical signals along the axon. Moreover, myelination plays a crucial role in in various neurological processes, encompassing motor coordination, sensory perception, and cognitive functions (Bercury et al., 2015).

Oligodendrocyte dysfunction and impaired myelination have been previously related with SZ (Kubicki et al., 2005; Takahashi et al., 2011). Briefly, in the PNS, Nrg1 promotes Schwann cell proliferation and differentiation (Birchmeier et al., 2008; S. Chen et al., 2006). Loss of function of Nrg1 signaling induces almost complete loss of Schwann cells, resulting in a reduction of myelin thickness in the axons (S. Chen et al., 2006). Conversely, the role of Nrg1 in oligodendrocytes is much more controversial. Some studies suggest that Nrg1 promotes oligodendrocyte proliferation, differentiation and survival (Canoll et al., 1996; Flores et al., 2000). Furthermore, it was suggested that the loss of ErbB4 signaling produces reduction in oligodendrocyte number, myelin thickness and conduction velocity in CNS axons (Roy et al., 2007). However, those studies suggesting a significant role for Nrg1 in brain myelination and oligodendrocyte function, haven't been consistently replicated by independent research groups in recent years. As a result, the exact role of Nrg1 in this process remains unclear.

Dendrite development

Nrg1 stimulates neurite outgrowth in multiple populations of ErbB4 expressing primary neurons, including hippocampal and thalamic neurons, among others (Gerecke et al., 2004; López-Bendito et al., 2006). Conversely, the role of the of Nrg1 and its intracellular signaling in cortical excitatory neurons is much less understood. Nonetheless, a few studies suggest that Nrg1 loss-of-function may impair dendritic development. In particular, constitutive deletion of the type III isoform of Nrg1, shows a reduced dendritic arborization in mouse (Y. Chen et al., 2010; Z. Zhang et al., 2017). Moreover, primary cortical neurons from this type III Nrg1 mutant show an impaired dendrite development at early stages of maturation. Nrg1 intracellular signaling can partially rescue the developmental deficits in dendrites (Y. Chen et al., 2010).

Synapse formation and excitatory-inhibitory circuitry homeostasis

The most consistent endophenotypes in SZ include specific changes in synaptic connections (Bjarnadottir et al., 2007; B. Li et al., 2007; Lisman et al., 2008). We and others have previously shown that the SZ risk gene Nrg1 is expressed in excitatory pyramidal neurons, whereas its specific receptor Erbb4 is mainly found in PV^+ chandelier and basket inhibitory interneurons (Fazzari et al., 2010). Several studies demonstrated that Nrg1/Erbb4 signaling plays an important role in the cortex, and specifically in the wiring of inhibitory cortical neurons (Y.-J. Chen et al., 2010; Y. Chen et al., 2010; Fazzari et al., 2010; Navarro-Gonzalez et al., 2021; Pedrique et al., 2010; Rahman-Enyart et al., 2020).

Erbb4 activation in inhibitory neurons is required for proper wiring of local inhibitory circuits, as it promotes the growth of inhibitory axons *in vitro* and the formation of GABAergic synapses both *in vitro* and *in vivo* (Fazzari et al., 2010; Navarro-Gonzalez et al., 2021; Rico et al., 2011). Mutant mice lacking ErbB4 show synaptic dysfunction, as the number of inhibitory synapses made by the basket and chandelier cells decrease (del Pino et al., 2013; Fazzari et al., 2010). These alterations in connectivity increase cortical excitability and disrupt synchronization between cortical regions (del Pino et al., 2013).

Nrg1 is equally important for the activity of cortical interneurons and inhibitory homeostasis. Indeed, Nrg1 was one of the first synaptogenic cues found to regulate cortical inhibitory circuits via the activation of ErbB4. Specifically, Nrg1 promotes, in ErbB4 expressing inhibitory cells, the formation of excitatory synapses in the dendrites

of inhibitory contacts in the axonal button (**Figure 6**) (Y.-J. Chen et al., 2010; Exposito-Alonso et al., 2020; Navarro-Gonzalez et al., 2021). Moreover, acute stimulation with Nrg1 enhances the release of GABA from ErbB4 expressing interneurons (Mei et al., 2008)**.**

Nrg1 also regulates the development of dendritic spines in pyramidal neurons. First, it was demonstrated that increased Nrg1 levels promote spine maturation (Barros et al., 2009). Later, it was proven that the role of Nrg1 in the dendritic spines is performed probably via its intracellular signaling, as a loss of dendritic spine was identified in Aph1bc$^{-/-}$ mice, in which the intracellular proteolytic processing of Nrg1 is impaired (Fazzari et al., 2014).

The Nrg1/ErbB signaling pathway has been implicated in the maturation of dendritic spines, crucial for effective synaptic transmission and the establishment of cortical circuits. It has been demonstrated that Nrg1/ErbB signaling promotes spine maturation by regulating the interactions between the NMDA receptors and the postsynaptic density protein 95 (PSD95). Thus, mice deficient in ErbB2/ErbB4 display an impaired dendritic spine maturation in hippocampal pyramidal neurons (Barros et al., 2009). Additionally, interruption of Nrg1/ErbB4 signaling destabilizes synaptic AMPA receptors, leading to a loss of NMDA receptors and dendritic spines, provoking a glutamatergic hypofunction (B. Li et al., 2007).

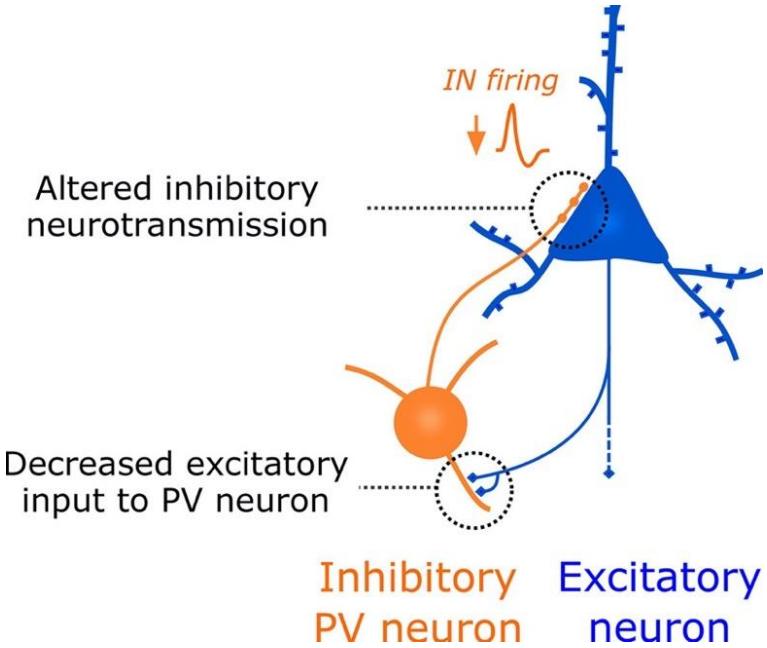


Figure 6. **Impaired cortical circuits in Nrg1 deficient mice.** ErbB4 is expressed by many parvalbumin-expressing (PV) chandelier and basket cells, where it localizes to axon terminals

and postsynaptic densities receiving glutamatergic input. In Nrg1 mutant mice, alterations in the connectivity between pyramidal neurons (blue) and inhibitory cells (orange), were observed. IN: interneuron (del Pino et al., 2013; Fazzari et al., 2010; Mei et al., 2014) (Adapted from Navarro-González et al., 2021).

Neuronal survival

In the adult brain, Nrg1 presents a neuroprotective role for cortical (B.-S. Li et al., 2003) and dopaminergic neurons (L. Zhang et al., 2004), among others cell types. Nrg1 can exert this role under neuronal stress conditions, such as brain injury or neurodegenerative diseases, by promoting neuronal survival and protecting against stress-induced cell death (Y. Li et al., 2007; Navarro-González et al., 2019; Shyu et al., 2004). Notably, it has been demonstrated that Nrg1 intracellular signaling is neuroprotective upon stroke both *in vitro* and *in vivo* (Navarro-González et al., 2019).

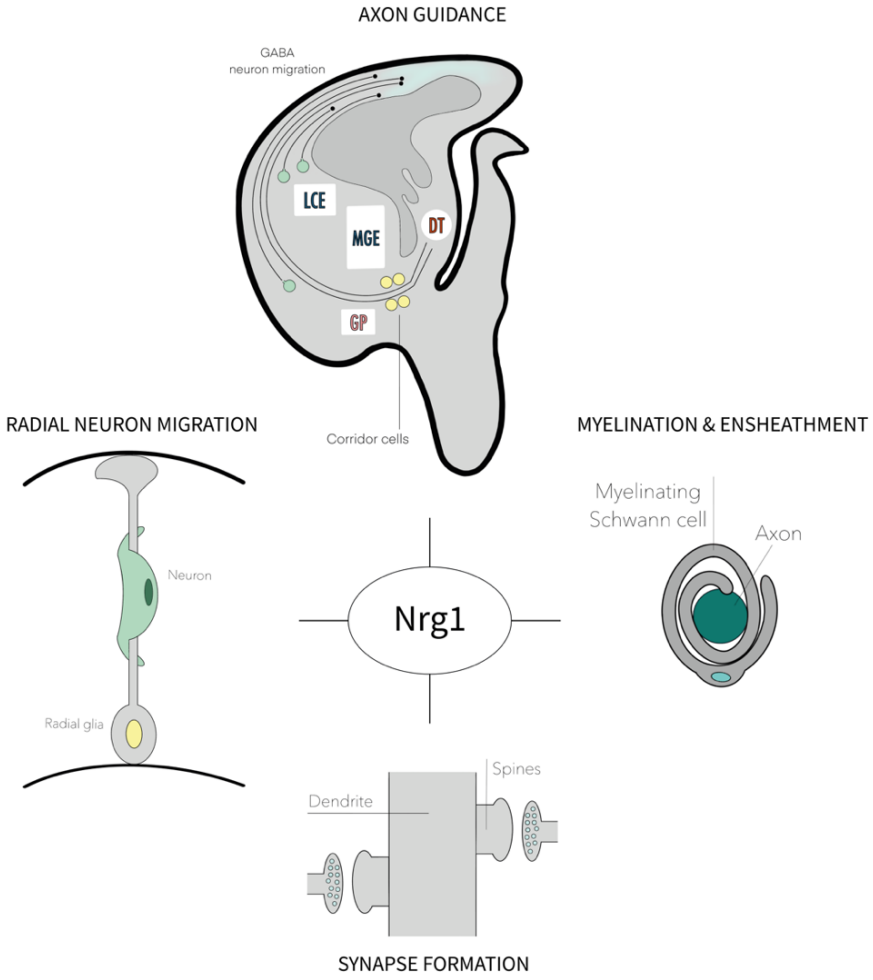


Figure 7. **Overview of the main Nrg1 functions in the central nervous system.** Among other functions, it has been described the relevant role of Nrg1 in neuronal migration, axon guidance, myelination and synapse formation.

Nrg1 as a potential therapeutic target

Because of its important role in the CNS and PNS, the potential therapeutic roles of Nrg1 have been widely studied, particularly in relation to neurodevelopmental disorders, such as SZ and autism. In addition, an expanding literature suggests that the neurotropic activity of Nrg1 may be useful in other neuropathologies, such as Alzheimer's disease (AD) and brain injury.

Some patients with SZ have shown reduced expression of certain components of the Nrg1/ErbB pathways, while other studies have seen an elevation in Nrg1/ErbB4 activity. Either way, diverse alterations in the Nrg1/ErbB signaling are expected to manifest as varied disruptions in synaptic function and circuitry connectivity homeostasis (Bjarnadottir et al., 2007; B. Li et al., 2007; Lisman et al., 2008). Accordingly, different mutants for Nrg1 or its receptor ErbB4 show alterations characteristic of SZ (del Pino et al., 2013; Fazzari et al., 2010; B. Li et al., 2007; Nakazawa et al., 2020; Navarro-Gonzalez et al., 2021; Stefansson et al., 2002; Yin et al., 2013). Thus, modulation of Nrg1/ErbB signaling pathways has shown promise in preclinical models of schizophrenia, suggesting its potential as a therapeutic target.

The function of Nrg1 in AD is an evolving area of research. Its involvement in AD has been the subject of study due to its influence on synaptic function and neuronal survival, processes that are altered in this neurodegenerative disease. It has been reported that Nrg1 concentration is increased in the cerebrospinal fluid (CSF) and plasma of AD patients (Mouton-Liger et al., 2020; Pankonin et al., 2009; Vrillon et al., 2022). It is difficult to determine whether Nrg1/ErbB4 signaling has beneficial or detrimental effects in AD. In CSF and AD brains, the enzyme BACE1 that cleaves amyloid precursor protein (APP) and pro-Nrg1, is increased. This enhances the Nrg1 secretion, leading to a Nrg1 accumulation in the neuritic plaques and consequently to neuroinflammation (Mouton-Liger et al., 2020).

Conversely, numerous studies show different positive effects of Nrg1 on this disease. Treatment with Nrg1 in aged mice models for AD rescues the reduction in dendritic spine density and promotes neurogenesis (Ryu et al., 2016). Moreover, the reduction of Nrg1-ErbB4 signaling in PV^+ interneurons could reactivate cortical plasticity (Huh et al., 2016; L. Shi et al., 2020). Taken together, these studies indicate that Nrg1

may be a promising non-invasive synaptic and AD biomarker, and its regulation could represent a new therapeutic target in AD.

Nrg1/ErbB signaling has been recognized not only as a risk factor in psychiatric disorders but also as a potential regulator for the recovery following brain injury. Several studies have demonstrated its ability to promote neuronal survival and regeneration after injury, potentially through its effects on synaptic plasticity and axonal growth. Thus, manipulation of Nrg1 signaling pathways has shown beneficial effects in animal models of stroke and traumatic brain injury, highlighting its therapeutic potential (Kataria et al., 2019).

Brain damage is the leading cause of disability in adults, presenting significant implications for cognitive and motor functions. This process is characterized by an immediate increase in excitotoxicity and neuronal loss, followed by the release of inflammatory mediators and oxidative stressors, which compromise neuronal integrity and consequently impair brain reorganization (Joy et al., 2021). Hence, efforts have been undertaken to identify potential targets that facilitate neuroprotection.

A large body of evidence supports the neuroprotective role of Nrg1 following brain injury. Briefly, several studies have indicated that Nrg1 treatment in mice results in prevented neuronal death, reduced injury size (Guo et al., 2006; Y. Li et al., 2007; Navarro-González et al., 2019; Noll et al., 2024a), decreased astrocytes and glia presence in the perilesional area (Alizadeh et al., 2017) increased oligodendrocyte proliferation (Canoll et al., 1996; Cui et al., 2023; Flores et al., 2000) and decreased release of inflammatory insults (Kataria et al., 2019; Noll et al., 2024a). All these studies provide evidence that Nrg1 represents a novel neuroprotective strategy. Furthermore, in clinical trials Nrg1 was previously shown to be safe in human patients, so it has a potential therapeutic value in treating individuals after acute ischemic stroke (Noll et al., 2024a).

Altogether, Nrg1 promises to be a therapeutic target for both neurodevelopmental disorders and brain injury, due to its ability to modulate synaptic function and promote neuronal survival.

OBJECTIVES

In the present thesis we aimed to understand the role of Nrg1 signaling in the development of cortical circuits by studying Nrg1's function in the excitatory neurons and specifically, in the development of callosal axons. This objective was pursued by taking advantage of multiple approaches, including gain- and loss-of function experiments in cultured primary cortical neurons, neuronal tracing in newborn Nrg1 knockout mice and callosal projecting neurons labeling by *in utero* electroporation.

The specific objectives were:

1. To determine the role of Nrg1 in the development of callosal projections.
2. To study the cell autonomous effect of Nrg1 on neurites development.
3. To study the molecular mechanisms underlying Nrg1's role in neuron development.
4. To assess the role of Nrg1 in the reprogramming of astrocytes to neurons as a novel therapeutic approach following brain injury.

MATERIALS AND METHODS

Animals

In this study, we crossed mice expressing Cre under Nestin promotor (B6.Cg-Tg(Nes-cre)1Kln/J; hereafter Nes-Cre) with conditional mutant mice for Nrg1 (Nrg1^{tm3Cbm}; aka Nrg1$^{flox/flox}$; MGI:2447761) that carry a "floxed" Nrg1 allele. In Nes-Cre; Nrg1$^{flox/flox}$ mice (Nrg1 KO), the embryonic expression of Cre in the CNS results in the developmental deletion of the exons 7–9, which abrogates the expression and the Nrg1 signaling starting from embryonic day 11 (Tronche et al., 1999). Nes-Cre; Nrg1$^{flox/flox}$ mice were viable, fertile and did not show major signs of alterations in our housing conditions. For neuronal cultures, Nes-Cre; Nrg1$^{flox/flox}$ mice were crossed with Nrg1$^{flox/flox}$ mice to obtain Nrg1 KO and control Nrg1$^{flox/flox}$ mice from the same litter.

CD-1 background mice were used for the Nrg1 overexpression studies *in vitro*, C57BL/6J females and pups for the gain- or loss-of-function *in vivo*, respectively. The Nrg1$^{flox/flox}$ conditional mouse model was used to study the effect of Nrg1, by producing a single cell loss-of-function model *in vitro* with the expression of a Cre containing plasmid in cultured neurons. Nes-Cre transgenic mice were employed for the loss-of-function *in vivo*, as well as the Nrg1 molecular mechanisms studies.

The animals were kept and cared in the animal facility of the Centro de Investigación Príncipe Felipe (Valencia, Spain). The maintenance and use of all animals were by guidelines established by the European Communities Council Directive (86/609/ECC) and the Spanish Royal Decree 53/2013. The experiments were supervised by the bioethics committee of the institute and performed in compliance with bioethical regulations of the European Union and Spain. Animals were group-housed with food and water *ad libitum* in standard housing conditions.

Nrg1 and astrocyte reprogramming constructs

The original constructs for the expression of GFP-tagged Nrg1-ICD and Nrg1-FL, from *Mus musculus*, were previously generated and fully described (Fazzari et al., 2014). Briefly, Nrg1-FL expresses the CRD-Nrg1 isoform of Nrg1, aka type III Nrg1. Nrg1-ICD expresses the entire intracellular domain of CRD-Nrg1 including the nuclear localization

signal. Nrg1-ICD therefore mimics the intracellular domain of Nrg1 resulting from the cleavage by gamma-secretase. Nrg1 was expressed under the cytomegalovirus (CMV) promoter for *in vitro* experiments. CAG promoter was used for *in utero* electroporation (IUE) and astrocytes reprogramming experiments.

The constructs for astrocyte-to-neuron reprogramming were previously described (Heinrich et al., 2010; Masserdotti et al., 2015). DsRed, Neurog2 and Ascl1 were expressed under the CAG promoter along with GFP, under an Internal Ribosome Entry Sites (IRES): pCAG-DsRed, pCAG-Ascl1-IRES-DsRed, pCAG-Neurog2-IRES-DsRed.

Primary neuronal culture and neurons transfection by electroporation

Primary cultures of cortical neurons were prepared from embryonic day 15-16 (E15-16) of CD-1 or C57BL/6 ($Nrg1^{flox/flox}$ and Nes-Cre) mice, as previously described (Navarro-González et al., 2019; Rodríguez-Prieto et al., 2021). In short, embryonic brains were dissected and placed into ice-cold Hank's solution (14175-095; ThermoFisher). with 7 mM HEPES and 0.45% glucose (15630-080; ThermoFisher). The tissue was trypsinized (25300054; ThermoFisher) at 37°C for 15 min. Cortices were washed with Hank's solution, dissociated by mechanical disaggregation in 5 mL of plating medium containing Minimum Essential Medium (MEM) (11095080; ThermoFisher), supplemented with 10% horse serum (26050088, Invitrogen) and 20% glucose (15630-080; ThermoFisher) (**Supplementary Material S1**). Cells were counted in a Neubauer chamber and plated into precoated dishes with poly D-lysine (P2636; Sigma-Aldrich), for a final concentration of 110,000 cells per well in 12-well plates.

The rest of the cells were transfected to express the gene of interest when needed. Transfection was performed with the NEPA21 electroporation system as previously described (Rodríguez-Prieto et al., 2021). Concisely, after disaggregation the adequate volume of the cell suspension was transferred into a new tube, centrifugated and resuspended in electroporation medium (Opti-MEM 31985-062; Invitrogen). The final volume corresponding to 1 million of cells was mixed in a cuvette with the desired amount of DNA per each electroporation condition (**Table 1**): pCMV-GFP, pLV-CMV-LoxP-DsRed-LoxP-eGFP (65726; Addgene. Kindly provided by Dr. Jacco van Rheenen); pLenti-Lifeact-tdTomato, pCMV-GFP-IRES-Cre (Fazzari et al., 2010), pCAG-Nrg1-FL-nGFP, pCAG-Nrg1-ICD-GFP (Bao et al., 2003; Fazzari et al., 2014), pCAG-GAP43-

Myc (Okada et al., 2022). The cuvettes were inserted in the electroporator under the pre-established conditions: poring pulse of 2 ms, 175 V, 50 Interval, 10 Decay Rate, positive Polarity, 2 times; transfer pulse of 50 ms, 20 V, 50 Interval, 40 Decay Rate, positive and negative Polarity, 5 times (**Supplementary Material S2**).

For all experiments *in vitro*, all the neurons were electroporated to express GFP to visualize the neuronal structure with the immunolabeling anti-GFP. For both Nrg1 loss- and gain-of function experiments, control neurons were co-cultured together with Nrg1 KO and Nrg1-FL/Nrg1-ICD overexpressing neurons in the same well to have an optimal internal control. Nrg1 KO neurons were obtained transfecting Nrg1 conditional neurons with pCMV-GFP-IRES-Cre to visualize the morphology in the green channel and to express Cre to abrogate Nrg1 expression. For gain-of-function experiments, wild type (WT) neurons were co-transfected to express pCMV-GFP to visualize neuronal morphology together with either Nrg1-FL or Nrg1-ICD. In our experimental conditions, the expression of either DsRed or Lifeact-tdTomato did not affect axonal growth and was simply used to distinguish control cells from Nrg1 KO and Nrg1-FL/Nrg1-ICD overexpressing neurons.

Transfected cells were mixed with 0.5 ml of plating medium and co-cultured with the previously plated non-electroporated neurons. The cells were placed into a humidified incubator containing 95% air and 5% CO_2. Two hours after plating, the plating medium was replaced with equilibrated neurobasal media supplemented with B27 (17504-044; Life Technologies) and GlutaMAX (35050038; Fisher Scientific) (**Supplementary Material S1**). Neurons were fixed at the fourth day *in vitro* (DIV4) with 4% paraformaldehyde (PFA) in PBS. Immunofluorescence was carried out as described below.

Table 1. **Plasmids structure and information.** DNA concentration to electroporate neuronal cultures for GOF and LOF *in vitro* experiments.

Plasmid	Structure information	DNA concentration (µg)	References
Plasmid for GFP-Ctrl	pCMV-GFP	3.5	11795 (Addgene)
Plasmid for DsRed	pLV-CMV-LoxP-DsRed	6	65726 (Addgene)
Plasmid for tdTomato	pLV-Lifeact-tdTomato	6	64048 (Addgene)
Plasmid for GFP-Cre	pCMV-GFP-ires-Cre	6	Fazzari et al., 2014
Plasmid for Nrg1-FL	pCAG-Nrg1-FL-nGFP	24	Fazzari et al., 2014
Plasmid for Nrg1-ICD	pCAG-Nrg1-ICD-GFP	24	Fazzari et al., 2014
Plasmid for GAP43	pCAG-GAP43-Myc	15	Okada et al., 2022

Neuronal transfection and mitochondrial staining

The primary cultured neurons for the peroxiredoxin (PRDX) studies were transfected at DIV4 with Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions, with pCAGIG constructs expressing both GFP and either PRDX3 wild type (WT) or PRDX3 p.D163E mutant. Upon transfection, mitochondria of transfected primary neuons at DIV5 and DIV12 were stained with MitoTracker® Red CMXRos (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, primary neurons were incubated with MitoTracker® Red CMXRos (100 nM) for 20 min in the dark (37°C, 5% CO_2) and fixed at the corresponding days in each case (DIV5 and DIV12). Immunofluorescence was carried out as described below.

Primary astrocyte culture

Astrocytes were cultured from P5-P7 cerebral cortex of C57BL/6 mice, as previously described (Heinrich et al., 2010). Briefly, after removal of the meninges and white matter, the cortices were dissected and dissociated mechanically with 3 ml of Hank's solution (14175-095; ThermoFisher). Thereafter, cells were centrifuged for 5 min at 300g, re-suspended, and plated in astrocyte medium consisting of DMEM/F12 (21331-020; Gibco), 3.5 mM glucose (15630-080; ThermoFisher), 10% horse serum (26050088, Invitrogen), penicillin/ streptomycin (15140122; ThermoFisher), and supplemented with B27 (17504-04410, Life Technologies), 10 ng/mL epidermal growth factor (EGF, Roche), and 10 ng/mL fibroblast growth factor 2 (FGF2, Roche). The culture flasks were shaken vigorously several times to remove the oligodendrocyte precursor cells.

Cells were confluent (80-90%) in uncoated plastic flasks after 6-7 days. Then, they were passaged by using trypsin/EDTA (25300054; ThermoFisher), and plated onto poly-D-lysine-coated lysine (P2636; Sigma-Aldrich) glass coverslips containing the same medium. Purity of these cultures was confirmed by immunocytochemistry, being the vast majority of the cells (80%) positive for glial fibrillary acidic protein (GFAP) and βIII-tubulin (**Table 2**).

Twenty-four hours after seeding, cells were either transfected with plasmids (data not shown) or infected with retroviral particles at DIV8, as described below.

Astrocytes retroviral transduction for direct neuronal reprogramming

Cells were infected about 24 hours after splitting (DIV7) as described. Because the use of retroviruses requires approval from government authorities and must be performed within a safety level 2 laboratory, retroviral transduction of cultured astroglia was carried out by my supervisor in the LMU (Germany).

For the expression of neurogenic transcription factors, the cDNA of selected genes was subcloned into self-inactivating retroviral vectors containing the actin promoter with the cytomegalovirus enhancer (pCAG), driving the expression of the genes of interest linked to a fluorescent reporter through an internal ribosomal entry site (IRES), as previously described (Heinrich et al., 2010; Masserdotti et al., 2015). Viral vectors were produced with a vesicular stomatitis virus pseudotyping at titers of 10^{6-9}. The Neurog2 and Ascl1 constructs were expressed to reprogrammed astrocytes to glutamatergic and GABAergic neurons, respectively (Heinrich et al., 2010; Masserdotti et al., 2015).

One day after transduction, astrocyte medium was removed and differentiation medium, consisting of DMEM/F12 medium with penicillin/streptomycin and B27, enriched with GlutaMAX (35050038; Fisher Scientific), 30 µM Forskolin (Sigma) and 1 µM Dorsomorphin (Sigma), was added to allow neuronal differentiation and survival. Cells were allowed to differentiate overnight at a CO_2 concentration of 9%. One week after inducing neuronal differentiation, cells were fixed with at DIV4 with 4% PFA in PBS. Immunofluorescence was carried out as follows.

Immunofluorescence

Immunocytochemistry was performed according to a standard protocol previously described (Navarro-González et al., 2019). Neurons were fixed with 4% PFA for 10 min, permeabilized for 10 min with PBS-0.1% Triton, and then blocked with 2% PBS-BSA. Primary and secondary antibodies were diluted in 2% PBS-BSA. The primary antibody incubation was made during 24h at 4ºC and the secondary during 2h at room temperature.

The antibodies used (**Table 2**) were anti-GFP 1/600 (GFP-1020; Aves Labs), anti-RFP 1/1000 (600-401-379; Rockland antibodies), anti-Nrg1 Type III 1/200 (ANR-113; Alomone labs), anti-GAP43 IgY 1/200 (#GAP43; Aves Labs), βIII-tubulin (T8203; Sigma). Secondary antibodies: anti-chicken-488 1/500 (A-11039; ThermoFisher), anti-

mouse-488 1/500 (A-21202; ThermoFisher), anti-rabbit-555 1/500 (A-31572; Life Technologies), anti-chicken-647 1/500 (N0701-AF647-S; NanoTag Biotechnologies). After staining, the coverslips were mounted in Mowiol for imaging.

Fluorescence imaging was performed using an EC PlnN 20x/0.5 DICII objective in a ZEISS observer Z1 with AxioCam MRm and Colibri 7 laser microscope. The Fiji-ImageJ software was used to perform the neurites morphology analysis.

Table 2. **Primary and secondary antibodies information.**

Primary Antibody	Species	Company	Reference	Dilution	
				ICC	IHC
anti-GFP chicken IgY	Chicken	Aves Labs	GFP-1010	1:600	1:500
anti-GFP mouse IgG2a	Mouse	Addgene	#114492	1:20	1:20
anti-RFP	Rabbit	Rockland antibodies	600-401-379	1:1000	1:500
anti-Nrg1 TypeIII	Rabbit	Alomone labs	ANR-113	1:200	-
anti-GAP43 IgY	Chicken	Aves Labs	Cat#: GAP43	1:200	1:300
anti-GFAP	Rabbit	DakoCytomation	Z0334	1:2000	-
anti-βIII tubulin	Mouse	Sigma	T8660	1:500	-

Secondary Antibody	Species	Company	Reference	Dilution	
				ICC	IHC
DAPI	-	Sigma-Aldrich	D9542-10MG	1:2000	1:2000
anti-Mouse IgG Alexa Fluor 488	Donkey	Thermofisher	A-21202	1:500	1:500
anti-Chicken Alexa Fluor 488	Goat	Thermofisher	A-11039	1:500	1:500
anti-Rabbit IgG Alexa Fluor 555	Goat	Life Technologies	A-31572	1:500	1:500
anti-Chicken Alexa Fluor 647	Chicken	Invitrogen	A21449	1:500	1:500

Nes-Cre mice characterization (E15 and P0): RNA extraction and RT-qPCR

To maintain the colony and select the animals, mice were genotyped by performing polymerase chain reactions (PCRs) according to manufacturer's protocol (MB36001-MB36003; Supreme NZYTaq II 2x Green Master Mix, NZYTech) and the product was runned in a 2.5% agarose gel to confirm that NRG1 gene was floxed and the Cre expression was adequate (**Table 3**).

Table 3. **Primer sequences used for mouse genotyping.**

Gene target	Primer name	Sequence
Nrg1	Nco1-s	TCCTTTTGTGTGTGTTCAGCACCGG
	M7-As	GCACCAAGTGGTTGCGATTGTTGCT
Cre locus	Cre 484 gene F	GCATTTCTGGGGATTGCTTA
	Cre 834 gene R	GTCATCCTTAGCGCCGTAAA
Ubiquitin C - Cre locus	UBCCre_25285_Trans_F	GACGTCACCCGTTCTGTTG
	UBCCre_oIMR7338_Intern_F	CTAGGCCACAGAATTGAAAGATCT
	UBCCre_oIMR7339_Intern_R	GTAGGTGGAAATTCTAGCATCATCC
	UBCCre_oIMR9074_Trans_R	AGGCAAATTTTGGTGTACGG

Neuronal cultures were obtained from embryos at E15 as described above. In short, the difference is that embryos are genotyped and cells are plated individually, obtaining 2 wells in 6-wells plates, with 400,000 cells in each one. Neurons were cultured in normoxic conditions and analyzed at DIV4. Neuronal cultures were homogenized with TRIzol (15596018; Fisher Scientific) and RNA was extracted with Direct-zolTM RNA minipreps (R2052; ZIMO) following the manufacturer's instructions.

Newborn mice (P0) were transcardially perfused with ice-cold PBS as described (Fazzari et al., 2014). One hemicortex was used for mRNA extraction as follows. RNA was extracted from the cortex according to the manufacturer's instructions, using TRIzol (15596018; Fisher Scientific) and a FastPrep-24 5G homogenizer (6005500; MP Biomedicals). RNA was quantified by absorbance at 260 nm using a NanoDrop ND-100 (Fisher Scientific).

In both cases, the cDNA was prepared using the High capacity cDNA reverse transcription kit (4368814; Fisher Scientific) and the quantitative PCR was performed with TB Green Premix Ex Taq Tli RNase H Plus 2x (RR420; Takara Bio) for NRG1-EGF, CRD-NRG1, Nrg1-TM, GAP43, RBFOX3 and GAPDH primer pairs, in a LightCyclerR 480 (Roche). The Ct was calculated in the LightCyclerR 480 Software. All values were normalized to the housekeeping gene GAPDH and to the mean value of the control samples.

The primers used for real-time polymerase chain reaction (RT-qPCR) amplification were as described in **Table 4**. The primer pairs used in this study were designed and validated by PrimerBank (https://pga.mgh.harvard.edu/primerbank/) as previously described (Navarro-González et al., 2019; Navarro-Gonzalez et al., 2021), except for

NRG1-EGF and NRG1-CRD primer pairs, that were previously published in Makinodan et al., 2012 (PMID: 22984073).

Table 4. **Primer sequences used to characterize NRG1 and GAP43 expression in Ctrl and Nrg1 KO mice.**

Gene target	Sequence	
NRG1-EGF	Forward	CATCTACATCCACGACTGG
	Reverse	TGAGGGGTTTGACAGGTC
NRG1-CRD	Forward	TCCACAAATACCCACTTTAGGC
	Reverse	CCAGACACCCACACAGAAGATG
NRG1-TM	Forward	AGTGCCCAAATGAGTTTACTGG
	Reverse	AGTTCCTCCGCTTCCATAAATTC
GAP43	Forward	TGGTGTCAAGCCGGAAGATAA
	Reverse	GCTGGTGCATCACCCTTCT
	Forward	AGGAGGAGAAAGACGCTGTA
	Reverse	GGCAACGTGGAAAGCCATT
RBFOX3	Forward	ATCGTAGAGGGACGGAAAATTGA
	Reverse	GTTCCCAGGCTTCTTATTGGTC
GAPDH	Forward	CGTCCCGTAGACAAAATGGT
	Reverse	TCGTTGATGGCAACAATCTC

Protein extraction from neuronal cultures, Western blot and quantification

For Nrg1 signaling studies, protein extraction and Western blot were carried out. Primary cultures of cortical neurons were prepared from E15 of Nrg1 Nes-Cre mice, as described above. The main difference was that these embryos were genotyped and plated individually, obtaining 3 wells in 6-wells plates with 400,000 cells for each one. Neurons were cultured in normoxic conditions and analyzed at DIV5.

The plated neurons were homogenized with RIPA 1x buffer (150 mM NaCl, 1% Nonidet P40, 50 mM Tris, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM PMSF, 0.1 mM Leupeptin, 2 mM Na2VO4, 100 mM NaF, 20 mM Na4P2O7), following the General Protocol for Western blotting (BioRad) as described (Navarro-González et al., 2021). For sample preparation, 15-20 µg of protein was diluted with 4X Laemmli buffer (1610747; BioRad) with 50 mM Dithiothreitol and incubated during 5 min at 90ºC for protein denaturalization. Equal amount of denatured samples were run on 12% acrilamide SDS-PAGE gels. Proteins from gels were transferred onto PVDF membranes (03010040001; Roche). Red Ponceau Staining was performed at RT

during 5 min to ensure the transfection homogeneity. Membranes were incubated with the primary antibodies (**Table 5**) and tubulin-HRP 1:50.000 (AC030; ABclonal), diluted in 1% BSA TBST buffer, at 4ºC overnight. The antibodies were blotted with 1:500 HRP conjugated secondary antibodies and the signal was developed using an ECL chemiluminescence detection kit (NEL105; PerkinElmer Life Sciences).

The signal was detected and imaged with the luminescent image analyzer Uvitec Q9 Alliance and quantified with ImageQuantTL software. The protein levels were normalized to tubulin as loading control.

Table 5. **Antibodies used for Western blotting.**

Primary Antibody	Species	Company	Reference	Dilution WB
anti-GAP43 IgY	Chicken	Aves Labs	Cat#: GAP43	1:5000
anti-Phospho-AKT (S473)	Rabbit	Cell Signaling	1674060P	1:80000
anti-AKT	Rabbit	Cell Signaling	1679272S	1:50000
anti-Phospho-JNK (T183/Y185)	Rabbit	Cell Signaling	9251S	1:10000
anti-JNK	Rabbit	Cell Signaling	9252S	1:2000
anti-Phospho-ERK (p44/42)	Rabbit	Santa Cruz	sc-7383	1:1000
anti-ERK	Rabbit	Santa Cruz	sc-514302	1:1000
anti-Tubulin-HRP	Mouse	ABclonal	AC030	1:50000

Anterograde tracing of the callosal projections and histology

For callosal projections tracing in P0 mice, Nes-Cre animals were perfused with 4% PFA in PBS, and their brains dissected and post-fixed overnight. In Guillermina López-Bendito's laboratory, small DiI (1,1′-Dioctadecyl 3,3,3′,3′-Tetramethylindocarbocyanine perchlorate) (D3911; Invitrogen) and DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine) (D7757; Invitrogen) crystals were inserted into the somatosensory cortices of both hemispheres, under a stereo fluorescence microscope (MZ10 F; Leica), as previously described (Guillamón-Vivancos et al., 2022).

The dye was allowed to diffuse at 37 ºC in PFA solution for two weeks. Vibratome sections (80 µm thick) were obtained and counterstained with the fluorescent nuclear dye DAPI (D9542-10MG; Sigma-Aldrich). Slices were imaged within the next 2 following days, to avoid greater crystal diffusion and therefore, to identify isolated axons.

Fluorescence imaging was performed using a fluorescent microscope Leica DM5000B. The Fiji-ImageJ software was used to perform the analysis.

In utero electroporation and histology

In utero electroporation was performed as described (Gil-Sanz et al., 2013; Mateos-White et al., 2020) in Cristina Gil-Sanz's laboratory. Pregnant mice (C57BL/6J) with E15 pups, were anesthetized with isoflurane and analgesic solution was injected subcutaneously. The abdominal region was shaved and after performing an abdominal incision was performed, exposing the two uterine horns. 0.5-2 µL of the particular endotoxin-free plasmid DNA solution (pCAG-IRES-GFP (PCIG), PCIG + pCAG-Nrg1-FL or PCIG + pCAG-Nrg1-ICD) as described in **Table 6**, was injected into one of the embryos' lateral ventricles. The electroporation conditions involved 5 pulses of 45 V of 80 ms, during 950 ms using forceps electrodes (Platinum Tweezertrode 5 mm Diameter; BTX) and a square wave electroporator (ECM 830 Square Wave Electroporation System; BTX). After surgery, the uterus was returned to the abdominal cavity, and the abdominal wall and skin were sutured. Pregnant females were allowed to give birth.

Two days old mice (postnatal day 2, P2) were transcardially perfused with PBS followed by freshly prepared 4% PFA in PBS as described (Mateos-White et al., 2020). The brains were cut using a vibratome in 100µm sections, ordered in series and processed in floating sections. Primary and secondary antibodies used were diluted in PBS with 0.25% Triton and 4% BSA. Incubation with the primary lasted for 48h at 4ºC, while the secondary antibodies were left overnight at 4ºC, both in agitation conditions. The antibodies and dilution used were as follows: anti-GFP 1/500 (GFP-1010; Aves Labs), anti-RFP 1/300 (600-401-379; Rockland antibody), anti-chicken-488 1/500 (A-11039; Thermo Fisher Scientific) anti-mouse-555 1/500 (A-31570; Invitrogen) (**Table 2**).

Images were taken with a ZEISS observer Z1 with AxioCam MRm and Colibri 7 laser microscope with a Pln 10x/0.25 Ph1 objective. The imaging analysis was carried out with the Fiji-ImageJ software.

Table 6. **Plasmids structure and information for *in utero* electroporation.** DNA concentration to perform *in utero* electroporation for the GOF *in vivo* experiments.

Plasmid	Structure information	DNA concentration (µg)	References
Plasmid for GFP-Ctrl	pCAG-IRES-GFP	0.5 - 2	Gil-Sanz et al., 2013
Plasmid for Nrg1-FL	pCAG-Nrg1-FL-nGFP	0.5 - 2	Fazzari et al., 2014
Plasmid for Nrg1-ICD	pCAG-Nrg1-ICD-GFP	0.5 - 2	Fazzari et al., 2014

Imaging

For all the *in vitro* experiments, a ZEISS observer Z1 microscope equipped with an AxioCam MRm camera and a Colibri 7 laser system was employed. For the axonal length/branching and dendrites Sholl analysis in the *in vitro* GOF and LOF experiments, images were taken with an EC PlnN 20x/0.5 DICII objective, in blind and under same exposure conditions within the same experiment. Approximately 10 photos per coverslip were taken. For the GAP43 puncta density experiments, a FLUAR 40x/1.3 Oil was used to perform 11 slices/0.25 interval Z-stacks of the distal axon (100 µm from the soma). For the GAP43 axonal length rescue experiments, an EC PlnN 20x/0.5 DICII objective was used to perform 5x5 fields, choosing 4 random fields per coverslip to have a whole sample representation.

For the PRDX protein-related analyses, in the intensity and colocalization studies, pictures were taken with a Leica Dmi8 microscope with 20X/0.3 NA Plan-Neof luar or 63X Plan-Apochromat/1.4 NA objectives. For the neurite damage analysis, fields of 3×3 photos were taken with a Zeiss Observer Z1 microscope with an AxioCam MRm and EC PlnN 20x/0.5 DICII objective.

For the astrocyte-to-neuron reprogramming experiments, digital pictures were obtained with an Axio Observer Z1 epifluorescence microscope, equipped with an AxioCam color camera. Retroviral vector-transduced cells were quantified from 3 randomly chosen 20x fields composed of 4x4 images, all under the same exposure conditions.

The images of the anterograde tracing were acquired with a Leica DM5000B microscope with a N PLAN 2.5x/0.07 and HCX PL Fluotar 10x/0.3 Ph1 objectives, within two days after immunofluorescence. To facilitate the analysis, photos were made under two exposure conditions and at two different magnifications: 2.5x for a slice overview and at 10x for a better resolution of the callosal projections. Approximately 4 sections per brain had an optimum dye signal.

For the *in utero* electroporation samples, all sections with fluorescence signal were scanned using a ZEISS observer Z1 with AxioCam MRm and Colibri 7 laser with an A Pln 10x/0.25 Ph1 objective, generating fields of the whole corpus callosum and stitching the images with the ZEISS ZEN Blue Microscopy software. Additionally, we generated,

with the EC PlnN 20x/0.5 DICII objective, 17 slices/1.4 interval Z-stacks for the visualization of the more advanced cortico-cortical projecting axons (top axons).

In all cases, images were processed and analyzed with Fiji-ImageJ and mounted with Inkscape software. GraphPad was used for statistical analysis.

Image analysis

The *in vitro* results performed by primary neuronal cultures, named the Nrg1 gain-of-function and loss-of-function and the GAP43 related experiments, were analyzed using the Fiji-ImageJ software.

For both, the Nrg1 gain-of-function and loss-of-function experiments, images were processed by subtracting background (rolling ball radius: 50 pixels) and setting manually a threshold until soma signal saturation. We used the NeuronJ plugin to track the longest and thinnest neurite, also named the axon, by measuring the length and branch density (number of axonal branches per axonal length). To study dendritic arborization, Sholl analysis was perform by using the Neuroanatomy plugin. The soma was set as the center and we considered a 30 μm start radius, 25 μm step size and 255 μm end radius.

For the GAP43 puncta density analysis, the most focused slice of the Z-stack was manually selected and processed by subtracting background (rolling ball radius: 60 pixels). We used the SynQuant plugin as a first step to normalize the detected puncta signal (400 max particle size and 10 threshold tuning). Then, we manually quantified the puncta within the distal axon (100 μm from the soma) signal. For the GAP43 axonal length rescue experiments, neurons were randomly chosen in each 5x5 field. Then images were processed by subtracting background (rolling ball radius: 25 pixels) and automatically modified until soma signal saturation (**Supplementary Material S3**). We used the NeuronJ plugin to track the length of the longest and thinnest neurite.

For the PRDX protein-related analyses, colocalization and intensity analyses were performed with ImageJ. All the pictures were equally processed and analyzed in three different regions: soma, proximal neurite (0–15 μm from soma) and distal neurite (15–30 μm from soma). The colocalization analysis was performed with the JACoP plugin, considering Pearson's correlation coefficient and Manders' M1 and M2 coefficients. For neurite damage analysis in mature neurons, the status of the biggest neurite was analyzed

in each neuron in a radius of 150 μm from the soma and classified as an intact neurite or a neurite with swelling or degeneration.

To analyze the effect of Nrg1 in the astrocyte-to-neuron reprogramming, we used the ImageJ software. First, we evaluated the reprogramming efficiency, by setting an equal threshold (**Supplementary Material S4**) and analyzing all the images in blind. All retroviral vector-transduced cells were quantified from 3 randomly chosen 4x4 20x fields per coverslip. We considered the efficiency by doing a percentage among the βIII-tubulin positive cells with neuronal morphology and the DsRed+GFP double infected cells. For the morphology analysis of the reprogrammed neurons, images were downsized for feasibility. They were processed by subtracting background (rolling ball radius: 50 pixels) and automatically modified and normalized until soma signal saturation (**Supplementary Material S4**). Axonal length quantification was performed using the plugin NeuronJ. Branch quantification was manually performed with the multipoint tool, considering all the projections from the soma.

For the *in vivo* analysis of the dye tracing of callosal axons in the Nrg1 KO mice model, we used the Fiji-ImageJ software. All the analysis was carried out in duplicate due the crystal insertion in the two somatosensory cortices of each brain. We considered the axonal front within the corpus callosum as the most advance bundle of non-individualize axons. We measured the distance between the midline, as an external non-variable reference, to the front. As a control for the intrinsic variability of the technique, we also measured the distance within the insertion zone and the front (data not shown). Additionally, the corpus callosum thickness was measured to assess the possibility of major impairments at earlier developmental stages. As another variability control, we complementarily analyzed the area of the dye insertion zone and its correlation with the observed phenotype.

For the *in vivo* analysis of our Nrg1 gain-of-function model, we analyzed the *in utero* electroporation images by using the Fiji-ImageJ software. We measured the distance between the electroporation zone and the top 10 more advance axons in the corpus callosum. This parameter allowed to reduce some of the variability intrinsic to the technique. To confirm the results, we measured the electroporation area and we did correlation analysis with our phenotype (data not shown).

In all the *in vivo* experiments, we classified the sections by using the DAPI staining and the Allen Brain Atlas: Developing Mouse Brain P1 (**Supplementary Material S5**). We compared only analogous regions of different brains.

Statistical analysis

All statistics were performed with GraphPad Prism software. All bar graphs show mean ± SEM. Significance is indicated with asterisks in each graph, being a p-value > 0.05 considered as not significant (ns). The statistical test used for each analysis is mentioned in all figure legends.

For the Nrg1 loss-of-function *in vitro*, named the axonal length and branching analysis, and the GAP43 puncta density studies, an unpaired t-test was used in all the cases. Welch's correction was additionally applied for the GAP43 puncta results. Conversely, for the Nrg1 gain-of-function *in vitro*, one-way ANOVA followed by a Tukey's multiple comparison test was used to compare Ctrl Vs Nrg1-FL and Nrg1-ICD overexpression. Also, for the GAP43 axonal length rescue experiments, a one-way ANOVA followed by a Tukey's multiple comparison test was used to compare Ctrl Vs Nrg1 KO and GAP43 expressing Nrg1 KO neurons. Individual neurons were considered as the sample size.

To analyze the dendritic arborization with a Sholl analysis, a mixed-effects analysis with Sidak's multiple comparisons test was performed for the *in vitro* loss-of-function experiments (Wilson et al., 2017). Conversely, a two-way ANOVA with a Tukey's post hoc was used for the *in vitro* gain-of-function experiments. Individual neurons were considered as the sample size.

To analyze the PRDX colocalization, two-way ANOVA was performed to compare the overlap coefficient of the PRDX WT and PRDX3 p.D163E mutant, within each position (soma, proximal and distal dendrites). Regarding the neurite integrity study, a Fisher's exact test was employed to analyze the differences in healthy or damaged neuronal morphology between control neurons, PRDX WT and PRDX3 p.D163E.

Regarding the astrocyte-to-neuron reprogramming experiments, two-way ANOVA was performed to compare the Ctrl, Nrg1-FL and Nrg1-ICD within each condition,

meaning the expression of the control plasmid only containing DsRed, DsRed+Neurog2 or DsRed+Ascl1.

For the protein expression analysis, we used an unpaired t-test with Welch's correction, to compare the expression in both Ctrl and Nrg1 KO mice. Independent animals were considered as the N.

For the loss-of-function *in vivo*, we used unpaired t-test in the dye-tracing samples and in the corpus callosum thickness analysis, being compared Ctrl and Nrg1 KO mice and taking as the N the different brain sections. Additionally, a Pearson correlation coefficient was computed to assess the relation between Nrg1 KO and control neurons.

Finally, for the gain-of-function *in vivo*, we carried out normality tests in the *in utero* electroporation samples, both for Ctrl vs Nrg1-FL and Ctrl vs Nrg1-ICD. As the Ctrl vs Nrg1-FL group did not pass the normality test, we compared the conditions by using a Kolmogórov-Smirnov test. On the opposite, due positive normality test, we used an unpaired t-test to compare the Ctrl vs Nrg1-ICD conditions. The individual axons were considered as N.

To perform the results representation, all the figures were designed using the open-source Inkscape software.

CHAPTER 1: PRIMARY NEURONAL CULTURES FOR NEUROPATHOLOGIES STUDIES

Chapter 1 highlights:

- We developed a robust *in vitro* model based on primary neuronal cultures that can be employed for studying diverse neuropathologies.
- We optimized the primary neuronal cultures by transfecting the neurons using electroporation, allowing us to have internal controls that reduce the experimental variability.
- The developed model is suitable to study neuronal morphology, integrity and survival in a simple manner thanks to the sparse labeling.
- We used primary neuronal cultures in a real case study, determining the role of the p.D163E PRDX3 mutation in developing neurons.

Publications related to Chapter 1:

1. **Rodríguez-Prieto, Á**., González-Manteiga, A., Domínguez-Canterla, Y., Navarro-González, C., & Fazzari, P. (2021). A Scalable Method to Study Neuronal Survival in Primary Neuronal Culture with Single-cell and Real-Time Resolution. Journal of visualized experiments: JoVE, (173), 10.3791/62759. https://doi.org/10.3791/62759

2. Martínez-Rubio, D., **Rodríguez-Prieto, Á**., Sancho, P., Navarro-González, C., Gorría-Redondo, N., Miquel-Leal, J., Marco-Marín, C., Jenkins, A., Soriano-Navarro, M., Hernández, A., Pérez-Dueñas, B., Fazzari, P., Aguilera-Albesa, S., & Espinós, C. (2022). Protein misfolding and clearance in the pathogenesis of a new infantile onset ataxia caused by mutations in PRDX3. Human molecular genetics, 31(22), 3897–3913. https://doi.org/10.1093/hmg/ddac146

Introduction

Primary neuronal cultures are a widely used model for studying different aspects in neuroscience research. These cultures involve isolating neurons from the mouse brains and growing them in a controlled environment, as described in the Materials and Methods section.

One of the key benefits of utilizing primary neuron cultures is their capacity to closely replicate the *in vivo* environment but in a more reductionist manner. This results in a more accurate representation of neuronal functions and disease mechanisms compared to immortalized cell lines. Primary neuronal cultures offer a cost-effective approach where experimental conditions can be precisely controlled and simplified. This allows to acquire a preliminary knowledge more quickly and with fewer ethical concerns compared to *in vivo* assays (Holloway, P. M., & Gavins, 2016; Rodríguez-Prieto et al., 2021; Tasca et al., 2015).

Primary neuronal cultures are valuable for investigating the mechanisms underlying various neuropathologies, including schizophrenia, Alzheimer's disease, Parkinson's disease and stroke. Additionally, they serve as a valuable platform for testing potential therapeutic compounds, allowing initial screenings before transitioning to *in vivo* studies.

Primary neuronal cultures: internal controls and sparse labeling

Here, we developed a robust *in vitro* model based on primary neuronal cultures, that can be employed for studying diverse aspects of neuronal development and survival in the context of a specific neuropathology.

The study of neuronal disorders requires experimental models that are amenable to genetic, molecular and cellular analyses. Primary cortical neurons are a very potent model for studying neuronal development and survival. Under the appropriate conditions, primary neurons will progressively develop their neurites, stablish synaptic contacts and present hallmarks of mature neurons (Fazzari et al., 2014; Kaech et al., 2006; Navarro-González et al., 2019). Therefore, this model is more reliable than immortalized cell lines in modeling the physiology of the neurons and more prone to manipulations than animal models (Rodríguez-Prieto et al., 2021).

The study of neuronal disorders requires experimental models that are amenable to genetic manipulation, and molecular and cellular analyses. Primary cortical neurons are

difficult to transfect and relatively fragile. Liposome-mediated transfection, via the Lipofectmine 2000 reagent, and electroporation are two commonly used techniques for neuronal transfection. Transfection with Lipofectamine involves a high density of transfected cells but often result in a high toxicity. Moreover, the intricate morphology of cortical neurons may complicate the single-cell resolution in high-density cultures.

Conversely, the neuron transfection by electroporation provides a flexible and inexpensive procedure, that allows the study of the influence of different genes and proteins with a reduced toxicity (Rodríguez-Prieto et al., 2021; J. Shi et al., 2018). Moreover, this method can be easily adapted to obtain an optimal transfection efficiency and density of transfected neurons, mixing electroporated with non-electroporated cells. The co-culture with naïve (non-electroporated) neurons allows to have a higher neuronal survival and robustness, besides a lower experimental variability (**Figure 8)** (Rodríguez-Prieto et al., 2021). In addition, neuronal transfection by electroporation enables sparse labeling to obtain single-cell resolution when needed. This facilitates both neurite analysis and the ability to automate image analysis. This approach can also be adapted to study multiple genes of interest by co-culturing neurons co-electroporated with different cytosolic fluorescent markers. Remarkably, the green fluorescent protein (GFP) may be co-transfected with specific markers to visualize neurites morphology along with different cellular structures (**Figure 8**) (Rodríguez-Prieto et al., 2021).

In conclusion, we developed a versatile, affordable and effective *in vitro* model utilizing primary neuronal cultures for studying neuronal morphology and survival.

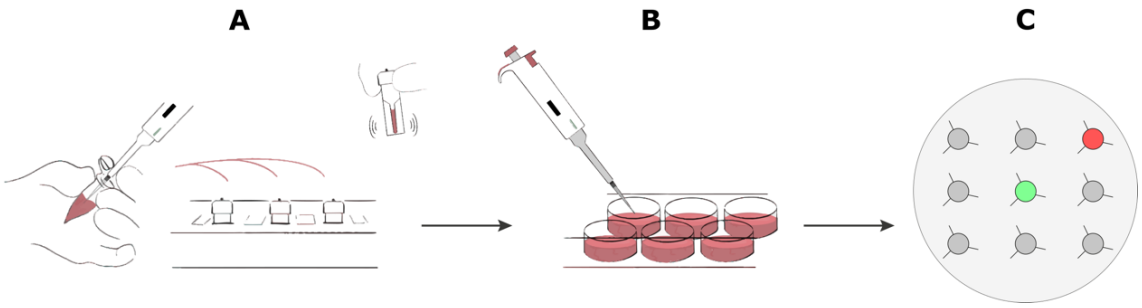


Figure 8. **Primary neuronal cultures as a suitable *in vitro* model.** After brain dissection both brain cortices are disaggregated. (A) For electroporation, the cells are mixed with DNA to undergo an electric pulse. (B) (C) The mix of naïve and electroporated cells enable to have internal controls and a flexible sparse labeling.

Primary neuronal cultures to study neuronal survival

Neuronal loss is at the core of many neuropathologies, including stroke. Different methods were developed to study the process of neuronal survival upon cytotoxic stress. In the field of stroke research, ischemic conditions can be simulated by either oxygen/glucose deprivation (OGD) or metabolic inhibition using different chemical agents. The OGD is a well stablish neurotoxic model commonly employed to study neuronal survival and the role of specific proteins under hypoxic conditions *in vitro*. This approach entails exposing the cultured primary neurons to a salt buffer similar to an artificial cerebrospinal fluid but deprived of oxygen and glucose. It challenges the neurons with multiple hits: absence of glucose and oxygen and lack of growth factors, vitamins, anti-oxidants, hormones, and other important components that are typically present in the neuronal culture medium and supplement (Navarro-González et al., 2019; Rodríguez-Prieto et al., 2021; Tasca et al., 2015).

We showed that our primary cortical neurons *in vitro* model is suitable for OGD neurotoxicity assay, taking advantage of sparse labeling of the neurons and the consequent possibility of automating quantification (Rodríguez-Prieto et al., 2021).

Mutations in PRDX3 cause an infantile onset ataxia: a case study as an example of applicability

Employing primary neuronal cultures for an *in vitro* approach, we studied the neuronal morphology and integrity in a new case study of infantile onset ataxia (Martínez-Rubio et al., 2022).

The brain is particularly vulnerable to oxidative stress. Elevated reactive oxygen species (ROS) levels are a common factor in the etiology of various neurodegenerative diseases, such as Alzheimer's and Parkinson's (Barnham et al., 2004). In mitochondria, the thioredoxin (TXN) and glutathione (GSH) systems are essential for maintaining ROS levels and preventing oxidative damage. The TXN system includes the peroxiredoxin 3 (PRDX3). Thus, PRDX3 is an antioxidant protein that acts by reducing hydrogen peroxide (H_2O_2) produced in the mitochondrial respiration, being essential for protecting cells from oxidative stress by controlling the cellular redox homeostasis (Cox et al., 2010; Martínez-Rubio et al., 2022).

PRDX3 has been previously implicated in various disorders, particularly those associated with oxidative stress. Mutations in the *PRDX3* gene have also been previously

linked to cerebellar ataxia. In our study, we investigated the molecular cause of an ataxia with infantile onset. Whole exome sequencing identified a novel mutation (p.D163E) that disrupts the function of the mitochondrial ROS defense system. Mitochondrial computational analysis indicated that the p.D163E PRDX3 mutation leads to significant mitochondrial alterations. Biochemical analysis suggested that this mutation results in an unstable protein structure prone to aggregation, which triggers unfolded protein responses in both mitochondria and endoplasmic reticulum. This mitochondrial dysfunction likely contributes to oxidative stress, ultimately leading to cerebellar neurodegeneration. To characterize the effect of this mutation on developing cortical neurons, we employed our primary cortical neuron *in vitro* experimental paradigm (Martínez-Rubio et al., 2022).

Results

Mutations in PRDX3 cause an infantile onset ataxia: a case study as an example of applicability

To characterize the pathogenesis of a new case of infantile-onset ataxia, we investigated the effects of the PRDX3 p.D163E mutation in developing and mature cultured neurons. To this aim, primary cultures of cortical neurons were isolated from embryonic day 15-16 mice, as detailed in the Materials and Methods section.

We investigated both developing neurons, at 5 days *in vitro* (DIV5), and more mature neurons at DIV12, after transfecting in both cases the neurons at DIV4 with a construct expressing both GFP and either PRDX3 wild type (WT) or p.D163E (**Figure 9, A and B**). While the WT-PRDX3 was expressed both in the soma and along the neurites, PRDX3 p.D163E expression was decreased (**Figure 9, C, upper graph**) and mainly found in the soma (**Figure 9, A and C, bottom graph**). Additionally, WT-PRDX3 and PRDX3 p.D163E were mainly located in mitochondria as revealed by co-labeling with MitoTracker (**Figure 9, D**).

To further investigate the role of the prolonged PRDX3 p.D163E expression in neurons, we examined the morphology of more mature primary cortical neurons at DIV12 Notably, while the morphology of neurons expressing WT-PRDX3 was not distinguishable from control neurons expressing GFP, neurons expressing mutant PRDX3 often displayed different degrees of neurite swelling and impaired morphology (**Figure 9, E and F**), a hallmark of neuronal degeneration.

In conclusion, our findings in Martínez-Rubio et al., 2022 demonstrated that exogenous expression of PRDX3 p.D163E was reduced and triggered alterations in neurite morphology and in mitochondria. These results support the expanding clinical spectrum of PRDX3-associated neurodegeneration and provide new insights into the pathological mechanisms underlying this novel form of cerebellar ataxia.

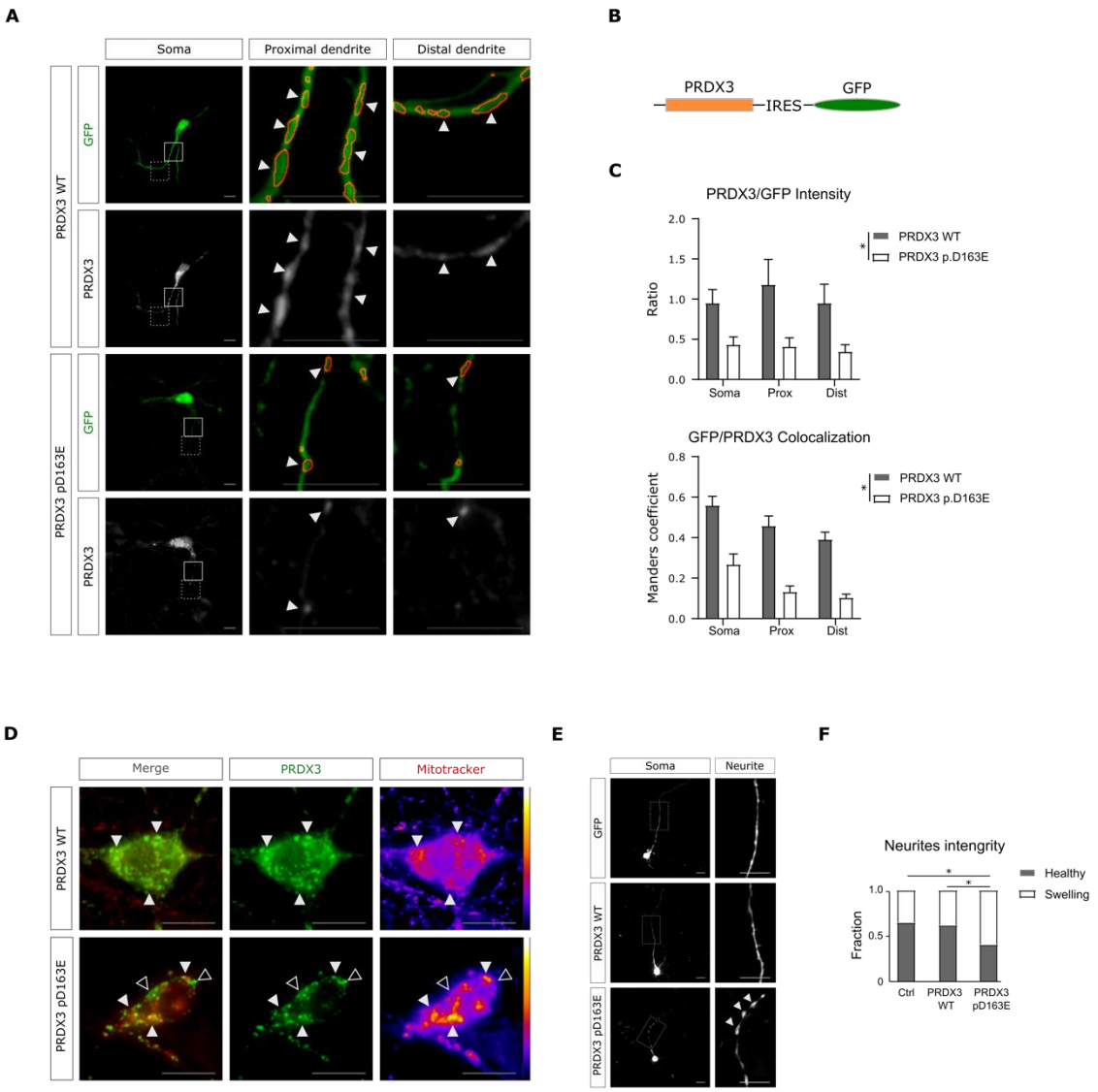


Figure 9. **PRDX3 p.D163E expression in primary neurons.** (A) Representative pictures of primary cortical neurons expressing at DIV5 either WT-PRDX3 or PRDX3 p.D163E. Boxed area shows the regions magnified and indicated as proximal and distal (solid and dashed lines, respectively). Arrowheads and red lines show the GFP positive regions that colocalized with PRDX3 protein in the neurites. Scale bar, 10 µm. (B) Schematic representation of the vector for the co-expression of PRDX3 and GFP linked by an internal ribosome entry site (IRES). (C) Upper graphs show either the ratio of the intensity of GFP and PRDX3 labeling. n = 20 control or mutant neurons. Two-way ANOVA test, * $p < 0.05$. Average ± SEM. Lower graph shows the colocalization expressed as Manders' Overlap Coefficient of PRDX3 over GFP labeling, overlapping areas are lined in red, n ≥ 15 control or mutant neurons. Two- way ANOVA test, * $p < 0.05$. Average ± SEM. (D) Representative pictures of primary neurons at DIV5 expressing either PRDX3 WT or p.D163E mutant co-labeled with MitoTracker. Full arrowheads indicate regions of co-localization. Scale bar, 10 µm. (E) Representative neurons at DIV12 expressing

either PRDX3 WT or PRDX3 p.D163E mutant labeled with GFP. The box indicates the area of the neurite magnified or the right panels. Arrowheads indicate axonal swellings. Scale bar, 10 μm. (F) Quantification of neurite integrity based on the neuronal morphology. Ctrl, n = 50; PRDX3 WT, n = 109; PRDX3 mutant, n = 109. Fisher's exact test, * $p < 0.05$.

Discussion

Primary neuronal cultures have become a vital tool in neuroscience. They offer a simplified, yet physiologically relevant model for studying neuronal development, function and survival in the context of diverse neuropathologies.

There are several advantages to using primary neuronal cultures. They are a cost-effective approach and raises fewer ethical concerns compared to *in vivo* studies. Additionally, the ability to manipulate the *in vitro* environment allows to model specific neuropathologies, like oxygen-glucose deprivation to mimic stroke conditions. While primary neuronal cultures offer significant advantages, they also have limitations, as they lack the complex 3D structure and intricate cell-cell interactions found in the intact nervous system. Additionally, neuronal transfection implies different levels of neurotoxicity and studies requiring single-cell resolution are often difficult to conduct (Holloway, P. M., & Gavins, 2016; Rodríguez-Prieto et al., 2021; Tasca et al., 2015).

To address these limitations, we have developed a novel experimental paradigm for primary neuronal cultures based on electroporation-mediated transfection. This method takes advantage of mixing both electroporated and naïve cultured neurons. This way, it allows a feasible regulation of cell density and, thereby, promotes an increase in neuronal survival. Additionally, this approach facilitates sparse labeling, enabling the distinction of individual cells, neurite morphology analysis and the application of automated imaging when needed (Rodríguez-Prieto et al., 2021).

Primary neuronal cultures in this study provided significant insights into the pathogenesis of a newly identified infantile-onset ataxia caused by the p.D163E mutation in the *PRDX3* gene. Our results highlight that the p.D163E mutant PRDX3 protein shows a reduced expression and altered distribution in the neuron, as well as it triggers morphological changes in neurite morphology and in mitochondria, as hallmarks of neuronal degeneration. Thus, our results underscore morphological consequences of PRDX mutations and its relevant role in maintaining mitochondrial function and cellular redox balance (Martínez-Rubio et al., 2022).

Further research is needed to elucidate the detailed molecular pathways by which PRDX3 dysfunction leads to neuronal cell death. Understanding the broader implications of PRDX3 mutations on mitochondrial diseases could also provide insights into other neurodegenerative disorders with similar pathological features. Future research should

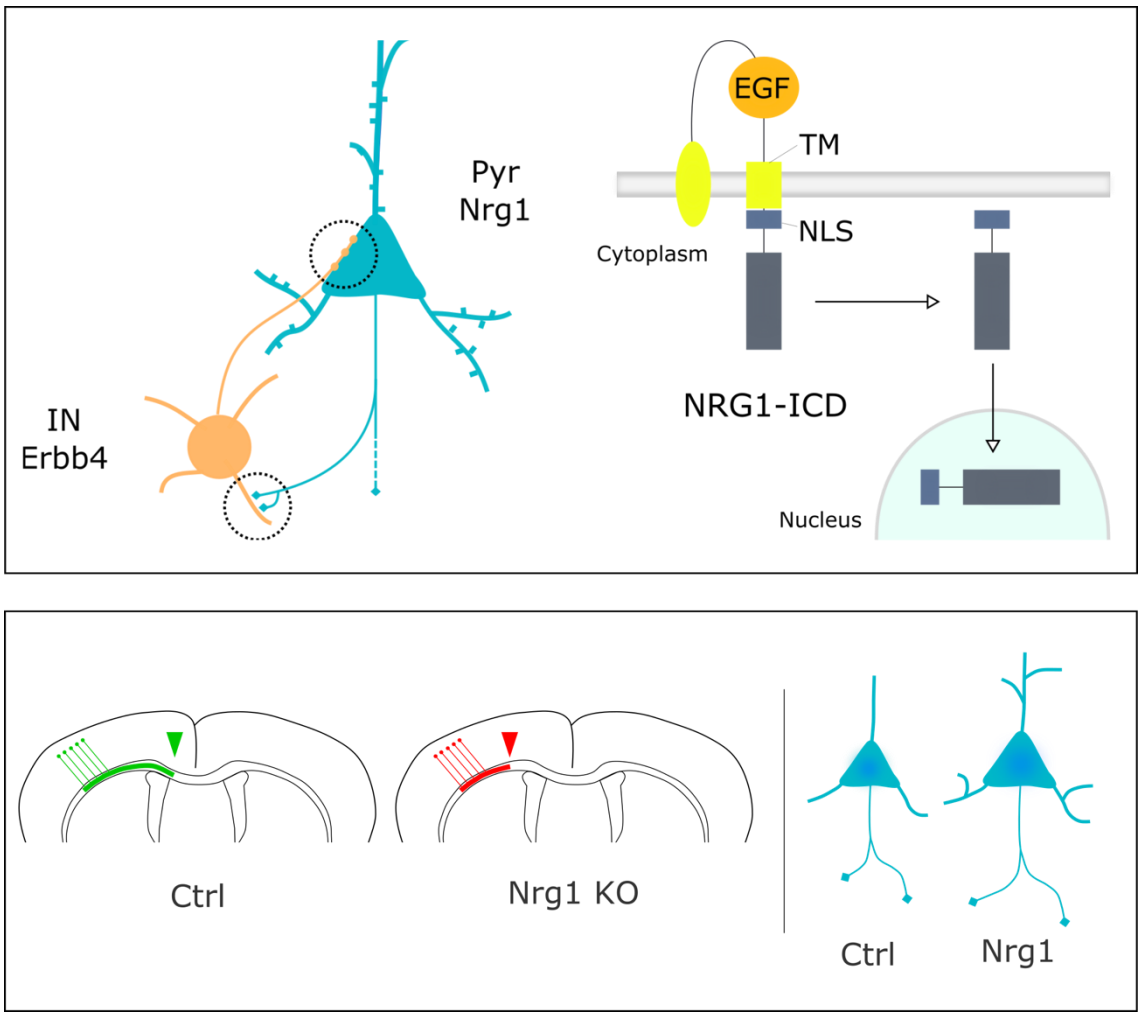
focus on developing *in vivo* models to validate these findings and explore potential therapeutic strategies.

In conclusion, primary neuronal cultures are a versatile and powerful tool for neuroscience research. Their cost-effectiveness, experimental control and closer but simpler representation of the *in vivo* environment make them valuable for studying neuronal function, disease mechanisms and potential therapeutic strategies. Our contribution to the understanding of the pathogenesis of a new case of infantile-onset cerebellar ataxia, caused by the PRDX3 p.D163E mutation, demonstrate the robustness and applicability of primary neuronal cultures in studying complex neurological diseases.

This experimental paradigm of primary neuronal cultures and transfection by electroporation, with internal controls and sparse labeling, has allowed us to specifically and rigorously investigate the role of Nrg1 in neurite development and, in particular, its cell autonomous effect. These results are shown in the next chapter.

CHAPTER 2: NRG1 INTRACELLULAR SIGNALING REGULATES THE DEVELOPMENT OF INTERHEMISPHERIC CALLOSAL CONNECTIONS

Chapter 2 graphical abstract:



Chapter 2 highlights:

- Nrg1 expression is required for the proper development of callosal axons *in vivo*.
- Nrg1 signaling is required and, specifically, the activation of its intracellular domain, is sufficient to promote axonal development *in vitro*.
- Nrg1 signaling promotes in a cell autonomous manner the development of long-range cortico-cortical connections between brain hemispheres *in vivo*.

Publication related to Chapter 2:

1. **Rodríguez-Prieto, Á.,** Mateos-White, I., Aníbal-Martínez, M., Navarro-González, C., Gil-Sanz, C., Domínguez-Canterla, Y., González-Manteiga, A., Del Buey Furió, V., López-Bendito, G., Fazzari, P. (2024). Nrg1 intracellular signaling regulates the development of interhemispheric callosal axons in mice. Life Science Alliance. https://doi.org/10.26508/lsa.202302250

Introduction

Schizophrenia and Nrg1

SZ is a neurodevelopmental disorder that affects cognitive processes and social behavior (Harrison et al., 2005; Lewis et al., 2009). Unlike other neuropathologies, the brains of SZ patients do not display obvious histological hallmarks. The most consistent endophenotypes in SZ include reduced neuropil, impaired functional connectivity between cortical areas and specific changes in synaptic connections (Arat et al., 2015; Fenlon et al., 2015; Hoptman et al., 2012; Innocenti et al., 2003; Kubicki et al., 2005; Mohr et al., 2000). Therefore, SZ is considered a pathology of abnormal wiring of cortical neurons. (Bjarnadottir et al., 2007; Harrison et al., 2005; Lewis et al., 2009; Lisman et al., 2008).

While the developmental etiology of SZ remains largely unresolved, it is well established that SZ has a strong genetic component (Harrison et al., 2005; Stefansson et al., 2002). The schizophrenia risk-gene *NRG1* is expressed in excitatory pyramidal neurons, whereas its specific receptor *ERBB4* is mainly found in inhibitory interneurons. Several studies demonstrated that Nrg1/Erbb4 signaling plays an important role in the cortex, and specifically in the wiring of inhibitory cortical neurons that express the Nrg1 receptor Erbb4 (Bjarnadottir et al., 2007; Y.-J. Chen et al., 2010; Fazzari et al., 2010, 2014; B. Li et al., 2007; Mei et al., 2008; Navarro-Gonzalez et al., 2021; Pedrique et al., 2010; Rahman-Enyart et al., 2020).

The vast majority of previous studies have focused on the role of Nrg1/Erbb4 signaling in interneurons. Conversely, the role of Nrg1 in excitatory neurons and the function of its reverse intracellular signaling are poorly studied and understood. The physiological mechanisms leading to the activation of Nrg1 intracellular signaling are unclear but *in vitro* studies have shown that it can be triggered by multiple stimuli, including binding to the ErbB4 receptor, neuronal depolarization and hypoxia (Bao et al., 2003; Mei et al., 2008; Navarro-González et al., 2019). Only a few studies suggested that Nrg1 loss-of-function may impair dendritic development in the excitatory neurons (Y. Chen et al., 2010; Z. Zhang et al., 2017). In particular, primary cortical neurons from type III Nrg1 mutant mice showed impaired dendrite development at early stages of maturation. Additionally, the Nrg1 intracellular signaling could partially rescue the developmental deficits in dendrites (Y. Chen et al., 2010).

Nonetheless, the Nrg1 effect on axonal development in pyramidal neurons remains unknown. Herein, in this study we investigated its role in excitatory neuron axonal development, focusing specifically on its intracellular signaling pathway. For this purpose, we employed the robust *in vitro* model detailed in the previous section, which leverages primary neuronal cultures and cellular transfection via electroporation. We employed this *in vitro* model because of its reproducibility and ease of neuronal morphology analysis, as previously described.

The corpus callosum is the main interhemispheric connection in the brain

One of the most consistent neurobiological findings in schizophrenia is the presence of WM abnormalities. WM is solely composed of axons and connects different brain regions. This is essential for efficient communication and synchronization between distant brain areas. Schizophrenia patients have been shown a WM reduction, which is thought to contribute to altered connectivity (Kubicki et al., 2005).

The CC is the largest WM structure in the brain, forming a bundle of cortico-cortical nerve fibers that connects the two cortical hemispheres. This structure is susceptible to structural defects during development, which often result in significant neuropsychological dysfunction. The development of precise contralateral connections is critical for most aspects of cortical function including basic brain activities and higher cortical functions. Indeed, the interhemispheric integration of brain function largely depends on the wiring of the callosal axons. Given its major functional relevance and complex development, it is not surprising that CC deficits were previously associated with neurodevelopmental disorders (Fenlon et al., 2015; Hoptman et al., 2012; Innocenti et al., 2003; Kubicki et al., 2005).

Converging evidence from morphological and electrophysiological studies conducted over the past two decades implicates abnormal callosal connections in schizophrenia. Specifically, morphological studies indicate an hypoconnectivity in the interhemispheric communication due a reduction of the CC (Hoptman et al., 2012; Innocenti et al., 2003; Kubicki et al., 2005). This suggests that a reduced development of CC would directly impair interhemispheric communication and information processing. However, the cellular and molecular mechanisms underlying the alterations of CC development in SZ remain largely undetermined.

Because of its previous association with SZ, we studied the role of Nrg1 in the development of callosal connections. Because the CC is the largest white matter structure in the brain, its study enabled a specific examination of the role of Nrg1 in axonal development. To this aim, we traced the callosal projecting neurons by *in utero* electroporation and anterograde tracing dye, in wild-type and Nrg1 knockout mice, respectively.

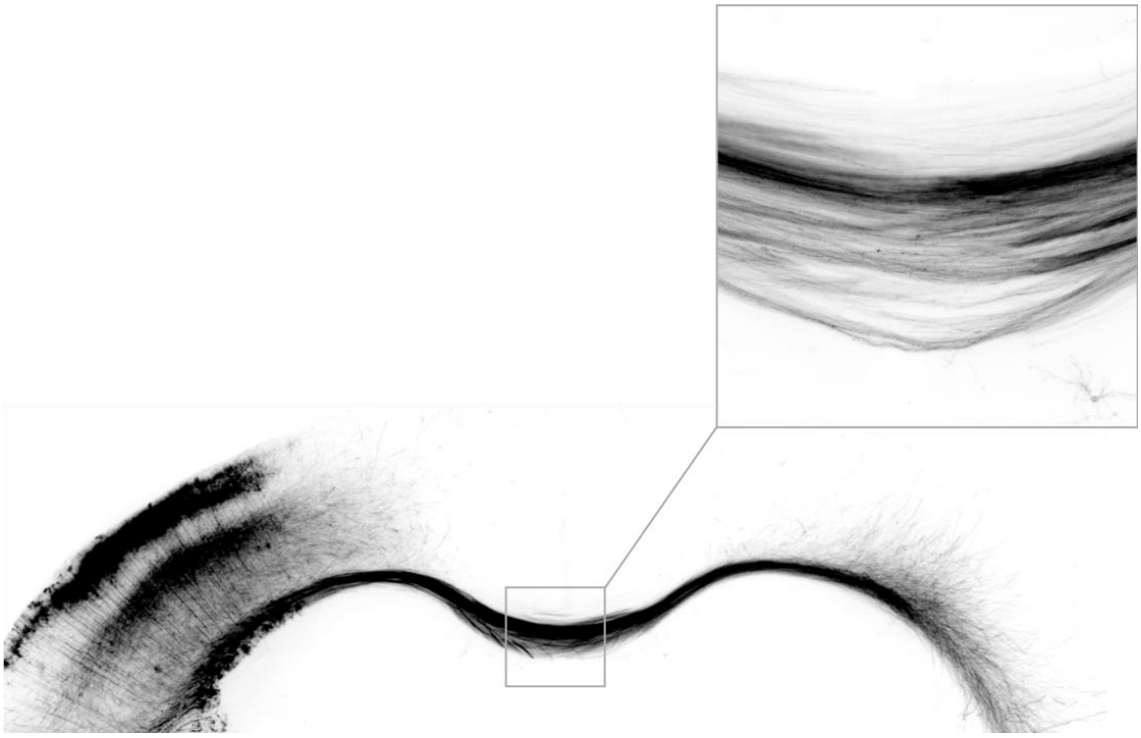


Figure 10. **Axons projecting through the corpus callosum.** Coronal section of a mouse brain at postnatal day 7 (P7). *In utero* electroporation was performed at embryonic day 15 (E15) for specific labeling of neurons in the cortical layers II and III, which are those that mainly projects through the corpus callosum. In the zoomed region, isolated axons crossing the midline.

Results

Nrg1 is required for the development of cortico-cortical projecting axons *in vivo*

The CC axons form the major interhemispheric connection. Its development is critical for cortical wiring and interhemispheric connectivity is reduced in SZ (David, 1994; Fenlon et al., 2015). Here, we tested the hypothesis that the SZ-risk gene Nrg1 may control the development of interhemispheric cortical axons in the mouse. To investigate the development of callosal axons, we generated a constitutive Nrg1 mutant mice, by expressing Cre recombinase under the Nestin promoter (**Figure 11, A**). We confirmed deletion of the *NRG1* gene by using RT-qPCR in E15 embryos (**Figure 11, B**) and newborn pups (P0) (data not shown). Once the model was characterized, we performed tracing experiments of callosal connections in P0 Nrg1 KO mice by labeling them with dye crystals (**Figure 11, C**) (López-Bendito et al., 2006).

To trace cortico-cortical callosal axons, we placed, in collaboration with López-Bendito's lab, small DiI and DiD crystals on the putative somatosensory cortices of control and Nrg1 KO brains (**Figure 11, C**). The dye was let to diffuse for two weeks. After sectioning the brains, the slices were imaged and classified on a rostro-caudal axis, using as reference the Allen Brain Atlas of Developing Mouse Brain at P1 (**Figure 11, D**).

Consistent with previous studies (López-Bendito et al., 2007; Mizuno et al., 2007; Wang et al., 2007), dye tracing in control mice at P0, showed that labeled somatosensory callosal axons were approaching the midline at P0 (**Figure 11, E**). Notably, we found that in Nrg1 KO brains the development of callosal axons is reduced as compared to control littermates (**Figure 11, E**). Specifically, by measuring the distance between the midline and the axon front (**Figure 11, E and Figure 12, A**), we observed that in Nrg1 KO mice the developing callosal axons were more distant from the midline in comparison to control axons (**Figure 11, E and F**). We reasoned that this phenotype may be due to a reduced growth of callosal axons in Nrg1 KO cortices.

To rule out that this observation might be biased by a difference in the size or diffusion of the dye crystals, we measured the diffusion area of the dyes in the cortex (**Figure 12, A and B**). We did not observe a relevant correlation between the cortical area labeled by the dye crystals and the distance of the axons from the midline (**Figure 12, B**). Notably, at the rostro-caudal level that we traced with dye crystals, we found that the

bundle of somatosensory callosal axons runs perpendicular to the anterior-posterior axis in both control and Nrg1 KO cortices. This observation suggests that the rostro-caudal orientation of somatosensory callosal axons is not overtly impaired in Nrg1 deficient brains (data not shown). Moreover, to exclude the possibility that the deficit in axon development was secondary to major impairments in earlier steps of callosal formation, we measured the thickness of the CC tract both in newborn and adult mice. We did not find obvious differences in the thickness of Nrg1 KO callosal structure compared to control littermates nor other major histological abnormalities (**Figure 12, C and D**).

Altogether, these results suggested that Nrg1 expression is required for proper development of callosal axons *in vivo*.

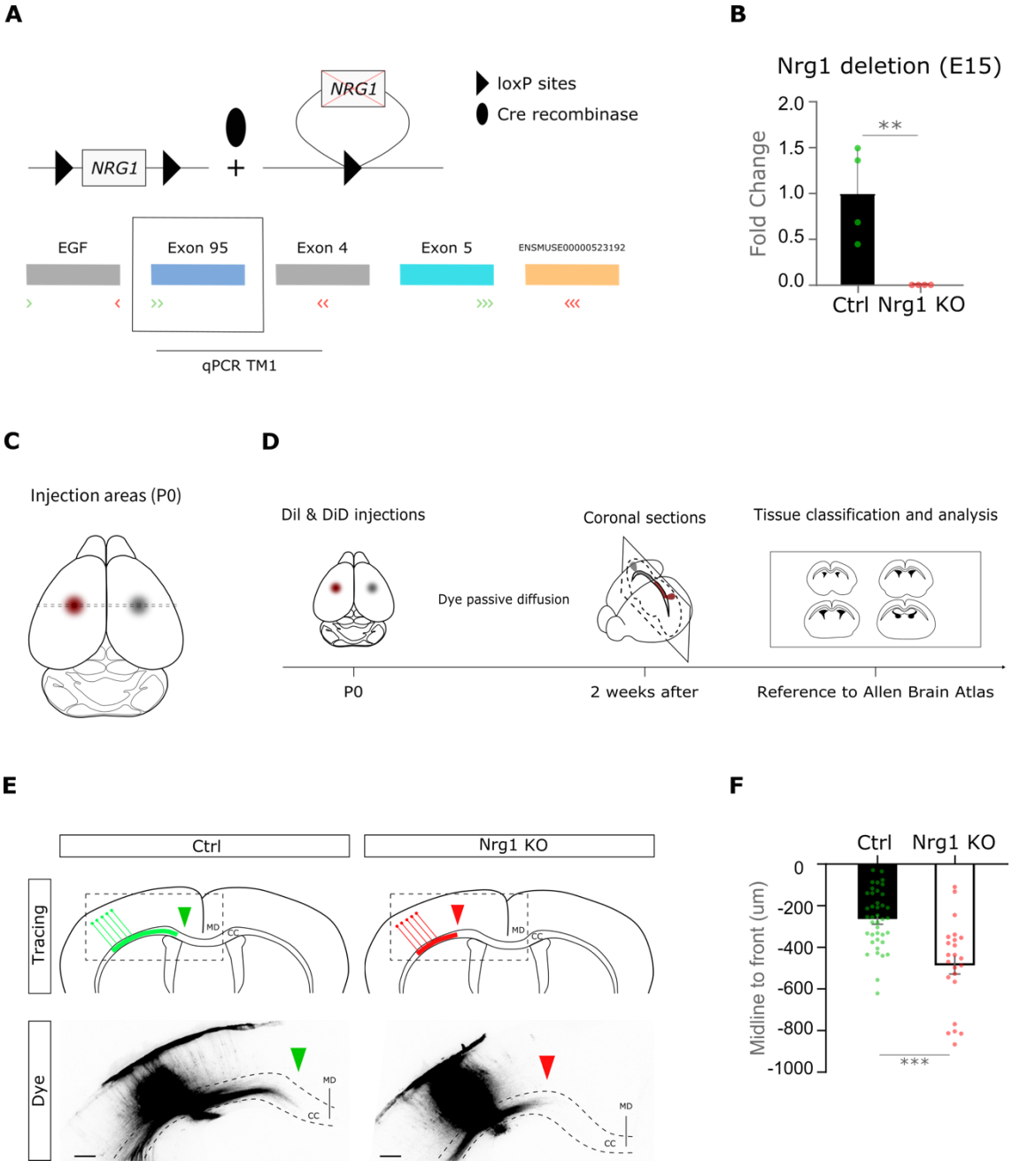


Figure 11. **Nrg1 regulates the development of callosal axons.** (A) We generated a constitutive Nrg1 mutant mice. Nrg1 deletion was driven by the expression of the Cre recombinase under a Nestin promoter. (B) Characterization of Nrg1 loss-of-function model *in vivo*. The graph shows the mRNA levels of Nrg1 in Nrg1 KO relative to control littermates at E15, quantified by qPCR. Ctrl = 3 and Nrg1 KO = 2, littermates. Unpaired t-test, *** $p < 0.001$. Average ± SEM. (C) Schema summarizing the experimental paradigm. In Nrg1 KO newborn mice, were carried out injections of Dil and DiD dye crystals (represented in red and gray, respectively) in both somatosensory cortices. The dashed lines indicate the rostro-caudal level. (D) Schema of the experimental workflow. We performed two crystal dye injections (Dil and DiD, in the somatosensory cortices of both hemispheres of newborn Nrg1 KO mice. We let the dye to diffuse for two weeks. After sectioning the brains, the slices were pictured and classified on a rostro-caudal axis. We took as reference the Allen Brain Atlas of Developing Mouse Brain at P1. (E) Schematic representation and images of cortico-cortical projecting axons, both in Ctrl and Nrg1 KO brains. Arrowheads indicate the axonal front within the corpus callosum. Boxed areas depict the magnified area in the images above. Dashed lines show the border of the CC. MD: midline; CC: corpus callosum. Scale bar, 200 µm. (F) Quantification of the position of the axonal front with respect to the midline (taken as the point zero). n = 40 sections, out of 8 brains, from three different litters for the Ctrl group and n = 23 sections, out of 5 brains, from three different litters for the Nrg1 KO condition. Unpaired t-test, ***$p < 0.001$. Average ± SEM.

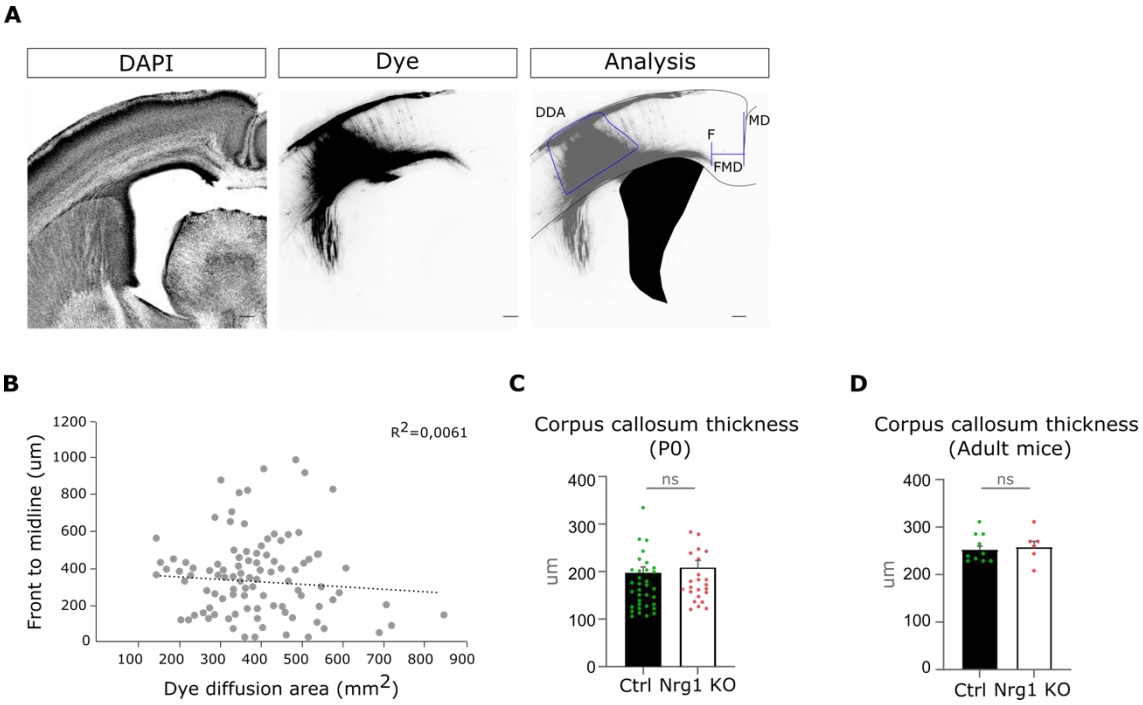


Figure 12. **The reduced growth of callosal axons in Nrg1 KO mice is not biased by the technical variability nor major impairments in earlier steps of callosal formation.** (A) Representative image of dye diffusion in our experiment setting. Schematic drawing of the analysis conducted: dye diffusion area (DDA), axonal front (F), midline (MD) and distance from front to midline (FMD). Scale bar, 100 μm. (B) The plot shows the lack of correlation between the diffusion of the dye and the position of the front of the callosal axons. We found no correlation between the two variables. n = 107, r = -0.08, Pearson correlation; R squared = 0.006. (C) The graph shows the quantification of the corpus callosum thickness in newborn (P0) Ctrl and Nrg1 KO mice. We failed to find any major impairment in this parameter. Ctrl n = 34 sections, out of 8 brains and Nrg1 KO n = 23 sections, out of 5 brains, compared with its internal controls from 3 litters. Unpaired t-test, p = 0.47 (ns). Average ± SEM. (D) The graph shows the quantification of the corpus callosum thickness in adult (8-10 months) Ctrl and Nrg1 KO mice. Ctrl n = 10 sections, out of 5 brains and Nrg1 KO n = 5 sections, out of 3 brains. Unpaired t-test, p = 0.52 (ns). Average ± SEM.

Nrg1 is cell-autonomously required for the growth of cortical axons *in vitro*

We reasoned that the phenotype observed in Nrg1 deficient mice could be attributed to a direct role of Nrg1 in excitatory neurons. However, cortical development is a complex process that requires the continuous interaction of multiple cell types in the brain (Noelia S. De León Reyes et al., 2020). Since Cre recombinase expression under the Nestin promoter drives Nrg1 deletion in all brain cells (Tronche et al., 1999), alterations in non-neuronal cell types might in principle cause the deficits in the development of callosal connections that we observed. Therefore, to directly determine the role of Nrg1 in axonal development we took advantage of a more reductionist *in vitro* model.

We established primary cultures of cortical neurons from $Nrg1^{flox/flox}$ mice. To obtain a spare labeling and single-cell resolution, we performed co-culture of naïve $Nrg1^{flox/flox}$ neurons with $Nrg1^{flox/flox}$ neurons expressing Cre to obtain Nrg1 deficient neurons (**Figure 13, A**). This experimental paradigm, presented in detail in the previous chapter, allowed us to evaluate the effect of Nrg1 deletion in cortical neurons developing together with non-mutant cells. We found that Nrg1 KO developing neurons exhibited a significant reduction in axonal length compared to control cells, with no change in the axonal branching density (**Figure 13, B, C and D**). This observation suggests that Nrg1 is cell-autonomously required for the development of cortical axons.

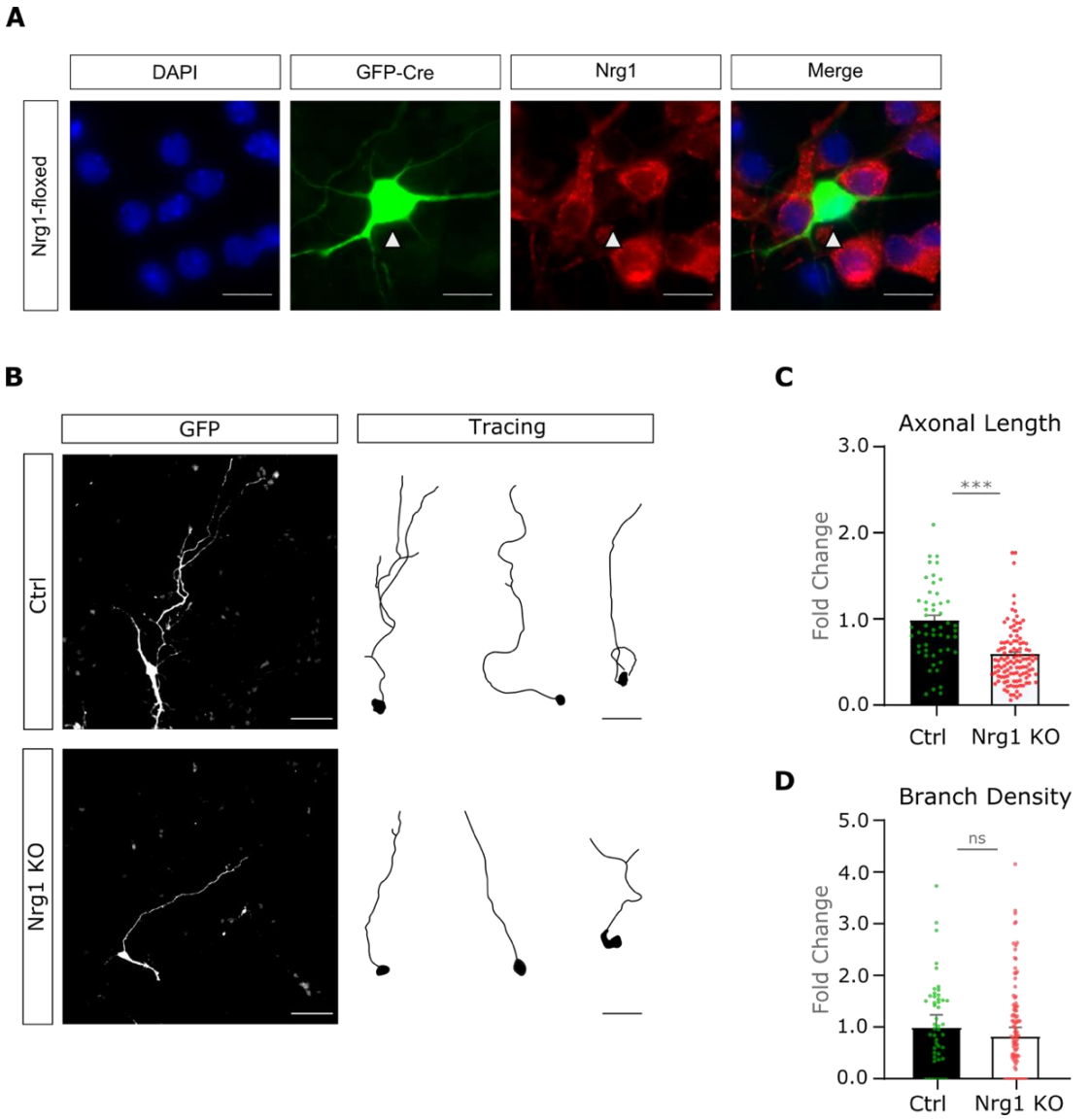


Figure 13. **Nrg1 is necessary for axonal elongation *in vitro*.** (A) Nrg1 deletion by Cre expression in Nrg1 floxed cultured neurons. Representative pictures of a GFP-ires-Cre expressing $Nrg1^{flox/flox}$ neuron at DIV4. Naïve $Nrg1^{flox/flox}$ neurons can be observed around the Nrg1 KO neuron, due a positive DAPI staining and Nrg1 Type III labeling. Staining with the Nrg1 Type III specific antibody confirms the Nrg1 deletion in our experimental setup (White arrowheads). Scale bar, 25 µm. (B) Representative pictures and drawings of control and Nrg1 KO cultured neurons. The neurons were electroporated to express either GFP-ires-Cre to perform single cell deletion to obtain Nrg1 KO neurons and co-cultured with control neurons. Scale bar, 50 µm. (C) The graph illustrates the quantification of the axonal length expressed in fold change as compared to control. Ctrl: n = 52, Nrg1 KO n = 116 neurons, both conditions out of three independent litters. Unpaired t-test, ***$p < 0.001$. Average ± SEM. (D) Quantification of the branch density (number of axonal branches per length), expressed in fold change as compared to control. Ctrl: n = 52, Nrg1 KO n = 116, both out of three independent litters. Unpaired t-test, $p = 0.26$ (ns). Average ± SEM.

In addition, to assess the potential effect of Nrg1 on dendritic development, we performed the morphometric Sholl analysis, under the same experimental conditions. Nrg1 KO neurons exhibited a slight reduction in dendritic arborization, with a significant decrease in the number of dendritic processes (**Figure 14, A and B**).

In conclusion, our results suggest that Nrg1 plays a role in dendrites development (**Figure 14, A and B**) but it is specially required for cortical axon elongation (**Figure 13, B and C**). This interpretation is consistent with the hypothesis that the deficit in callosal axon growth may be caused by the deletion of Nrg1 in callosal projecting neurons (**Figure 11**).

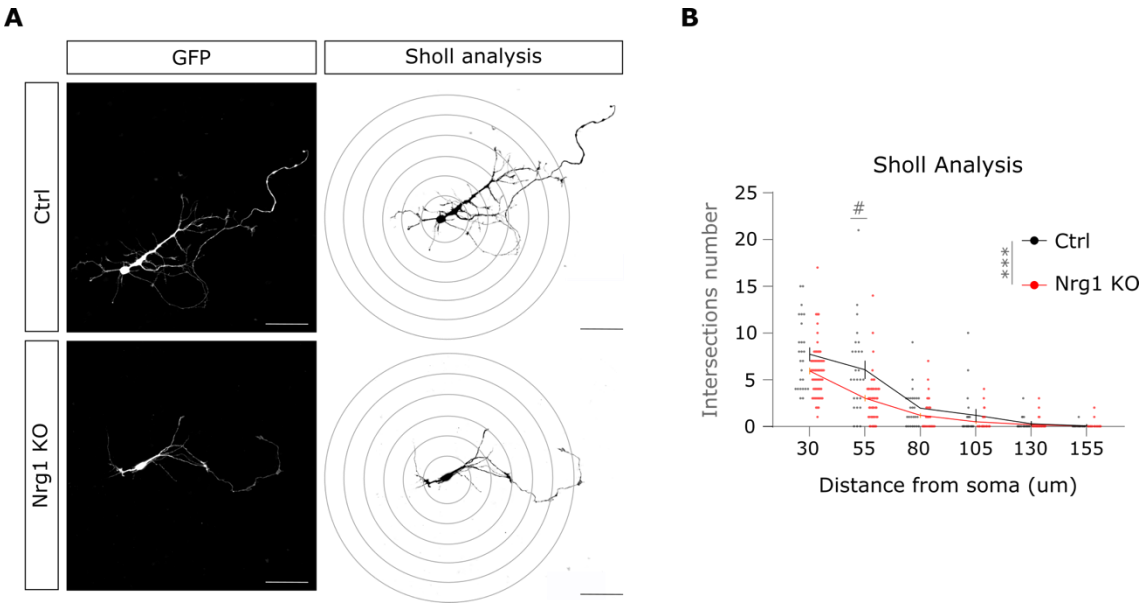


Figure 14. **Nrg1 plays a role in dendritic development.** (A) Representative pictures of control and Nrg1 KO cultured neurons. The neurons were electroporated to express either GFP-ires-Cre to perform single cell deletion to obtain Nrg1 KO neurons and co-cultured with control neurons. Drawings of the Sholl analysis on the right. The soma was set as the center and we considered a 30 µm start radius, 25 µm step size and 255 µm end radius. Scale bar, 50 µm. (B) The graph illustrates the quantification of the dendritic intersections number trough each radius from the soma. Ctrl: n = 26, Nrg1 KO n = 64 neurons, out of three independent litters. Mixed-effects analysis with Sidak's multiple comparisons test. Ctrl Vs Nrg1 KO at 30 µm p = 0.20; 50 µm * p < 0.05; 80 µm p = 0.43; 105 µm p = 0.63; 130 µm p = 0.98; 155 µm p = 0.99. Average ± SEM.

The Nrg1 intracellular signaling is sufficient to promote axonal growth *in vitro*

Since Nrg1 loss-of-function impaired axonal growth (**Figures 11 and 13**), we next asked whether Nrg1 expression was sufficient to promote axonal development. To address this point, we performed gain-of-function experiments in a single-cell experimental paradigm similar to the one described above. Namely, we cultured naïve primary cortical neurons with neurons transfected to express type III Nrg1, hereafter Nrg1-FL (**Figure 15, B**). Nrg1-FL is one of the major isoforms of Nrg1 and, like most Nrg1 isoforms, contains a transmembrane domain and a long intracellular domain (**Figure 15, B**). Notably, the expression of Nrg1-FL in cortical neurons increased axonal elongation (**Figure 15, C**) but not its branching density (**Figure 15, D**), as compared to control neurons.

Nrg1-FL undergoes a stepwise processing which ends with the cleavage of the Nrg1 transmembrane domain by gamma-secretase. As a result, the intracellular domain of Nrg1 is released in the cytosol and it translocates to the nucleus (**Figure 15, B**) (Bao et al., 2003; Y. Chen et al., 2010; Fazzari et al., 2014; Navarro-González et al., 2019). To determine the role of Nrg1 intracellular signaling in axonal elongation we expressed Nrg1-ICD in cortical neurons. It was previously demonstrated that the expression of Nrg1-ICD effectively mimics the activation of Nrg1 intracellular signaling (Bao et al., 2003; Fazzari et al., 2014; Navarro-González et al., 2019). Here, we found that the expression of Nrg1-ICD was sufficient to promote axonal growth (**Figure 15, C**), but not its branching (**Figure 15, D**), in developing cortical neurons.

To further investigate the potential role of Nrg1 intracellular signaling in dendritic development, we performed Sholl analysis under the same experimental conditions. Interestingly, we observed no significant differences, suggesting that neither Nrg1-FL nor signaling through Nrg1-ICD, regulate dendrites development (**Figure 16, A and B**). This observation suggests a molecular mechanism by which Nrg1 expression specifically promotes axonal elongation via the activation of Nrg1 intracellular signaling.

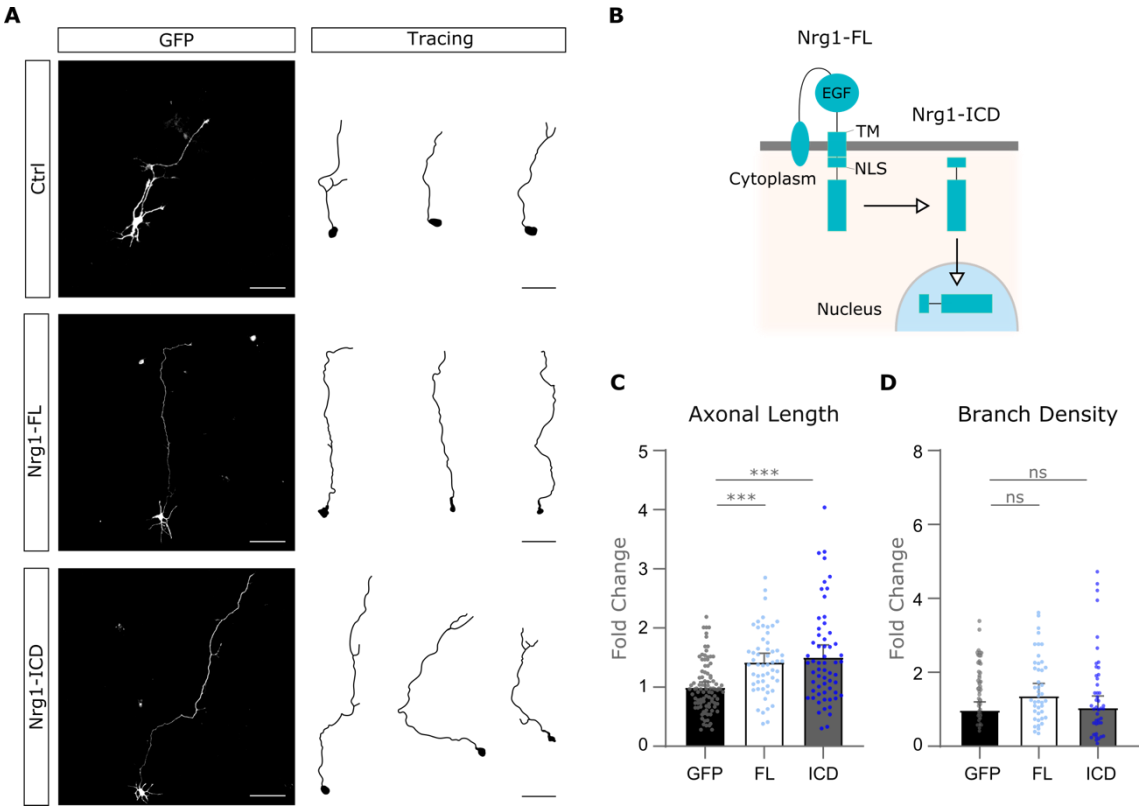


Figure 15. **Nrg1 intracellular signaling promotes axonal elongation.** (A) Representative images and drawings of control and Nrg1 overexpressing cultured neurons. The neurons were transfected to express either Nrg1-FL-GFP or Nrg1-ICD-GFP and co-cultured with control neurons. The cells were fixed at DIV4. Scale bar, 50 µm. (B) Schematic representation of the structure of Nrg1-FL: on the left in its full conformation (also named Nrg1-FL), which includes an epithelial growth factor domain (EGF), a transmembrane domain (TM), a cysteine-rich domain (CRD) and a nuclear localization signal (NLS); on the right, the intracellular Nrg1 (Nrg1-ICD). (C) Quantification of the axonal length of Nrg1 expressing neurons shown in fold change as compared to control. Ctrl: n = 97, Nrg1-FL overexpressing neurons: n = 57, Nrg1-ICD overexpressing neurons: n = 60, all of them out of three independent neuronal cultures. One-way ANOVA and post hoc with Tukey's test, *** $p < 0.001$. Average ± SEM. (D) Quantification of the branch density (number of axonal branches per length), expressed in fold change as compared to control. Ctrl: n = 78, Nrg1-FL overexpressing neurons: n = 55, Nrg1-ICD overexpressing neurons: n = 60, all of them out of three independent neuronal cultures. One-way ANOVA and post hoc with Tukey's test. Ctrl Vs Nrg1-FL $p = 0.10$ (ns); Ctrl Vs Nrg1-ICD $p = 0.93$ (ns). Average ± SEM.

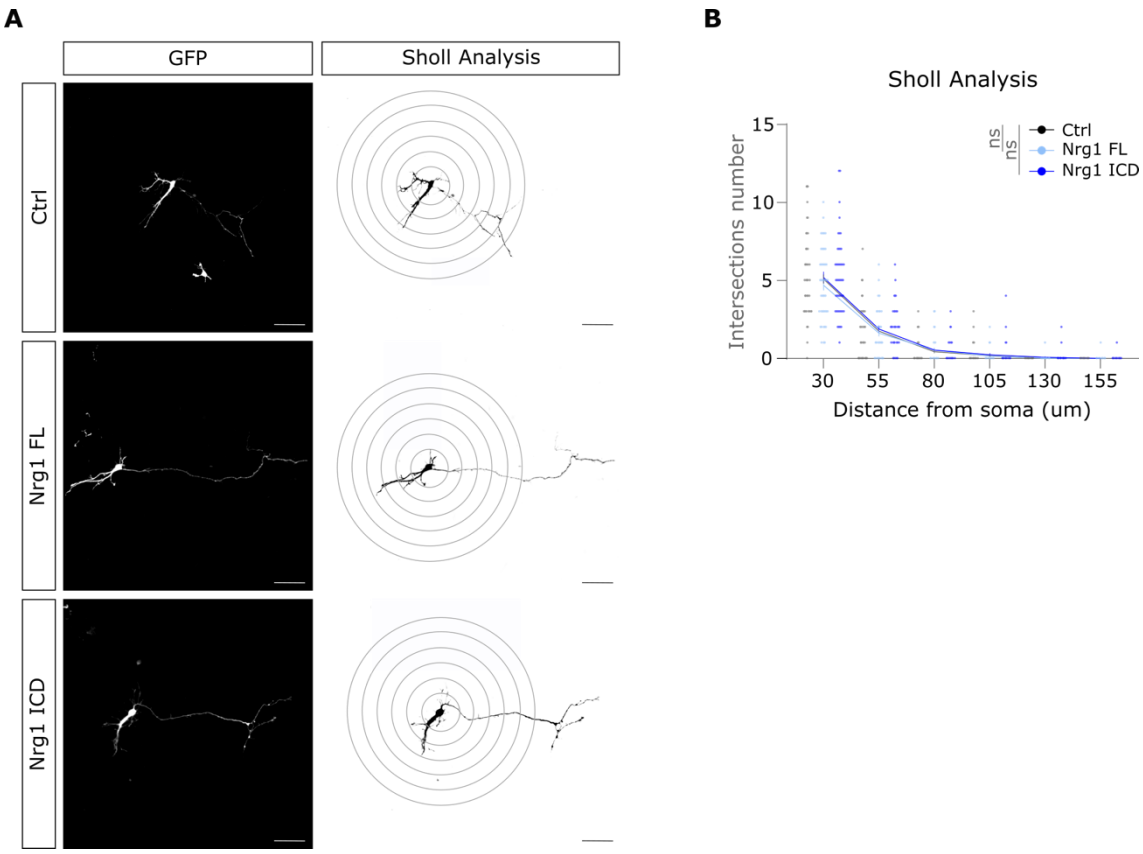


Figure 16. **Nrg1 intracellular signaling does not play a role in dendritic development.** (A) Representative images of control and Nrg1 overexpressing cultured neurons. Drawings of the Sholl analysis on the right. The soma was set as the center and we considered a 30 µm start radius, 25 µm step size and 255 µm end radius. Scale bar, 50 µm. (B) The graph illustrates the quantification of the dendritic intersections number trough each radius from the soma. Ctrl: n = 70, Nrg1-FL overexpressing neurons: n = 52, Nrg1-ICD overexpressing neurons: n = 46, all of them out of three independent neuronal cultures. One-way ANOVA and post hoc with Tukey's test. In all cases, $p > 0.05$ (ns). Average ± SEM.

Nrg1 signaling enhances the elongation of callosal axons *in vivo*

We showed that Nrg1 is required for the development of interhemispheric projections (**Figure 11**) and our *in vitro* experiments suggested that Nrg1 signaling cell-autonomously promotes axonal elongation *in vitro* (**Figure 15**). Therefore, we next wondered whether the activation of Nrg1 signaling was sufficient to promote the outgrowth of the callosal axons also *in vivo*. To determine the role of Nrg1 signaling *in vivo*, we performed gain-of-function experiments by using *in utero* electroporation (IUE), in collaboration with Gil-Sanz's lab. This experimental approach is particularly effective for studying the development of cortico-cortical callosal axons because it allows targeting specifically the upper cortical layers that contain the majority of contralateral projecting cortical neurons (Noelia S. De León Reyes et al., 2020; Mateos-White et al., 2020). Thus, we performed IUE at E15.5 to obtain the expression of Nrg1 to layer 2/3 callosal neurons (**Figure 17, A**) (Mateos-White et al., 2020). We let the mother give birth and P2 brains were sectioned. The slices were imaged and classified on a rostro-caudal axis, using as reference the Allen Brain Atlas of Developing Mouse Brain at P1 (**Figure 17, A**).

Specifically, we electroporated cortical neurons to express either Nrg1-FL or GFP as control. Because the exact timing of axonal development may vary from litter to litter, we expressed Nrg1-FL or GFP in littermates to provide an internal control (**Figure 18, A and D**) We performed the analysis of electroporated at P2 because, in our experimental settings, the electroporated callosal axons are approaching the midline at this stage. We measured the distance between the electroporation zone and the more advanced axons in the corpus callosum (**Figure 17, B**). Notably, we found that the expression of Nrg1-FL increased axonal elongation in callosal projecting neurons as compared to control littermates (**Figure 18, A to C**).

To investigate the signaling mechanism involved in this process, we also performed gain-of-function experiments expressing Nrg1-ICD to selectively determine the role of Nrg1 intracellular signaling (**Figure 18, D to F and Supplementary Figure S6**) Consistent with the *in vitro* experiments (**Figure 15**), we found that the activation of Nrg1 intracellular signaling was sufficient to promote the growth of callosal axons. Altogether, these results suggest Nrg1 promotes the formation of interhemispheric callosal connections by activating Nrg1 intracellular signaling.

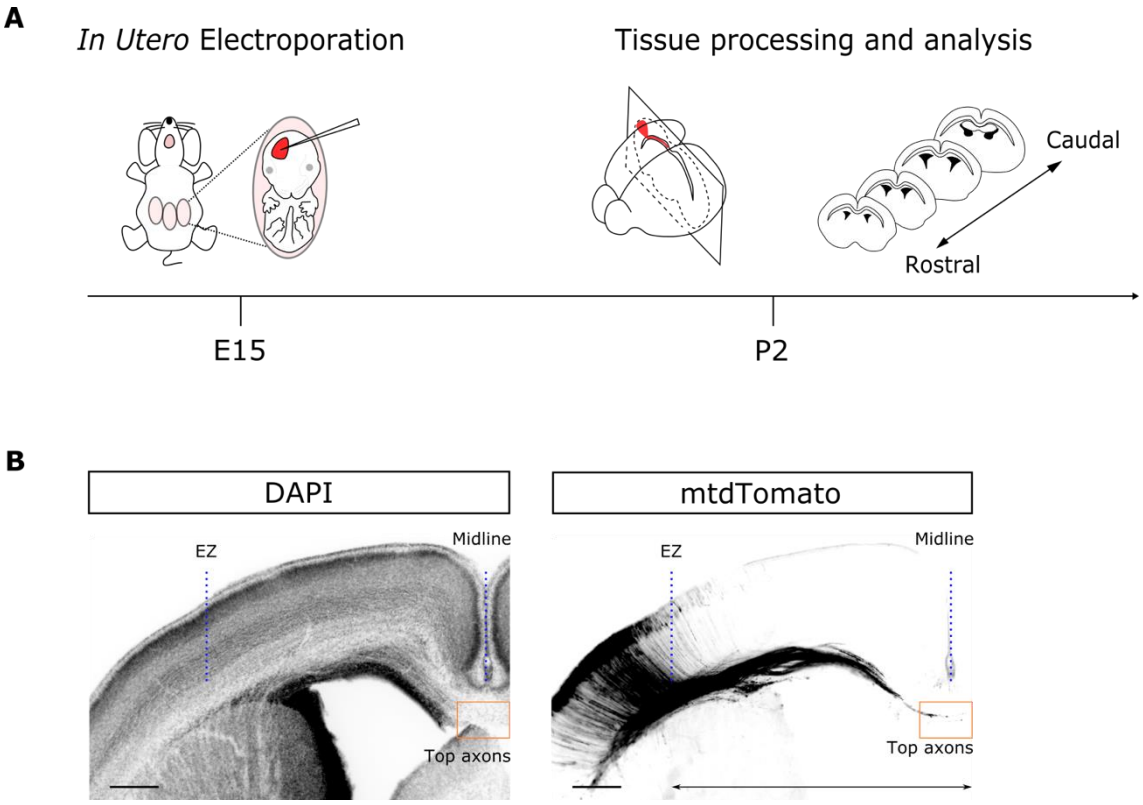
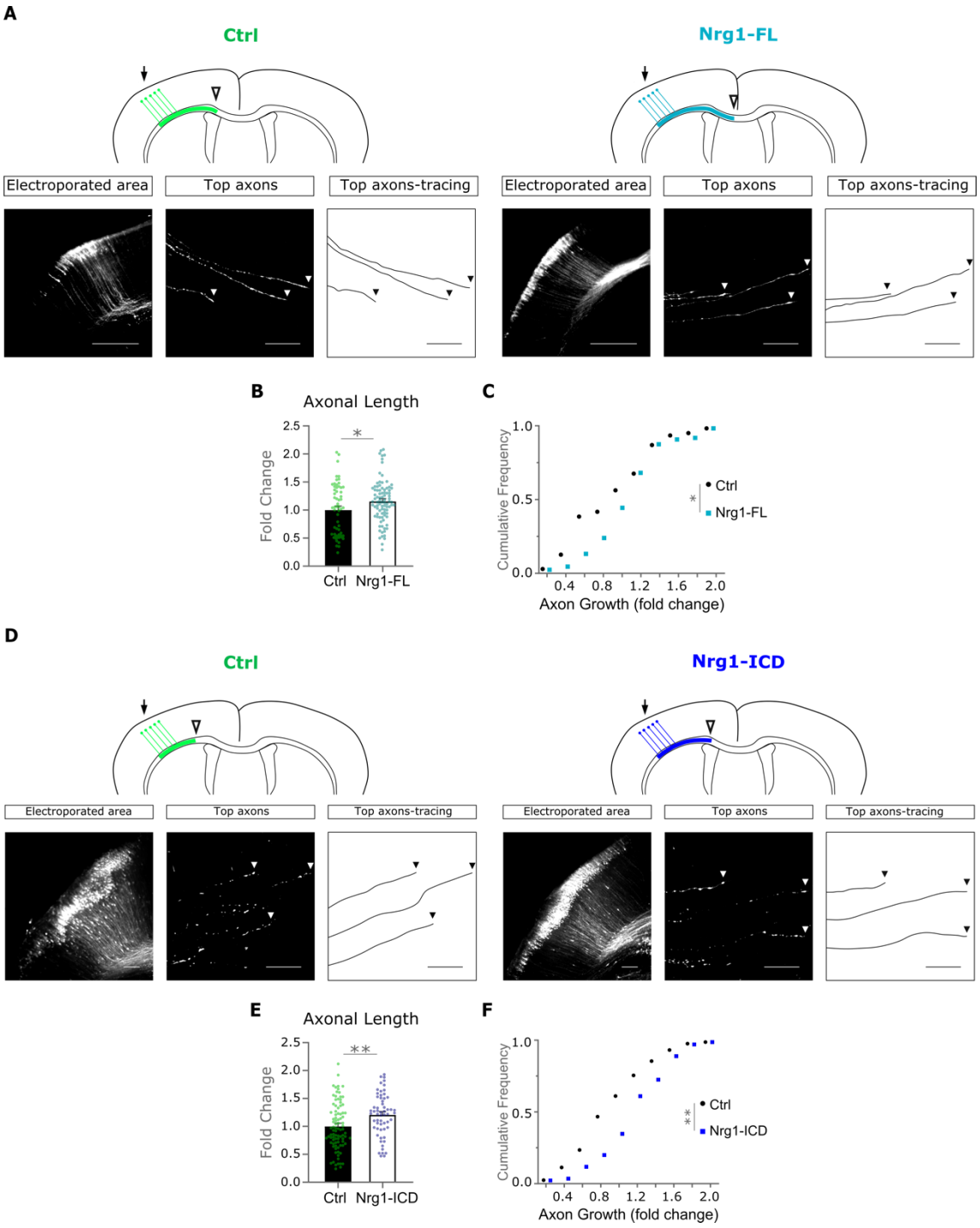


Figure 17. **Experimental paradigm of Nrg1 *in utero* electroporation.** (A) Experimental workflow: *in utero* electroporation at E15 for labeling cortico-cortical projecting neurons in the cortex and tissue processing at P2 for further scanning. Finally, all the sections were classified on a rostro-caudal axis, using as reference the Allen Brain Atlas: Developing Mouse Brain, to compare similar brain regions. (B) Tilescan images of a coronal section at P2. The DAPI staining helps to identify the rostro-caudal level. The labeling shows the callosal projections from electroporated L2/3 neurons approaching the midline. The boxed area in red highlights the more advance axons (Top axons). The line defines the midline position. We measured the distance between the electroporation zone and the more advanced axons within the corpus callosum. By taking this parameter, we managed to normalize part of the variability intrinsic to the technique used. EZ: Electroporated zone. Scale bar, 300 μm.


A
Ctrl
Nrg1-FL
Electroporated area
Top axons
Top axons-tracing
Electroporated area
Top axons
Top axons-tracing
B
Axonal Length
Fold Change
2.5
2.0
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Nrg1-FL
C
Cumulative Frequency
1.0
0.5
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Axon Growth (fold change)
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Figure 18. **Nrg1 signaling promotes the development of callosal axons *in vivo*.** (A) Schematic representation of developing callosal axons at P2 in control littermates and in neurons expressing Nrg1-FL. Arrows indicate the electroporated area and the empty arrowheads show the location of the more advanced axonal tips (Top axons). The images below show the electroporated area, the top axons and their tracing. The arrowheads indicate the tip of the axons. Scale bar, 50 μm. (B, C) Quantification of axonal extension in Nrg1-FL expressing neurons relative to control littermates. The bar graph shows Mean ± SEM (B) while the plot shows the Cumulative frequency (C). Ctrl n = 60 axons, out 6 sections from 4 brains; Nrg1-FL n = 90 axons, out 9 sections from 4 brains. Kolmogórov-Smirnov test, * $p < 0.05$. (D) The drawing shows the callosal axons at P2 in control littermates and in Nrg1-ICD expressing neurons. Arrows indicate the electroporated area and the empty arrowheads show the location of the more advanced axonal tips (Top axons). The pictures show representative images of the electroporated area, the top axons and their tracing. The arrowheads indicate the tip of the axons. Scale bar, 50 μm. (E, F) The bar graph shows Mean ± SEM (E) while the plot shows the Cumulative frequency (F). Ctrl n = 90 axons, out 9 sections from 3 brains; Nrg1-ICD n = 60 axons, out 6 sections from 3 brains. Both conditions are from one litter, with internal controls within littermates. Unpaired t-test, ** $p < 0.01$.

Discussion

Schizophrenia is a neurodevelopmental disorder that impacts around 1% of the global population but still presents a major socio-economic impact. SZ diagnosis proves challenging due to the absence of clear histological markers in the brains of patients, unlike other neurological pathologies. Furthermore, effective treatments remain elusive as the underlying molecular mechanisms of the disorder are yet to be fully elucidated. One of the most consistent findings in SZ research is the presence of WM abnormalities, particularly within the CC (Arat et al., 2015; David, 1994; Fenlon et al., 2015; Kubicki et al., 2005). The CC, composed solely of axons, acts as the major interhemispheric connection between cortical regions, facilitating efficient communication and synchronization of information, being its development is critical for most aspects of cortical function.

It is well-established that SZ has a strong genetic component. Since *NRG1* was identified as a significant SZ risk gene (Harrison et al., 2005; Stefansson et al., 2002), numerous studies have sought to understand Nrg1's role in brain wiring and cortical inhibition. Several studies have demonstrated that Nrg1 is pivotal in various stages of cortical development and in wiring inhibitory circuits (Y.-J. Chen et al., 2010; Y. Chen et al., 2010; Fazzari et al., 2010; Navarro-Gonzalez et al., 2021; Pedrique et al., 2010; Rahman-Enyart et al., 2020). Interestingly, while most studies in preclinical models have focused on the loss of Nrg1/ErbB4 signaling, others have shown that exogenous expression of Nrg1 can also be detrimental to cortical wiring and lead to SZ-like symptoms (Agarwal et al., 2014; Olaya et al., 2018). These results suggest that an optimal level of Nrg1 is required to maintain homeostasis of excitatory/inhibitory circuits in the cortex (Agarwal et al., 2014).

The focus of past research has largely been on the role of Nrg1/ErbB4 signaling in interneurons. Regarding this, it was shown that Nrg1 controls the migration of interneuron precursors from the ganglionic eminence to the cortex (Flames et al., 2004). Moreover, Nrg1 is important for the activity of cortical interneurons and inhibitory homeostasis. Indeed, Nrg1 was one of the first synaptogenic cues found to regulate cortical inhibitory circuits via the activation of ErbB4. Specifically, Nrg1 promotes, in ErbB4-expressing inhibitory cells, the formation of excitatory synapses in the dendrites and inhibitory contacts in the axonal buttons (Y.-J. Chen et al., 2010; Mei et al., 2008; Navarro-Gonzalez et al., 2021).

In contrast, the role of Nrg1 in excitatory neurons is poorly studied and understood. Only a few studies showed that Nrg1 loss-of-function impair dendritic development (Y. Chen et al., 2010; Z. Zhang et al., 2017). Additionally, it was shown that Nrg1 regulates, trough the ErbB4 activation, the development of dendritic spines in pyramidal neurons, (Barros et al., 2009; Fazzari et al., 2014). In summary, most studies have focused on the role of the forward (or canonical) Nrg1/Erbb4 signaling in dendrites, while the role of Nrg1 in axonal development remains largely unknown.

Our study identifies a novel role for Nrg1 intracellular signaling in excitatory neurons. We specifically showed that Nrg1 intracellular signaling is necessary and sufficient to promote the axonal development of callosal projections *in vivo* and *in vitro*. Our *in vivo* experiments showed that Nrg1 signaling is required for the development of callosal connections in the mouse. Single-cell deletion of Nrg1 in primary neuronal culture confirmed the role of Nrg1 in axonal development suggesting a cell-autonomous effect.

Likewise, we did not observe a reduction in the corpus callosum thickness in Nrg1 null mice. This suggests that CC development is delayed but not severely disrupted in the absence of Nrg1. Nevertheless, this delay in the growth of cortical callosal connections observed in Nrg1-deficient mice could have significant functional and behavioral consequences. Interhemispheric integration of brain function depends largely on the wiring of the callosal axons and the development of precise contralateral connections is critical for most aspects of cortical function, including basic brain activities and higher cortical functions (Noelia S. De León Reyes et al., 2020; Fenlon et al., 2015). Future studies, such as electrophysiological recordings to determine the interhemispheric correlation of neuronal activity, will be required to more specifically address the functional consequences of Nrg1 loss.

Several studies have shown that Nrg1-deficient mice exhibit SZ-related behavioral changes (Nakazawa et al., 2020; Stefansson et al., 2002). However, given the multiple roles that Nrg1 plays in brain development, it will be very difficult to determine the specific contribution of the deficits we observed in callosal development to the behavioral alterations. Nevertheless, we speculate that the delay in callosal development may contribute, at least in part, to the SZ-like symptoms in Nrg1 mutant mice.

Mechanistically, our experiments indicated that the activation of Nrg1 intracellular signaling is involved in axonal growth both *in vitro*, in primary cultures, and *in vivo* in

callosal projecting neurons. This growth-promoting activity may be cell autonomous since the expression of Nrg1-ICD to activate Nrg1 intracellular signaling in single cells is sufficient to promote axonal growth, both *in vitro* and *in vivo*. The downstream effectors of Nrg1 intracellular signaling are still unknown, although it was proposed that the localization of Nrg1-ICD to the nucleus is required for its function.

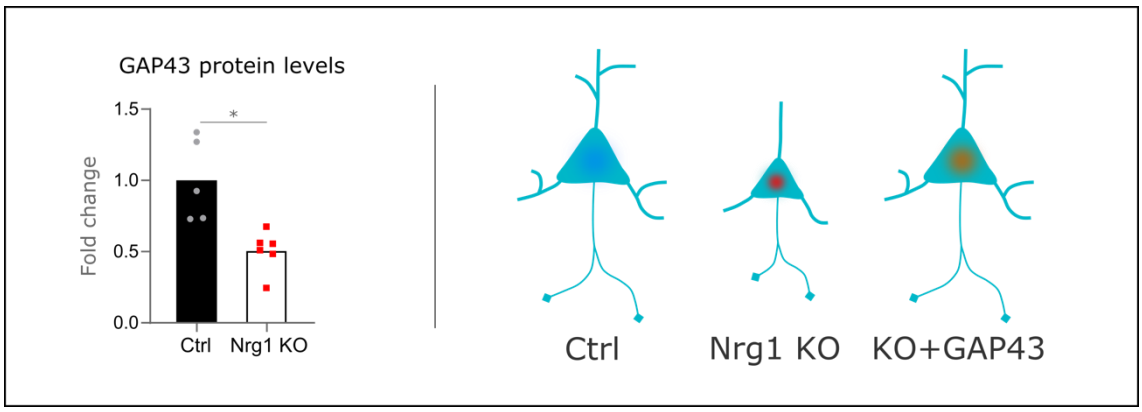
Our current study focuses primarily on the early stages of callosal development and does not address the role of Nrg1 in later stages of interhemispheric connectivity. While our observation suggests that Nrg1 expression by IUE accelerates callosal axon growth, this may not necessarily translate into improved interhemispheric connectivity at later stages. Nrg1-induced accelerated growth could be counterbalanced later by pruning and target refinement. Alternatively, Nrg1-expressing neurons might overshoot their targets and connect to inappropriate ones. To explore the function of Nrg1 at later stages, we employed a gain-of-function approach in P8 mice using *in utero* electroporation (data not shown). Unfortunately, we were unable to obtain consistent results due to a low sample size caused by high maternal infanticide of the electroporated mice. Further studies should be conducted to investigate the contributions of Nrg1 to interhemispheric synaptic wiring and function.

In conclusion, our work showed a novel role of Nrg1 in the development of callosal axons, the major contralateral connection in the brain. These results provide the first evidence that Nrg1 is important for the development of cortical axons and callosal projections *in vitro* and *in vivo*. Interestingly, our experiments suggest that the activation of Nrg1 intracellular signaling is sufficient to promote the growth of callosal axons, possibly via a cell-autonomous mechanism in contralateral projecting callosal neurons. This is particularly relevant because the role of Nrg1 in excitatory neurons and the function of its reverse intracellular signaling are poorly studied and understood. Given the importance of callosal connections for brain function in physiological conditions and in SZ, we speculate that this alteration, together with the previously reported synaptic deficits, may contribute to the behavioral SZ- relevant phenotype of Nrg1-deficient mice.

Altogether, our study provides a novel perspective on the role of Nrg1 and its intracellular signaling in SZ, highlighting the need for further research into the functional and behavioral impacts of disrupted Nrg1 signaling on cortical connectivity.

CHAPTER 3: GAP43 IS A PUTATIVE EFFECTOR OF NRG1 SIGNALING IN AXONAL GROWTH

Chapter 3 graphical abstract:



Chapter 3 highlights:

- Nrg1 effect on different key regulators of axonal outgrowth.
- GAP43 protein expression is strongly depleted in Nrg1 KO mice.
- GAP43 forced expression rescues the impaired axonal development observed in Nrg1-deficient neurons.

Publication related to Chapter 3:

3. **Rodríguez-Prieto, Á.,** Mateos-White, I., Aníbal-Martínez, M., Navarro-González, C., Gil-Sanz, C., Domínguez-Canterla, Y., González-Manteiga, A., Del Buey Furió, V., López-Bendito, G., Fazzari, P. (2024). Nrg1 intracellular signaling regulates the development of interhemispheric callosal axons in mice. Life Science Alliance. https://doi.org/10.26508/lsa.202302250

Introduction

In the previous chapter, we demonstrated the relevance of Nrg1 intracellular signaling in the axonal growth of pyramidal neurons. Here, we examined the specific axonal growth pathways regulated by Nrg1. Namely, we analyzed the activation of key growth pathways in Nrg1-deficient neurons. Specifically, we investigated the role of GAP43, a protein that we found downregulated in absence of Nrg1.

Main signaling pathways involved in axon development and regeneration

During neuronal development, intricate signaling pathways precisely orchestrate axon initiation and extension. As a response to external stimuli, these pathways control the dynamic behavior of the cytoskeleton within the growth cone, while simultaneously regulating gene expression in the neuronal cell body. Neuronal development hinges on the intricate regulation of the actin and microtubule cytoskeleton, which regulates neuronal polarization, axon formation, elongation and stabilization (Curcio et al., 2018; Polleux et al., 2010).

A diverse array of critical cytoskeletal regulators has been implicated in controlling axonal development. Among others, notably the Rho and Ras families of small GTPases have been shown to be involved in axon specification and extension, as they control the dynamic assembly, disassembly and reorganization of the actin and microtubule cytoskeletons (Hall et al., 2010; Polleux et al., 2010).

The Rho GTPases can either promote or inhibit axon extension depending on the type of effector. Activation of Ras leads to a phosphatidylinositol 3-kinase (PI3K)-mediated cascade of small GTPases regulating axon initiation, elongation and stabilization (Dupraz et al., 2019; Hall et al., 2010; Polleux et al., 2010). Additionally, Ras also activates the RAF/MEK/ERK cascade, playing a critical role in promoting axon elongation (Hall et al., 2010; Polleux et al., 2010). Sequentially, PI3K activates the protein kinase B, also called AKT. The activated form of AKT is enriched in growth cones, regulating the axon formation by regulating the protein translation trough the effector mammalian target of rapamycin (mTOR) (Hall et al., 2010; Polleux et al., 2010). In addition, recent findings have revealed another implicated pathway in which the c-Jun amino-terminal kinase (JNK) signaling regulates neurites formation, axonal outgrowth and stabilization by phosphorylation of various cytoskeletal proteins involved in axon dynamics (Hall et al., 2010; Okada et al., 2022; Polleux et al., 2010).

Various signaling pathways crucial for neurite outgrowth during neural development are downregulated in adulthood. This inactivity presents a significant hurdle in CNS repair after brain injury. Consequently, researchers have directed their efforts towards understanding these pathways with the aim of reactivating them. These studies have shed light on the role of Nrg1 in the signaling cascades involved in axonal development (Curcio et al., 2018; Z. Ding et al., 2021; Guo et al., 2010; Hao et al., 2021).

Studies in both *in vitro* and *in vivo* models of CNS injury have revealed that Nrg1 signaling regulates key signaling pathways, including the PI3K/Akt/mTOR and RhoA/Cofilin axes, that are essential for axon growth, stabilization and regeneration (Z. Ding et al., 2021; Guo et al., 2010; Hao et al., 2021). Nevertheless, the precise mechanisms by which Nrg1 signaling regulates these pathways or other major downstream molecular mechanisms critical for axonal growth, still remain unknown.

Growth-Associated Protein 43: a key protein in neuron development

The growth-associated protein 43 (GAP43), also known as neuromodulin, is a protein widely present in the CNS, being highly expressed during development and down-regulated upon maturation. This protein is highly expressed specifically in the growth cones of developing axons, contributing in numerous aspects of axonal development, including elongation, stabilization, branching and guidance. Because of this role, GAP43 is employed as a classical molecular marker that indicates axon growth and regeneration (Allegra Mascaro et al., 2013; Chung et al., 2020; Fallini et al., 2016; Okada et al., 2021).

GAP43's role in axonal elongation and branching takes place by its interaction with a variety of cytoskeleton-related proteins, including actin and tubulin. In specific, several studies confirm that GAP43 promotes axonal elongation through its ability to regulate actin dynamics, depending on the expression levels and post-translational modifications including phosphorylation (Chung et al., 2020; He et al., 1997; Okada et al., 2021).

GAP43 acts as a multifaceted regulator of the actin cytoskeleton, exerting both stimulatory and inhibitory effects through multiple pathways. Phosphorylated GAP43 stabilizes actin filaments, consequently promoting growth cone motility and axon elongation by three mechanisms: 1) recruiting actin-regulating proteins by forming clusters of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), the major driver of actin oligomerization; 2) interacting with the neural cell adhesion molecule-180 (NCAM-180) and spectrin; 3) stabilizing actin filaments in a protein kinase C (PKC)-dependent manner.

In contrast, m-calpain-mediated cleavage of GAP-43 leads to an increase of actin monomers, reducing actin assembly and consequently provoking growth cone collapse (Chung et al., 2020; Okada et al., 2021).

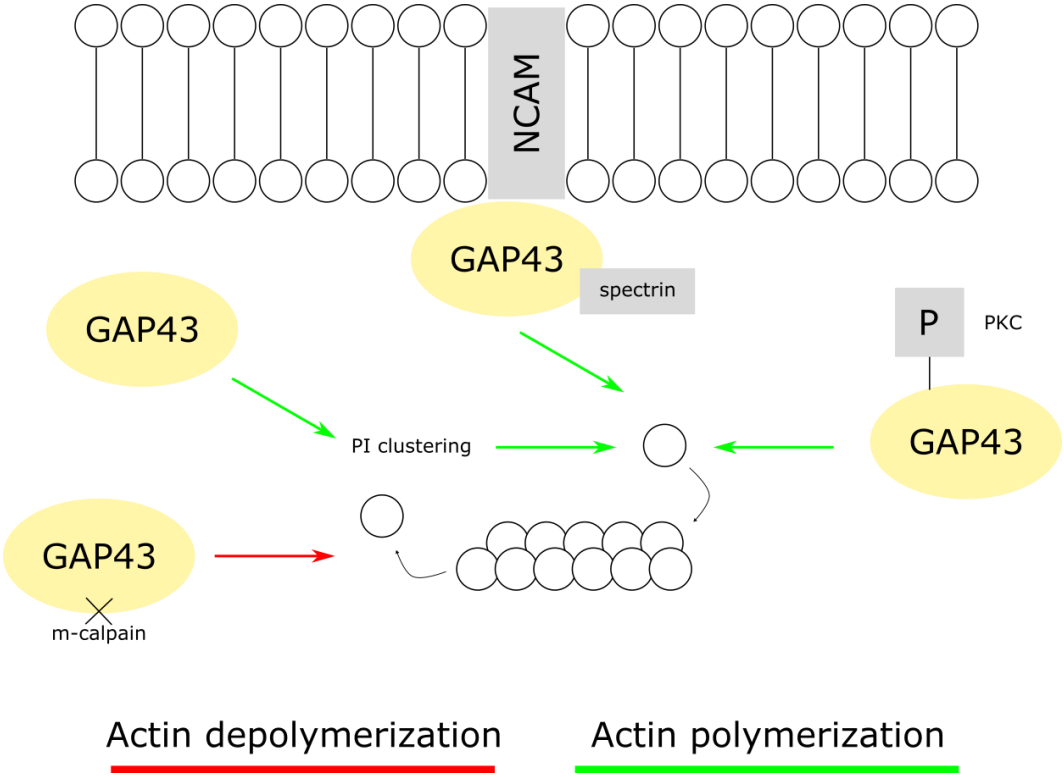


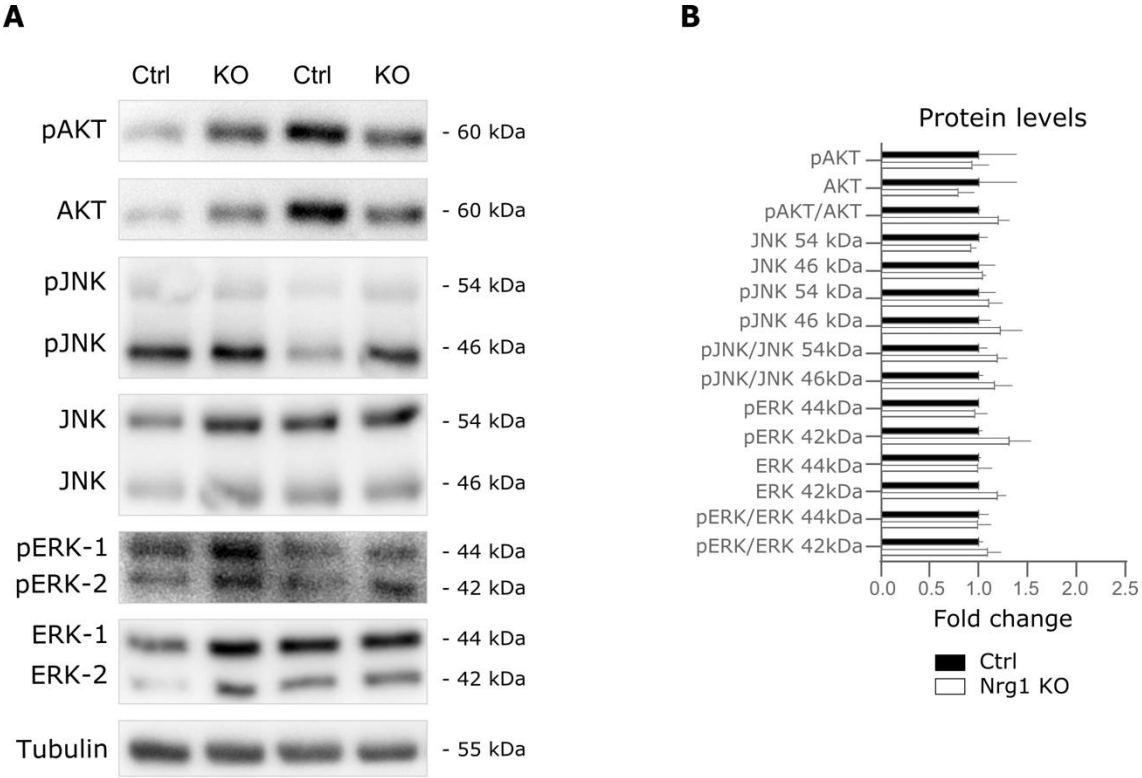
Figure 19. **The mechanisms of actin cytoskeleton regulation by GAP43.** Schematic representation of the stimulatory effect in actin polymerization by GAP43 and its different effectors (Adapted from Okada et al., 2021).

Results

Effect of Nrg1 loss on axonal growth pathways: GAP43 protein level is impaired in Nrg1 KO mice

Because Nrg1 loss reduced axonal growth, we hypothesized that Nrg1 deletion might affect the expression of key regulators of axonal development. To further explore the mechanisms behind Nrg1 signaling, we examined the effect of Nrg1 loss on key pathways involved in axonal growth, including AKT, JNK, ERK, and GAP43 (Chung et al., 2020; Hall et al., 2010; Okada et al., 2021, 2022; Polleux et al., 2010).

To investigate Nrg1's role in the molecular pathways involved in axonal development, we performed protein extraction and Western blot from high density primary neuronal cultures of E15 Nrg1 KO and WT mice. Our results suggested that Nrg1 deletion does not overtly affect the expression and activation of the AKT, JNK or ERK signaling pathways in our experimental model (**Figure 20, A and B**). In contrast, Nrg1 ablation in primary cortical neurons from Nrg1 KO mice resulted in a significant decrease in GAP43 protein expression levels as compared to control littermates (**Figure 20, C and D and Supplementary Figure S7**).



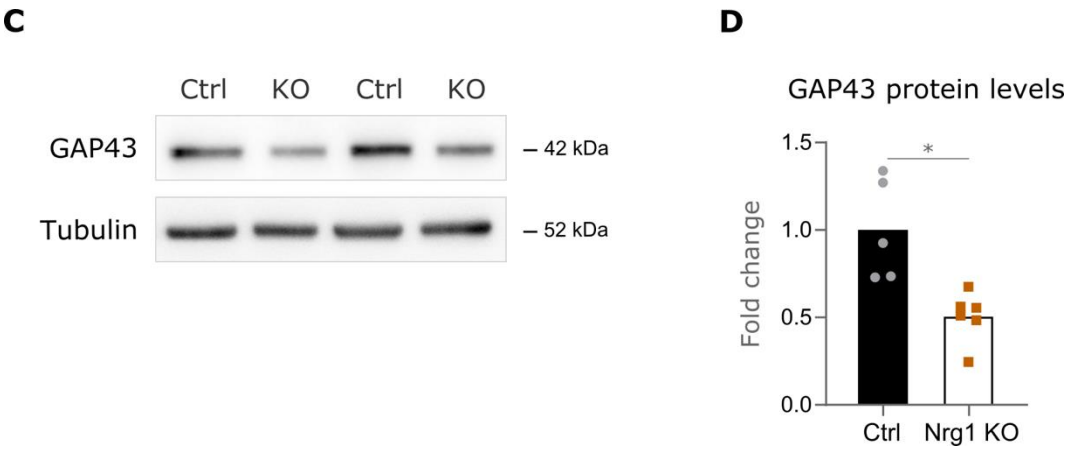


Figure 20. **Effect of Nrg1 loss on axonal growth pathways: GAP43 protein expression is highly reduced in Nrg1 KO mice.** (A) Representative images of Western blot (WB) analysis showing the expression levels of total and phosphorylated AKT, JNK, and ERK proteins in control ($Nrg1^{flox/flox}$) and Nrg1-deficient (Nes-Cre; $Nrg1^{flox/flox}$) neurons at DIV4. Tubulin levels are shown as loading control. (B) Quantification of total and phosphorylated AKT, JNK, and ERK protein levels from the WB analysis, normalized to Tubulin. Ctrl n = 2; Nrg1 KO n = 3, littermates from one single litter. Unpaired t-test, in all cases $p > 0.29$. Average ± SEM. (C) WB analysis of GAP43 protein levels in control and Nrg1-deficient neurons at DIV4. Tubulin levels are shown as loading control. (D) Quantification of GAP43 protein levels normalized to Tubulin. Ctrl n = 5, Nrg1 KO = 6, littermates from two different litters. Statistical significance was determined using an unpaired t-test with Welch's correction, * $p < 0.05$. Average ± SEM.

Nrg1 does not regulate GAP43 mRNA expression

To elucidate whether Nrg1's regulatory effect on GAP43 occurs at the protein or gene level, we performed RT-qPCR. First, we confirmed the deletion of the *NRG1* gene by using RT-qPCR in E15 embryos of our Nes-Cre driven Nrg1 KO model (**Figure 21, A and B**). Under the same experimental conditions, we failed to see a decrease in GAP43 mRNA levels (**Figure 21, C**).

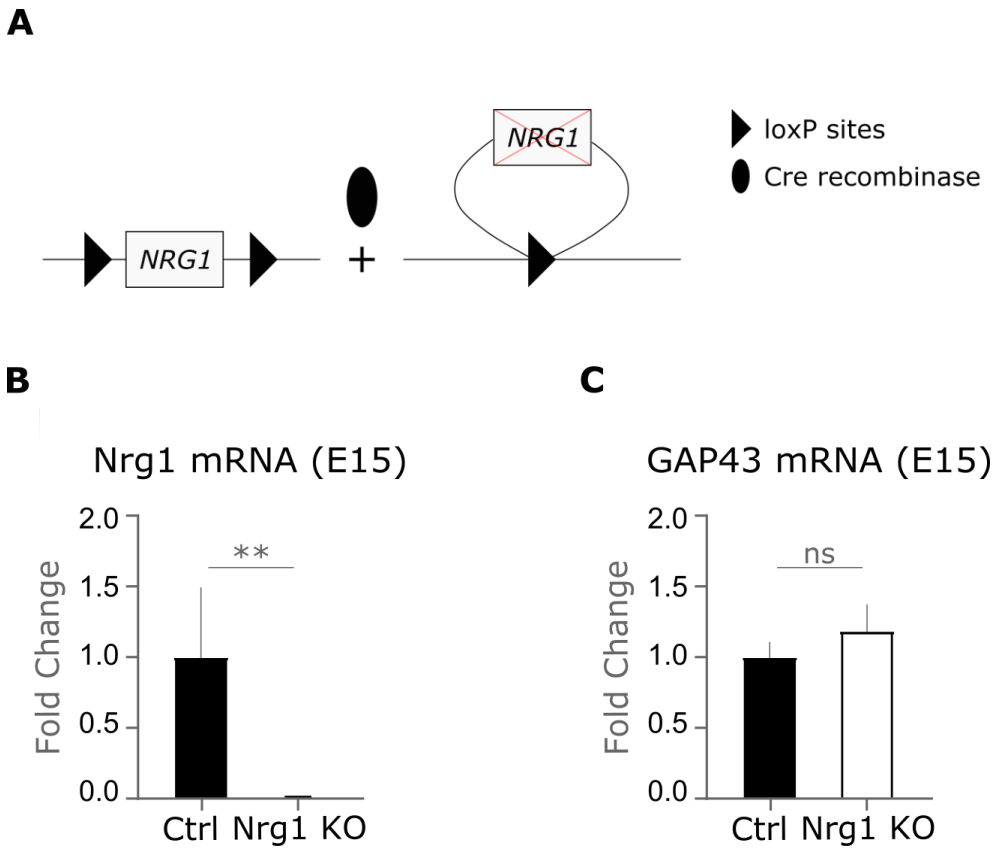


Figure 21. **In Nrg1 KO mice, GAP43 mRNA expression is not impaired**. (A) The constitutive Nrg1 mutant mice, driven by the expression of the Cre recombinase under a Nestin promoter. (B) Characterization of Nrg1 loss-of-function model. The graph shows the mRNA levels of Nrg1 in Nrg1 KO relative to control littermates at E15, quantified by RT-qPCR. Ctrl = 3 and Nrg1 KO = 2, littermates from one litter. Unpaired t-test, ** $p < 0.01$. Average ± SEM. (C) Quantification of the GAP43 mRNA levels by RT-qPCR in our Nrg1 loss-of-function model. Ctrl = 3 and Nrg1 KO = 2, littermates from one litter. Unpaired t-test, $p > 0.05$ (ns). Average ± SEM.

GAP43 expression is decreased in the axons of Nrg1-deficient neurons *in vitro*

Following up on our findings, we sought to confirm the observed significant decrease in GAP43 protein levels in Nrg1 KO mice by immunofluorescence. Additionally, we specifically examined GAP43 expression levels in developing axons. To achieve this, we performed primary cultures of cortical neurons from Nrg1$^{flox/flox}$ mice, under the same experimental conditions described in Chapter 2.

First, we performed experiments to validate the anti-GAP43 antibody by immunofluorescence. Namely, we overexpressed a c-Myc-tagged GAP43 and verified the correct labeling using both its c-Myc tag and the GAP43 specific antibodies. Notably, the c-Myc staining perfectly overlapped with GAP43 labeling, supporting the specificity of the staining (**Figure 22, A**). Next, we analyzed the GAP43 puncta density within the distal axon. We found a significant decrease in GAP43 protein expression levels as compared to control (**Figure 22, B and C**).

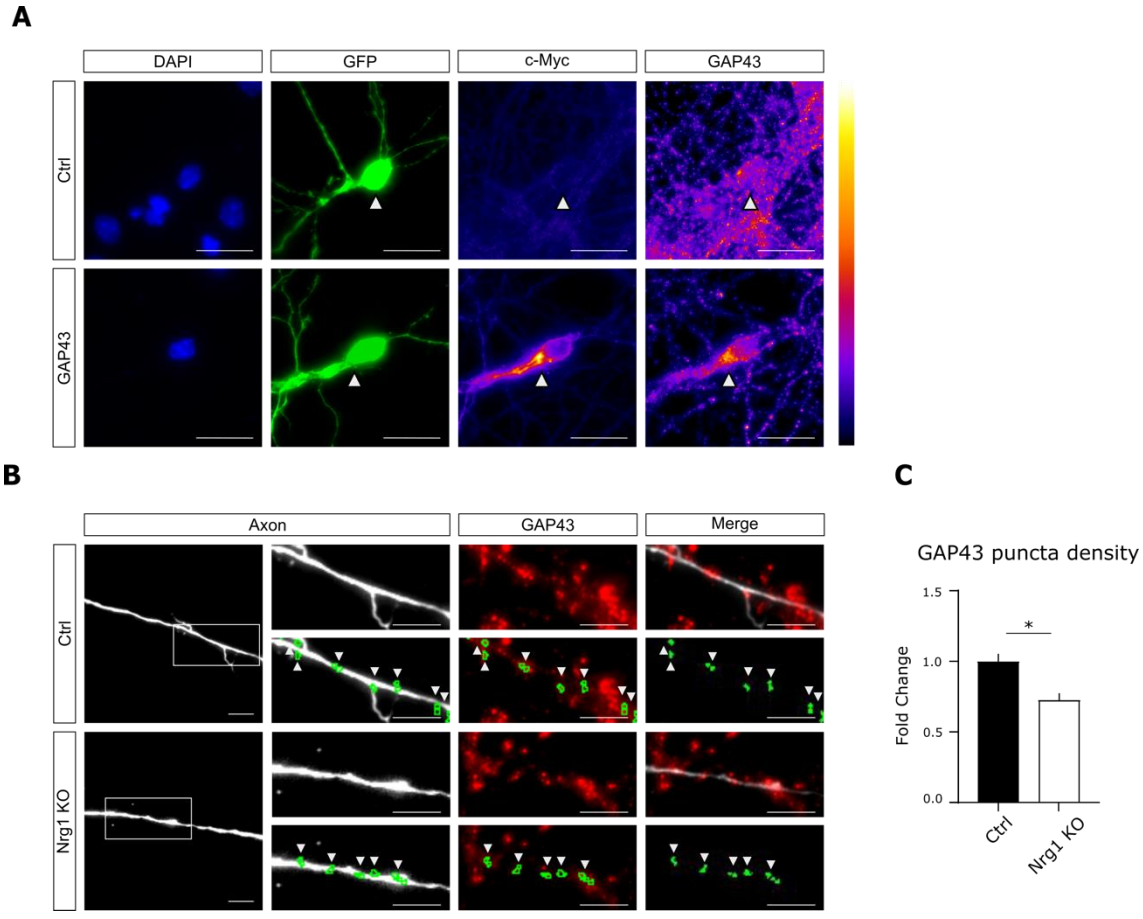


Figure 22. **GAP43 expression is significantly reduced in the axons of Nrg1 KO developing neurons *in vitro*.** (A) GAP43 plasmid characterization. Staining with antibodies for the plasmid tag (c-Myc) and GAP43 confirm the GAP43 expression in our experimental setup (White arrowheads). Scale bar, 25 μm. (B) Representative pictures of control and Nrg1 KO cultured neurons. The boxed area depicts the magnified area in the images on the right. White arrowheads indicate the GAP43 puncta that co-localize with the axon. Scale bar, 10 μm. (C) The graph illustrates the quantification of the GAP43 puncta density within the distal axon (100 μm from the soma), expressed in fold change as compared to control. Ctrl n = 56 and Nrg1 KO n = 54, neurons from three independent experiments. Unpaired t-test with Welch's correction, * $p < 0.05$. Average ± SEM.

GAP43 expression rescues axonal growth in Nrg1-deficient neurons

To functionally assess the role of GAP43 in Nrg1 signaling, we examined its ability to rescue the growth defects observed in Nrg1-deficient neurons (**Figure 23 and Figure 24**). We performed primary cultures of cortical neurons from $Nrg1^{flox/flox}$ mice as described previously (see Materials and Methods section for details). Briefly, we did co-culture of naïve $Nrg1^{flox/flox}$ neurons with $Nrg1^{flox/flox}$ neurons expressing Cre to obtain Nrg1 deficient neurons. We co-expressed Cre along GAP43 to perform the rescue condition.

Notably, we found that restoring GAP43 expression in developing cortical neurons rescued axonal elongation in Nrg1-deficient neurons (**Figure 23, A and B**) but didn't affect its branching (**Figure 23, C**). To further investigate the potential role of GAP43 in dendrites development, we performed Sholl analysis under the same experimental paradigm. Interestingly, we observed no significant differences (**Figure 24, A and B**). Taken together, these findings suggest that GAP43 may be a relevant downstream effector of Nrg1 signaling, specifically by promoting axonal elongation in developing cortical neurons.

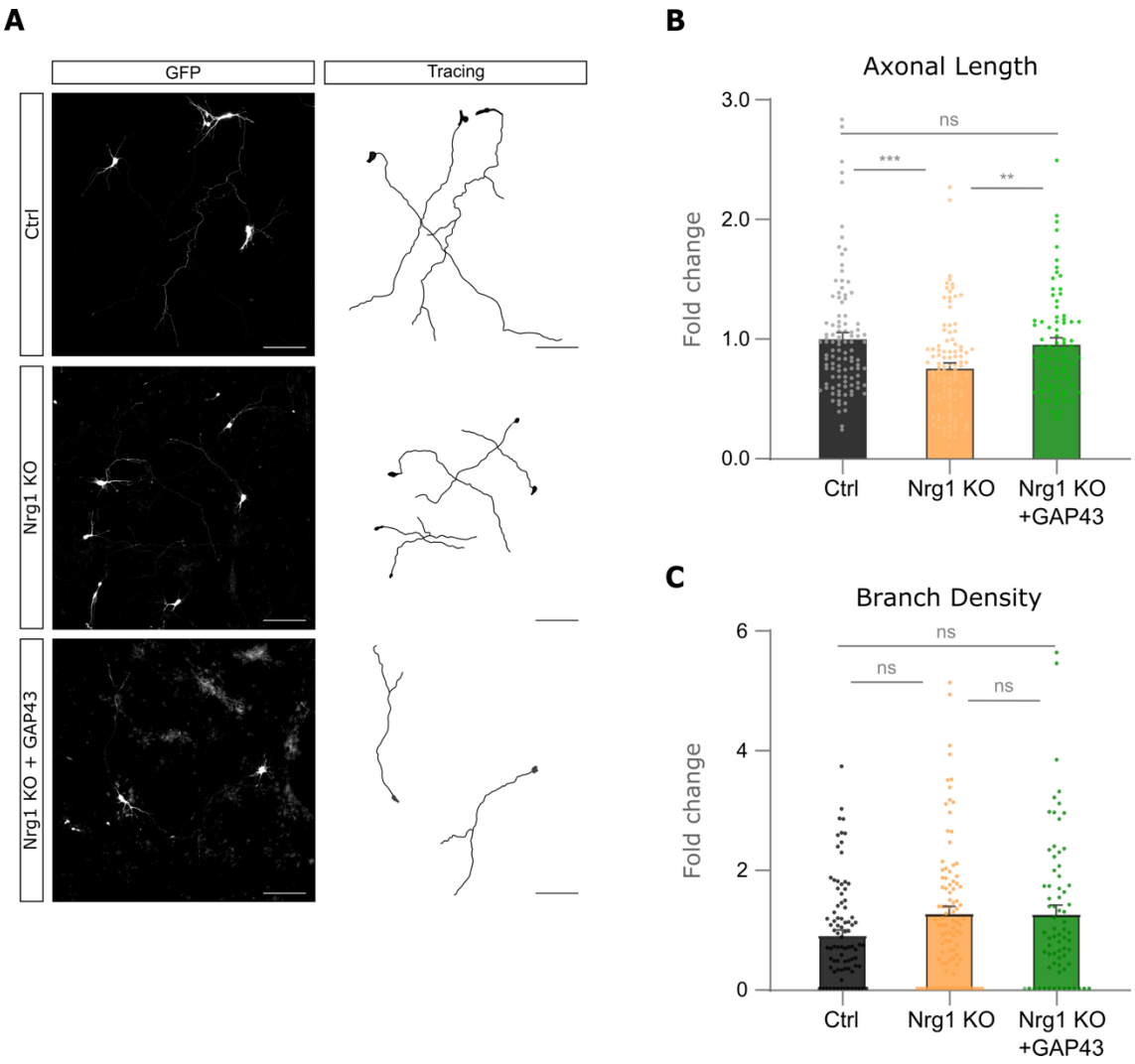


Figure 23. **GAP43 expression rescues axonal growth in Nrg1-deficient neurons.** (A) Representative images and corresponding tracings of immunofluorescence staining in control, Nrg1-deficient (Nrg1 KO), and Nrg1 KO neurons expressing GAP43 (Nrg1 KO+GAP43) at DIV4. Scale bar, 100 µm. (B) Quantification of axonal length in control, Nrg1 KO, and Nrg1 KO+GAP43 neurons, expressed in fold change. Ctrl n = 104, Nrg1 KO n= 105, GAP43 n = 78, neurons from two independent experiments. Statistical significance was determined using One-way ANOVA test with Tukey's post hoc: ** $p < 0.01$, *** $p < 0.001$. Average ± SEM. (C) Quantification of branch density in control, Nrg1 KO, and Nrg1 KO+GAP43 neurons, expressed in fold change relative to control. Ctrl n = 93, Nrg1 KO n= 98, GAP43 n = 73, neurons from two independent experiments. Statistical significance was determined using One-way ANOVA test with Tukey's post hoc. In all cases, $p > 0.05$ (ns). Average ± SEM.

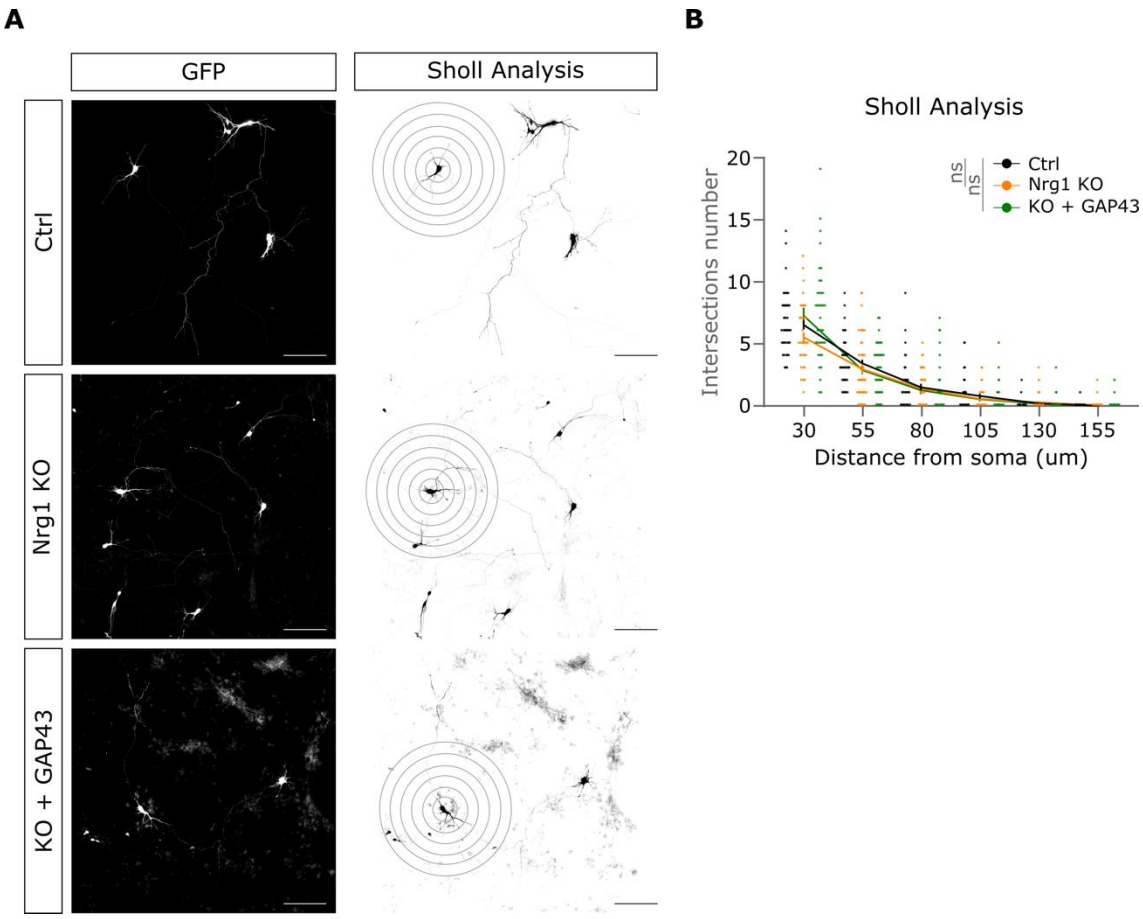


Figure 24. **GAP43 expression in Nrg1-lacking neurons does not affect the dendritic branching during development.** (A) Representative images and schematic drawings of neurons from control, Nrg1-deficient (Nrg1 KO), and Nrg1 KO neurons expressing GAP43 (Nrg1 KO+GAP43), including the drawings used for Sholl analysis of the dendrites. Cells were fixed at DIV4. Scale bar, 50 µm. (B) Graphs showing the quantification of dendrite ramification in control, Nrg1 KO and Nrg1 KO+GAP43, based on Sholl analysis. The soma was set as the center and we considered a 30 µm start radius, 25 µm step size and 255 µm end radius. Ctrl n = 45, Nrg1 KO n= 44, Nrg1 KO+GAP43 n = 37, individual neurons from two different experiments. One-way ANOVA followed by a Tukey's multiple comparison test was used to compare Ctrl Vs Nrg1 KO and GAP43 expressing Nrg1 KO neurons. In all cases, $p > 0.05$ (ns). Average ± SEM.

Discussion

The intricate regulation of the actin and microtubule cytoskeleton is essential for neuronal development. This orchestration governs neuronal polarization, axon formation, elongation and stabilization. GAP43 is a protein highly expressed in the CNS, particularly during neuronal development. It plays a critical role in axonal growth, regeneration and plasticity, by specifically regulating actin dynamics in response to extracellular signals. Notably, GAP43 expression is downregulated upon maturation but it can be re-induced in response to specific neurodegenerative diseases or brain injury. This characteristic positions GAP43 as a promising target for axonal regeneration (Chung et al., 2020; Okada et al., 2021; Polleux et al., 2010).

The GAP43 functions highly depend on its expression levels and phosphorylation status. In neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases and Amyotrophic Lateral Sclerosis, GAP43 expression is often altered. Therefore, several studies have explored the therapeutic potential of modulating its activity to compensate for the neuronal loss intrinsic to these neurodegenerative diseases (Chung et al., 2020).

Additionally, GAP43 expression has been shown to be upregulated in response to axonal injury, which suggests that it may play a role in axonal regeneration. GAP43 is also essential in mature neurons to switch into a growth state after a brain insult. The potential to induce a growth state in surviving neurons, protecting them and promoting functional network restoration, is being explored as a therapeutic avenue for brain repair (Allegra Mascaro et al., 2013; Chung et al., 2020).

Despite the therapeutic potential of GAP43, a complete understanding of its role in axon dynamics during development is necessary to further elucidate its dysregulation in the different neurodegenerative disorders. Moreover, effective and safe methods for delivering GAP43-targeting therapies after brain injury need to be developed.

In this work, we showed in Chapter 2 that Nrg1 is necessary to promote axonal growth during neuronal development, both *in vitro* and *in vivo* approaches. In addition, our data showed that Nrg1 deletion in neuronal cultures led to a significant decrease in the expression of GAP43. Interestingly, GAP43 expression could cell-autonomously rescue the decrease in axonal development observed in Nrg1 KO developing cortical neurons. These findings suggest that GAP43 may be a relevant downstream effector of Nrg1 signaling in axonal development.

However, there are limitations to consider. First, here we did not observe a significant decrease in dendritic arborization in Nrg1 KO developing neurons, which contrasts with our findings in Chapter 2. This discrepancy may be due to experimental variability. To definitively assess GAP43's potential role in dendrite development, a larger sample size under this experimental paradigm would be necessary. Second, our work on GAP43 mechanism relied solely on *in vitro* approaches. Future studies should investigate this mechanism *in vivo*, potentially using models of CNS injury for a more comprehensive understanding. Finally, although we observed a substantial decrease in GAP43 protein levels in Nrg1 KO mice, this decrease wasn't mirrored by a change in GAP43 mRNA levels, suggesting that Nrg1 regulates GAP43 expression post-transcriptionally. Further research is needed to elucidate the specific post-transcriptional mechanisms by which Nrg1 regulates GAP43 protein translation or stability.

Interestingly, local protein synthesis, where mRNA is translated within the axon itself, plays a crucial role in axon development (Shigeoka et al., 2019; H. H.-W. Wong et al., 2017). Notably, it was previously described that GAP43 mRNA is transported to axons and locally translated, suggesting a role for this process in regulating GAP43 function (Fallini et al., 2016). Investigating the potential role of Nrg1 in regulating local protein synthesis during axon development would be a valuable next step.

Moreover, we employed a candidate gene experimental approach, focusing on well-established pathways previously implicated in axonal outgrowth. However, it is likely that other effectors will play an important role in Nrg1 signaling during axonal development.

Although future studies will be required to further investigate the growth-associated pathways downstream of Nrg1 signaling and specifically the GAP43's role, our findings enrich the understanding of Nrg1 signaling and sheds light on the intricate interplay between Nrg1 and GAP43 in the context of axonal growth.

CHAPTER 4: NRG1 ROLE IN REPROGRAMMED NEURONS AS A POTENTIAL THERAPEUTIC TARGET

Chapter 4 highlights:

- Neurog2 and Ascl1 expression promote astrocyte-to-neuron reprogramming *in vitro*.
- Induced reprogrammed neurons acquire neuronal features and morphology.
- Nrg1 expression in reprogrammed neurons do not enhance reprogramming efficiency but may promote neuronal development.

Introduction

Brain damage: etiology and treatments

Brain injury is the leading cause of adult disability worldwide, classified into traumatic brain injury (TBI) and non-traumatic causes, such as stroke (Najem et al., 2018). Both disrupt brain function and share similar pathophysiological events across four consecutive phases after the injury: hyperacute, acute, subacute and chronic (Galgano et al., 2017).

Within the first few hours, the hyperacute phase sets in. This period involves immediate massive cell death triggered by energy depletion and excitotoxicity. The acute phase follows, typically lasting for the first week. During this time, an inflammatory response starts as microglia and astrocytes are recruited around the injury to remove damaged tissue. Notably, excessive inflammation can also be detrimental, increasing cellular oxidative stress and inducing neuronal loss. The next phase, the subacute phase, lasting weeks to months, is characterized by neuronal sprouting and the formation of new connections, offering the highest potential for plasticity and recovery. However, due to the limited regenerative capacity of the CNS, is generally impossible a full recovery of the brain functionality, leading to a chronic phase characterized by cognitive and motor impairments (Carmichael, 2016; Curcio et al., 2018).

Current options for treating strokes focus on restoring blood flow, while attempts to address TBIs are primarily neuroprotective. However, no treatment is completely effective, so a great deal of focus is being applied to understand the underlying mechanisms and to develop new therapeutic approaches.

Direct neuronal reprogramming as a novel therapeutic approach following brain injury

Part of the chronic phase in brain injuries is due the consequent neuronal loss that persists because the adult mammalian brain lacks neurogenesis. In recent decades, neuronal replacement therapies have primarily explored two promising approaches, using either exogenous or endogenous cell sources. The first approach is based on cell transplantation. However, while it appears to be a straightforward method, it presents some significant limitations. These limitations include a low survival rate due to various factors, such as poor vascularization of the injured area and immune rejection. The second strategy, by direct reprograming of local glial cells into neurons, overcomes these drawbacks (Bocchi et al., 2022).

The direct neuronal reprogramming approach leverages expressing neurogenic factors to directly convert local non-neuronal cells, such as astrocytes, into functional neurons. Following neuronal loss, some astrocytes can become detrimental, promoting inflammation and scar formation. Reprogramming these astrocytes into neurons could offer the replace of the lost neurons while simultaneously mitigate inflammation and scar tissue. Furthermore, the reprogramming of astrocytes into neurons extends beyond the postnatal period, being achievable from fully differentiated astroglia of the adult cerebral cortex. Therefore, this strategy holds immense potential for regenerative brain therapies (Bocchi et al., 2022).

In recent years, protocols for glia-to-neuron reprogramming have seen significant improvements. This progress is partly due to the identification of more effective neurogenic factors, leading to a higher reprogramming efficiency. Additionally, reprogrammed neurons have been shown to acquire a fully differentiated morphology and functionality (Bocchi et al., 2022).

Notably, selective expression of neurogenic transcription factors dictates the neurotransmitter identity of the astroglia-derived neurons. Expression of the dorsal telencephalic fate determinant neurogenin 2 (Neurog2) directs cortical astroglia to generate glutamatergic neurons (Berninger et al., 2007; Heinrich et al., 2010). On the contrary, expression of the Achaete-scute homolog 1 (Ascl1) drives cortical astrocytes to differentiate into putative GABAergic neurons (**Figure 25**) (Masserdotti et al., 2015).

Thus, although neuronal reprogramming is challenging and still under development, it has already shown promising results in *in vitro* models of brain damage.

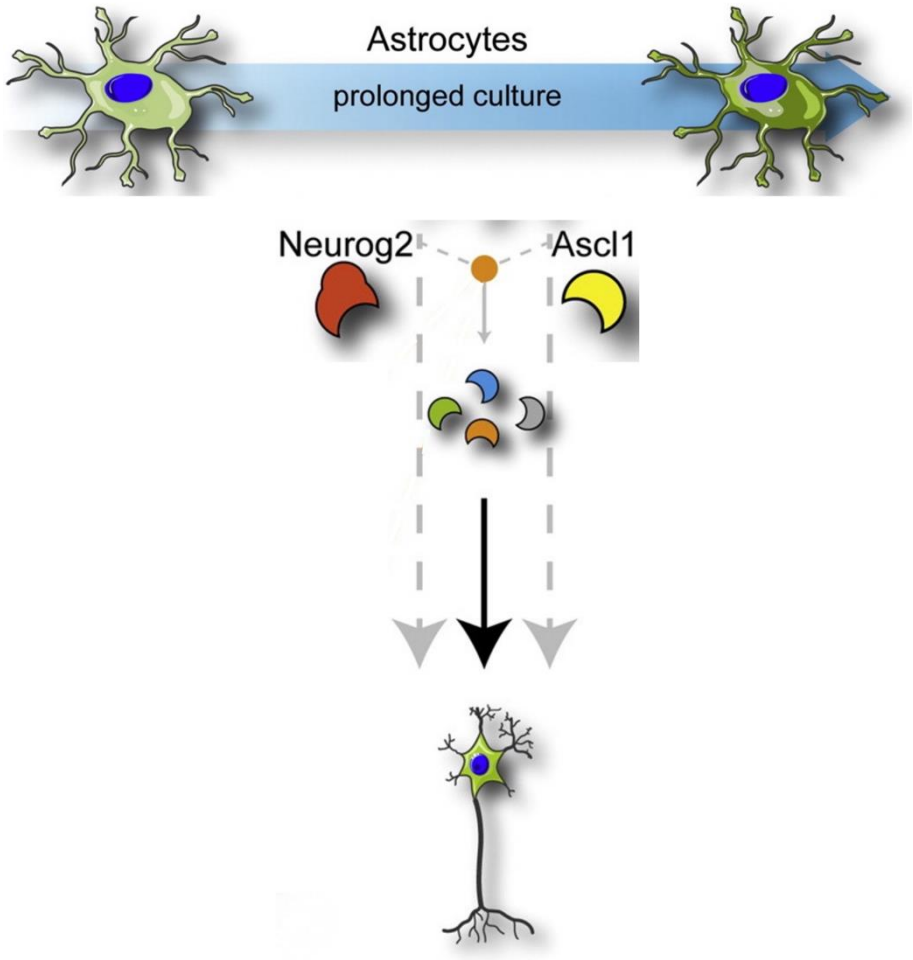


***Figure 25*. Direct glial-to-neuron reprogramming as a novel therapeutic approach for brain repair**. Astrocyte cultures from the postnatal mouse cortex are reprogrammed *in vitro* into different subtypes of neurons by the forced expression of neurogenic transcription factors. Neurog2 drives the development of glutamatergic neurons, whereas Ascl1 specifies the fate of neural progenitors towards GABAergic neurons (Adapted from Masserdotti et al., 2015).

Nrg1 as an emerging target in CNS repair: Nrg1 function in the Neuronal Stem Cells

Nrg1 is a multifaceted protein in the CNS, essential for both neuronal development and cortical circuitry wiring. Studies have shown its involvement in neuronal migration, oligodendrocyte development, axonal myelination, dendrite development, and excitatory/inhibitory synapse formation (Mei et al., 2014, 2008). These functions of Nrg1 in the nervous system development suggest a potential therapeutic role in brain injury repair. This notion is supported by recent evidence highlighting Nrg1's neuroprotective and neuromodulatory effects on CNS repair (Kataria et al., 2019).

Nrg1 treatment in rodent models of brain injury results in increased neuronal survival, reduced injury size (Guo et al., 2006; Y. Li et al., 2007; Navarro-González et al., 2019; Noll et al., 2024a), diminished astrocytes and microglia presence in the perilesional area (Alizadeh et al., 2017), increased oligodendrocyte proliferation (Canoll et al., 1996; Cui et al., 2023; Flores et al., 2000) and decreased neuroinflammation and oxidative stress (Kataria et al., 2019; Noll et al., 2024a). Furthermore, Nrg1 has been also described to promote neurite outgrowth upon injury *in vitro* (Hao et al., 2021; Kataria et al., 2019).

Specifically, demyelination is a crucial indicator of pathology following brain injury. Remyelination, a vital regenerative process, can then enhance functional recovery. In this sense, Nrg1 has been shown to enhance the emergence and growth of oligodendrocytes by promoting its generation from neural precursor cells (NPCs) and oligodendrocyte precursor cells (OPCs) (Calaora et al., 2001; Kataria et al., 2019; Pirotte et al., 2010). This regulation proves beneficial for brain injury recovery by stimulating oligodendrogenesis (Calaora et al., 2001; Cui et al., 2023; Flores et al., 2000) and also by promoting neuronal proliferation and survival (Cui et al., 2023; Kataria et al., 2019; Noll et al., 2024b; Pirotte et al., 2010).

This combined evidence positions Nrg1 as a promising therapeutic candidate for CNS insults (Cui et al., 2023; Kataria et al., 2019; Noll et al., 2024b; Pirotte et al., 2010). Our presented findings on Nrg1's ability to cell-autonomously promote axonal growth, along with existing research, suggest that its intracellular signaling, known to regulate gene expression in the nucleus (Bao et al., 2003), has a positive effect on neuronal maturation, growth and survival (Fazzari et al., 2014; Navarro-González et al., 2019). Here, we tested the working hypothesis that Nrg1 intracellular signaling might also be relevant in the context of astrocyte-to-neuron reprogramming, by promoting either the efficiency of reprogramming or the subsequent development of the induced neurons.

Results

The laboratory of Professor Götz in Munich (Germany), works to elucidate the key mechanisms of neurogenesis, in the developing and adult brain, with the aim to reactivate those mechanisms after brain injury. They have developed the newest insights in direct glia-to-neuron reprogramming, as a novel therapeutic approach following brain injury (Bocchi et al., 2022; Gascón et al., 2016; Heinrich et al., 2010; Masserdotti et al., 2015; Mattugini et al., 2019).

Given Nrg1's established roles in neuronal development and survival, we investigated it signaling in the development and integration of reprogrammed neurons. During a two-month stay at Professor Götz's lab, we expressed Nrg1 in primary mouse astrocyte cultures along with reprogramming factors to assess how Nrg1 intracellular signaling affects reprogramming efficiency, survival and morphological development of the induced neurons.

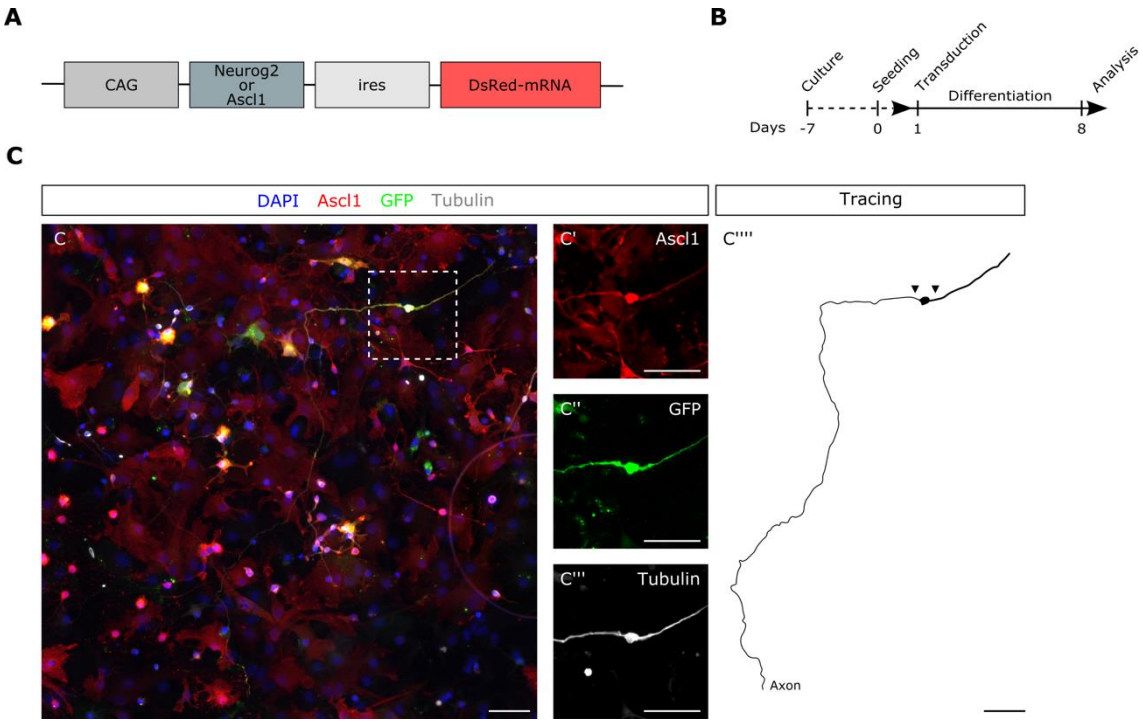
Professor Götz's lab has shown that forced expression of reprogramming factors like Ascl1 and Neurog2 in primary mouse astrocyte cultures can specifically convert glial cells into GABAergic and glutamatergic neurons, respectively (Heinrich et al., 2010; Masserdotti et al., 2015). Specifically, primary cultures of astrocytes were prepared from the gray matter of the cerebral cortex of mice at postnatal days 5-7, as previously described in the Materials and Methods section. After 7 days of proliferation, astrocytes were plated and transfected (data not shown) or transduced with a plasmid encoding either control (pCAG-DsRed), Ascl1 (pCAG-Ascl1-IRES-DsRed) or Neurog2 (pCAG-Neurog2-IRES-DsRed; i) alone as control; ii) together with pCMV-Nrg1-FL-nGPF; iii) with pCMV-Nrg1-ICD-GFP to activate specifically the intracellular signaling (**Figure 26, A and B**).

Nrg1 role in astrocyte-to-neuron reprogramming efficiency

Nrg1 has been demonstrated to play a neuroprotective role in cortical neurons (B.-S. Li et al., 2003). This neuroprotective function is particularly evident under conditions of neuronal stress, such as those encountered during brain injury (Navarro-González et al., 2019). Morphological criteria and expression of canonical neuronal markers were used to assess the Nrg1's effect on the efficiency of astrocyte-to-neuron reprogramming, neuronal survival and their subsequent development into mature neurons.

To assess the role of Nrg1 in astrocyte-to-neuron reprogramming efficiency, we attempted astrocyte transfection and transduction using our generated retroviruses. Due to low transfection efficiency, in this thesis only presents the results obtained from retroviral transduction of cortical astroglia. Cultured astrocytes were transduced to express either Nrg1-FL-GFP or Nrg1-ICD-GFP along with the neurogenic factors Ascl1 or Neurog2 (**Figure 26, A to C**). We evaluated the reprogramming efficiency at DIV8 by calculating the percentage of cells with neuronal morphology that were both βIII-tubulin positive (neuronal marker) and DsRed+GFP double-infected. This same experimental paradigm was employed to investigate the specific role of Nrg1 intracellular signaling (**Figure 26, C and D**).

Here, we present the different individual experiments separately due to high variability. We failed to see an increase in either reprogramming efficiency or the survival of the new neurons upon Nrg1 expression (**Figure 26, D**).



D

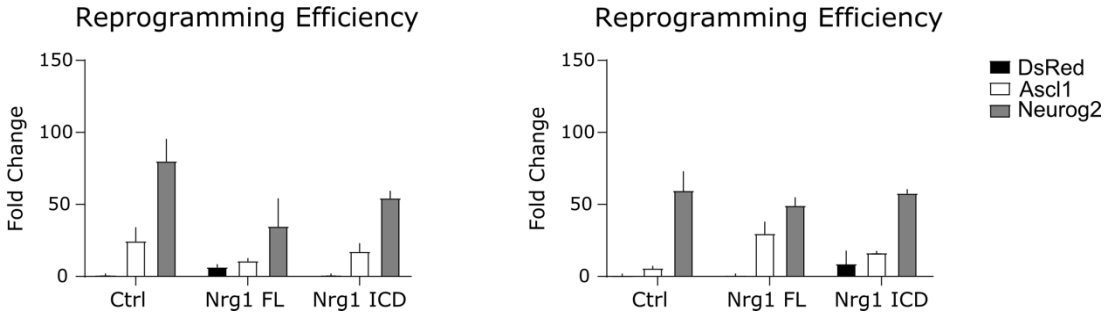


***Figure 26*. Nrg1 does not improve astrocytes-to-neuron reprogramming efficiency.** (A) Schematic representation of retrovirus with expression cassette for miRNAs. Ascl1 and Neurog2 neurogenic factors are expressed in vectors containing DsRed, while Nrg1-FL and Nrg1-ICD are expressed along with a GFP tag. DsRed alone serves as the control for the Ascl1 and Neurog2 transcription factors expression. GFP alone serves as the control for the Nrg1 conditions. (B) Schema summarizing the experimental paradigm. (C) Schematic representation and image of a reprogrammed neuron by forcing the Ascl1 expression in cultured astrocytes. Boxed area depicts the reprogrammed neuron and the magnified areas in the images on the right (panels C', C'' and C'''). The C''' cropped panel shows that the reprogrammed neuron is positive for the neuronal marker βIII-tubulin. A tracing in panel C'''' depicts the reprogrammed neuron's morphology, with the number of branches starting from the soma indicated by black arrowheads. Scale bar, 50 μm. (D) Quantification of the Nrg1's effect on reprogramming efficiency in control, Ascl1 and Neurog2 expressing astrocytes. Results from two independent P7 brains. The N was considered as 3 randomly chosen 20x fields, composed of 4x4 images, per condition. Two-way ANOVA was performed to compare Ctrl, Nrg1-FL and Nrg1-ICD within each condition, meaning the expression of the control plasmid only containing DsRed (Ctrl), DsRed+Ascl1 or DsRed+Neurog2. In all cases, $p > 0.05$. Average ± SEM.

Nrg1 role in reprogrammed neurons development

Our presented results demonstrate that Nrg1 is crucial for cortical axon development. Specifically, Nrg1's intracellular signaling is both necessary and sufficient to promote axon growth in a cell-autonomous manner. To determine whether Nrg1 signaling also promotes neurite development in reprogrammed neurons, we analyzed axonal extension and total branch number from the soma.

We employed the same experimental paradigm described above. Cultured astrocytes were transduced to express either Nrg1-FL-GFP or Nrg1-ICD-GFP along with the neurogenic factors Aslc1 or Neurog2 (**Figure 26, A to C**). Due to the observed

variability between experiments, here we present the results of each experiment separately. We did not observe any consistent effect of Nrg1 on neurite development in mature reprogrammed neurons, positive for the neuronal marker βIII-tubulin, at DIV8 (**Figure 27, A and B**).

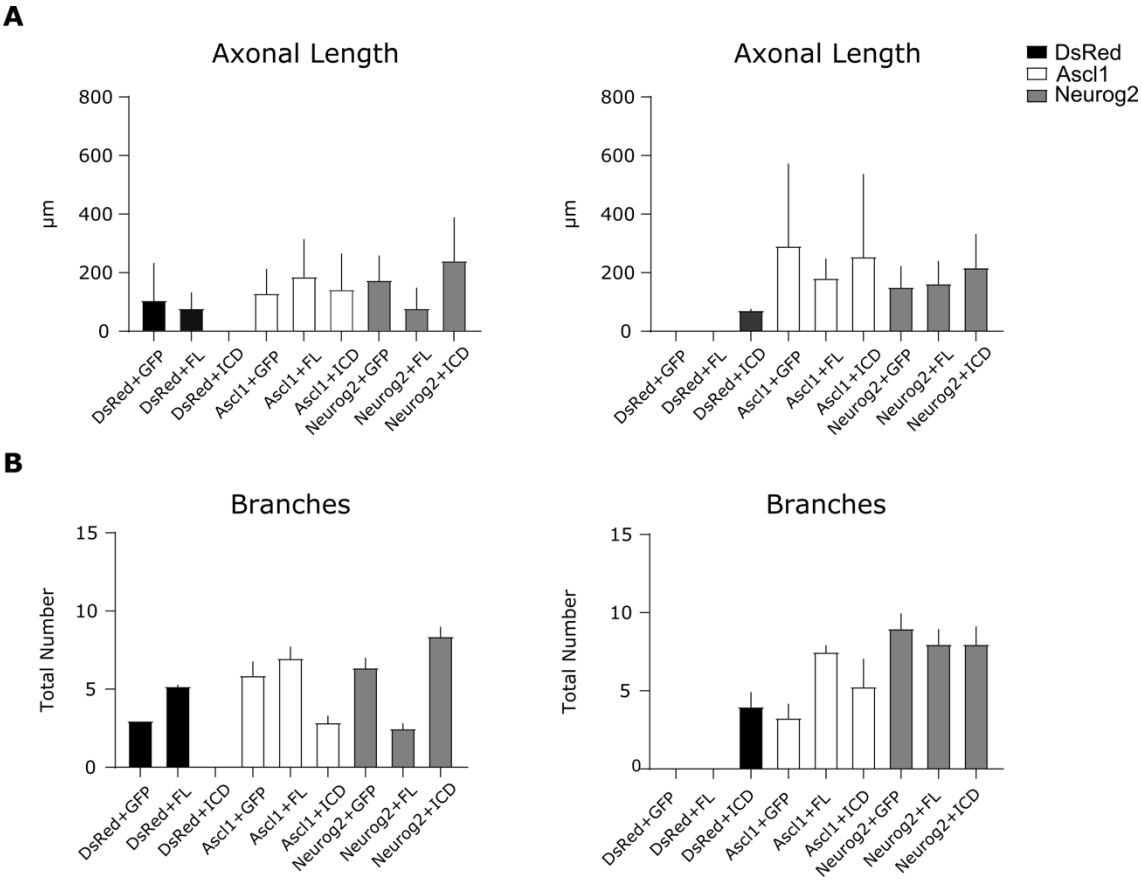


***Figure 27*. Nrg1 could promote neurites development in reprogrammed neurons.** (A) Quantification of the axonal length of Ctrl, Nrg1-FL and Nrg1-ICD in reprogrammed neurons shown in raw values (µm). (B) Quantification of the neuronal processes directly growing from the soma. (A) (B) Both quantifications are from two independent P7 brains. The N was considered as 3 randomly chosen 20x fields, composed of 4x4 images, per condition. Two-way ANOVA was performed to compare Ctrl, Nrg1-FL and Nrg1-ICD within each condition, meaning the expression of the control plasmid only containing DsRed (Ctrl), DsRed+Ascl1 or DsRed+Neurog2. In all cases, $p > 0.05$. Average ± SEM.

Discussion

Nrg1 is a versatile protein within the CNS, playing critical functions in both the development of neurons and in the wiring of the cortical circuits. Because of this, Nrg1 has been widely implicated as a risk factor for psychiatric disorders. However, recent research suggests it may also play a modulatory role in the CNS injury and repair processes (Kataria et al., 2019). Our findings presented in this thesis further support that Nrg1's intracellular signaling is both necessary and sufficient for axonal development.

While there are currently no completely effective treatments for brain injury, neuronal replacement therapies offer a promising approach. In specific, direct reprogramming of local glial cells into neurons is a promising strategy for neuronal replacement. Following neuronal loss, a hallmark of brain injury, some astrocytes contribute to harmful inflammation and scar formation. Reprogramming these astrocytes into neurons offers a potential two-fold benefit: replacing lost neurons and mitigating scar formation while reducing inflammation. Recent years have witnessed significant advancements in glial-to-neuron reprogramming protocols, leading to increased reprogramming efficiency and improved cell survival. Moreover, research demonstrates that reprogrammed neurons can achieve full differentiation, acquiring both the morphology and function of mature neurons. Notably, these induced neurons can develop specific neuronal subtypes and axonal projection identities congruently with their laminar location (Bocchi et al., 2022).

Considering the combined evidence, Nrg1 emerges as a highly promising therapeutic candidate for treating CNS injuries (Kataria et al., 2019). Our findings on Nrg1's ability to promote axonal growth, along with existing research on its role in the proliferation and differentiation of NPCs and OPCs (Calaora et al., 2001; Canoll et al., 1996; Cui et al., 2023; Flores et al., 2000; Pirotte et al., 2010), as well as its neuroprotective properties (Kataria et al., 2019; Navarro-González et al., 2019; Noll et al., 2024a), strongly support further investigation into its potential application in direct neuronal reprogramming strategies.

Thus, building upon the well-established roles of Nrg1 in neuronal development and survival, this study explored its potential role in the reprogramming and integration of newly generated neurons. We investigated Nrg1 signaling by expressing it alongside reprogramming factors in primary mouse astrocyte cultures. Morphological criteria and

expression of canonical neuronal markers were used to assess the Nrg1's effect on the efficiency of astrocyte-to-neuron reprogramming and the morphological development of the induced neurons. Nrg1 did not exhibit a neuroprotective effect on the survival of reprogrammed neurons and did not increase the reprogramming rate. However, our observations suggested a potential role for Nrg1 in promoting neurite outgrowth within reprogrammed neurons. This lack of conclusive results might be partly due to the limited number of individual experiments and the high degree of variability. We cannot definitively rule out the possibility that Nrg1 promotes neurite outgrowth in reprogrammed neurons, as we have seen some effect particularly through its intracellular signaling.

The observed effects on Nrg1's effect on reprogrammed neuronal development require further validation due to the inherent difficulties associated with the experiments. To address the large experimental variability observed, repeating the experiments with a larger sample size is crucial. Additionally, employing a different experimental strategy would allow for the visualization of individual neurons, facilitating a more detailed morphological analysis similar to those presented in Chapter Two.

In addition, it should be noted that direct reprogramming is a promising but also a challenging approach still presenting major limitations. While *in vitro* studies have shown promise for direct neuronal reprogramming, understanding its true potential for treating brain injuries requires *in vivo* approaches. This is because the diseased brain environment significantly impacts reprogramming efficiency and how well new neurons integrate into existing circuits. *In vivo* experiments do not yet show consistent results. Previous studies attempt used retroviruses for gene delivery, but these were less effective *in vivo* compared to *in vitro* methods. Since then, various approaches to improve efficiency have been explored, including combining factors like growth pro-survival efectors (Bocchi et al., 2022).

Additionally, limited cell division in the adult mouse brain, even post-injury, prompted the use of lentiviruses adeno-associated viruses (AAVs), due to their ability to transduce non-dividing cells. This strategy broadens the pool of potential targets, leading to a greater number of induced neurons. While AAVs offer advantages in terms of integration risk, further research is needed due to potential off-target that could lead to adverse effects (Bocchi et al., 2022).

Challenges remain despite the progress in efficiency. Ensuring newly formed neurons are the correct subtype and establishing proper connections with existing circuits are crucial for function and it is still under investigation. Myelination of new axons, another essential aspect, also needs to be addressed (Mattugini et al., 2019).

Therefore, further *in vivo* studies are necessary to demonstrate consistent results and confirm the integration of newly formed neurons into functional circuits. Furthermore, future research is needed to establish more precise methods for generating induced neurons. The CRISPRa technology and the development of blood-brain barrier-permeable AAV capsids hold significant promise, as these tools could enable non-invasive and cell-type specific reprogramming. While overcoming these challenges require further research and refinement of astrocyte-to-neuron reprogramming, this approach for brain injury repair presents exciting prospects for future therapeutic applications.

Our study offers an initial evidence supporting the potential of Nrg1 effect in astrocyte-to-neuron reprogramming, a promising but still evolving approach for brain injury repair. Our findings, as demonstrated earlier in this manuscript, reveal that Nrg1 promotes axonal development. This strongly suggests its potential to enhance neurite outgrowth in reprogrammed neurons. Specifically, further investigations are needed to confirm the Nrg1's role in promoting neurite development in the reprogrammed neurons.

GENERAL DISCUSSION

Cortical wiring in the etiology of SZ

Schizophrenia (SZ) is a very complex neurodevelopmental disorder. Diagnosing and treating it is difficult because there are no clear histological markers and the underlying molecular mechanisms remain unknown (Harrison et al., 2005). One of the most consistent findings in SZ research is the presence of white matter abnormalities, particularly within the corpus callosum (CC) (Arat et al., 2015; David, 1994; Fenlon et al., 2015; Kubicki et al., 2005). The CC, composed entirely of axons, acts as a critical bridge between brain regions, facilitating efficient communication and synchronization of information between cortical hemispheres. Because of this, proper CC development is essential for most aspects of cortical function and it is not surprising that CC deficits were associated with neurodevelopmental disorders (David, 1994; Fenlon et al., 2015; Hoptman et al., 2012; Innocenti et al., 2003). The cellular and molecular underpinnings of the alterations of CC development in SZ remain largely undetermined. The most straightforward explanation is that a reduced development of CC would directly impair interhemispheric communication and information processing.

The role of the SZ-risk gene *NRG1*

There is also strong evidence for a major genetic component related to SZ. Several studies have shown that Nrg1-deficient mice exhibit SZ-related behavioral changes (Nakazawa et al., 2020; Stefansson et al., 2002). Since *NRG1* was identified as a significant SZ risk gene (Harrison et al., 2005; Stefansson et al., 2002), most studies attempted to understand the role of Nrg1 in brain wiring and in cortical inhibition. Collectively, these studies showed that Nrg1 plays a pivotal role in various steps of cortical development and in the wiring of the inhibitory circuits (Y.-J. Chen et al., 2010; Y. Chen et al., 2010; Fazzari et al., 2010; Navarro-Gonzalez et al., 2021; Pedrique et al., 2010; Rahman-Enyart et al., 2020).

Regarding cortical interneurons, it was shown that Nrg1 controls the migration of interneurons precursors from the ganglionic eminence to the cortex (Flames et al., 2004). Postnatally, Nrg1 is important for the activity of cortical interneurons and inhibitory homeostasis (Mei et al., 2014, 2008). Specifically, we and others found that Nrg1

promotes, in ErbB4 expressing inhibitory cells, the formation of excitatory synapses in the dendrites of inhibitory contacts in the axonal button (Y.-J. Chen et al., 2010; Mei et al., 2008; Navarro-Gonzalez et al., 2021). Besides, Nrg1 regulates the development of dendritic spines in pyramidal neurons, probably via its intracellular signaling (Barros et al., 2009; Fazzari et al., 2014).

Interestingly, while most studies in preclinical models have focused on loss of Nrg1/ErbB4 signaling, others have shown that exogenous expression of Nrg1 can also be detrimental to cortical wiring and lead to SZ-like symptoms (Agarwal et al., 2014; Hahn et al., 2006; Olaya et al., 2018; Yin et al., 2013). These findings suggest that maintaining an optimal level of Nrg1 is crucial for the proper balance of excitatory and inhibitory circuits in the cortex (Agarwal et al., 2014).

In contrast to its well-studied role in interneurons, the function of Nrg1 in pyramidal neurons remains unclear. Only a few studies suggested that Nrg1 loss-of-function may impair dendritic development (Q. Zhang et al., 2016). In particular, constitutive deletion of the type III isoform of Nrg1, showed reduced dendritic arborization in mouse embryo (Y. Chen et al., 2010). Moreover, primary cortical neurons from a type III Nrg1 mutant showed impaired dendrite development at DIV3, an early stage of maturation. Nrg1 intracellular signaling could partially rescue the developmental deficits in dendrites (Y. Chen et al., 2010). Overall, most research has focused on the canonical Nrg1/ErbB4 signaling pathway in interneurons, leaving the role of Nrg1 in excitatory neurons, particularly in axonal development, largely unexplored.

Nrg1 intracellular signaling in the excitatory neurons and corpus callosum

Because of its previous association with SZ, we studied the role of Nrg1 in the excitatory neurons, specifically in the development of callosal connections. In addition, since the CC is the largest white matter structure in the brain, its study allowed us to investigate the role of Nrg1 in axonal development in a specific manner, as this Nrg1's function remain largely unaddressed. This way, our study identified a novel role for Nrg1 intracellular signaling in excitatory neurons. We showed that Nrg1 intracellular signaling is necessary and sufficient to promote the axonal development of callosal projections. Notably, because in our experimental settings the deletion of Nrg1 is acute, we can reasonably rule out the caveat of possible early developmental deficits unrelated to the specific process of axonal elongation.

The observed delay in the growth of callosal connections in Nrg1-deficient mice could have significant functional and behavioral consequences. Interhemispheric brain function integration relies heavily on the proper wiring of callosal axons. Also the development of precise contralateral connections is critical for most aspects of cortical function, including basic brain activities and higher cortical functions (Noelia S. De León Reyes et al., 2020; Fenlon et al., 2015). The formation of CC projections involves a tightly regulated sequence of events (Fenlon et al., 2015). This process begins in the embryo with the specification of contralateral projecting neurons and the axonal extension toward the midline, continuing postnatally with the innervation of the contralateral side. In mice, CC axons reach their contralateral target and arborize around P7-P10 (Mizuno et al., 2007; Wang et al., 2007), a critical period for cortical wiring that involves a peak in synaptogenesis and the concomitant activity-dependent apoptosis of cortical inhibitory interneurons (Favuzzi et al., 2019; F. K. Wong et al., 2018). Finally, redundant CC connections are pruned after weaning until P30 (N. S. De León Reyes et al., 2019).

While we did not observe major impairments in corpus callosum thickness in Nrg1 null mice, the developmental delay of callosal axons could still lead to long-lasting deficits in interhemispheric wiring due to the complex interplay between interhemispheric and local intracortical connections. These deficits could potentially affect cognitive and behavioral functions. Given the multiple roles that Nrg1 plays in brain development, it will be very difficult to determine the specific contribution of the deficits we observed in callosal development to the behavioral alterations. Future studies employing *in vivo* loss-of-function approaches at later developmental stages, electrophysiological recordings to assess interhemispheric neuronal activity correlation and behavioral tests, will be necessary to more definitively address the functional consequences of Nrg1 loss. Nevertheless, we speculate that the delay in callosal development may at least partially contribute to the SZ-like symptoms observed in Nrg1 mutant mice.

Our current study focuses primarily on the early stages of callosal development and does not address the role of Nrg1 in later stages of interhemispheric connectivity. While our observation indicated that Nrg1 expression accelerates callosal axon growth, this may not necessarily translate into improved interhemispheric connectivity at later stages. Nrg1-induced accelerated growth could be counterbalanced later by pruning and target refinement. Alternatively, Nrg1-expressing neurons might overshoot their targets and

connect to inappropriate ones. Further studies of Nrg1 expression at further developmental stages should be conducted to investigate the contributions of Nrg1 to interhemispheric synaptic wiring and function.

We complemented our *in vivo* findings with a robust *in vitro* model using primary neuronal cultures and electroporation, with internal controls and sparse labeling. This experimental paradigm was used to further asses the neuronal development in a more reductionist manner. This way, we confirmed that Nrg1 regulates axonal development in a cell-autonomous manner through its intracellular signaling pathway. However, further validation could be achieved using *in vitro* and *in vivo* experiments with a specific Nrg1-ICD mutant.

Nrg1 downstream mechanisms: a role for GAP43

Mechanistically, the precise downstream effectors of Nrg1 intracellular signaling remain elusive. Our data revealed that Nrg1 deletion in neuronal cultures led to a significant decrease in the expression of GAP43 protein levels, a well-known player in axonal growth and regeneration (Allegra Mascaro et al., 2013; Chung et al., 2020; Fallini et al., 2016; Okada et al., 2021). Interestingly, GAP43 expression could cell-autonomously rescue the decrease in axonal development in Nrg1 KO primary cortical neurons *in vitro*. These findings suggest that GAP43 might be a relevant mediator of Nrg1 signaling in axonal development.

Here, we did not further explore GAP43's potential role in dendrite development or *in vivo* approaches. Future research should investigate this mechanism *in vivo*, potentially using models of CNS injury for a more comprehensive understanding. Notably, while we observed a substantial decrease in GAP43 protein levels in Nrg1 knockout mice, GAP43 mRNA levels remained unaltered, suggesting that Nrg1 may regulates GAP43 expression post-transcriptionally. Further research is needed to elucidate the specific post-transcriptional mechanisms by which Nrg1 regulates GAP43 local protein synthesis, protein translation or stability. Moreover, we cannot exclude that others effectors may play an important role in Nrg1 signaling during axonal development.

Nrg1 signaling in astrocyte-to-neuron reprogramming

Nrg1 signaling is not only involved in the etiology of psychiatric disorders but it is also a potential modulator in brain injury and repair (Kataria et al., 2019). Nrg1 can promote functional recovery after stroke by enhancing neuronal survival and mitigating

neuroinflammatory responses (Kataria et al., 2019; Navarro-González et al., 2019; Noll et al., 2024a). Here, we investigated the potential role of Nrg1 in direct astrocyte-to-neuron reprogramming, a novel approach of neuronal replacement for brain repair. We found that Nrg1 most likely does not have a neuroprotective effect on the survival of reprogrammed neurons and does not increase the reprogramming rate but may promote neurite outgrowth in the reprogrammed neurons. Our experiments require further validation with a larger sample size due to the observed high experimental variability. Additionally, due the large advances in direct reprogramming *in vivo*, it would be interesting to re-evaluate our results using Nrg1 KO mice for an *in vivo* approach.

Exploring the protective and neuromodulatory roles of Nrg1 and GAP43 in cortical regeneration following brain damage, particularly within the context of therapeutic approaches like direct reprogramming, would be a valuable future direction to follow.

Main conclusions

In conclusion, our study identifies a novel role for the Nrg1 schizophrenia-risk gene in the development of cortical circuits. We specifically demonstrated the function of Nrg1's intracellular signaling in excitatory neurons. Our findings show that Nrg1 is both necessary and sufficient to promote the axonal development of callosal projections *in vivo*. Additionally, our *in vitro* experiments in primary cortical neurons demonstrated that single-cell deletion of Nrg1 impairs axon development. Conversely, single-cell expression of Nrg1-FL or Nrg1-ICD, to selectively activate Nrg1 intracellular signaling, were sufficient to promote axon growth. Mechanistically, we found that Nrg1 signaling regulates the expression of the GAP43 protein, a key regulator of axonal growth.

Altogether, our study provides a novel perspective on the role of Nrg1 and its intracellular signaling in SZ, highlighting the need for further research into the functional and behavioral impacts of disrupted Nrg1 signaling on cortical connectivity.

CONCLUSIONS

In this thesis, we aimed to study the role of Nrg1 in cortical circuits development. We specifically focused on understanding its cell-autonomous function in the excitatory neurons.

The main conclusions of this thesis are:

1. We have developed a robust *in vitro* model using primary neuronal cultures and electroporation, with internal controls and sparse labeling. This model facilitates the study of neuronal morphology, integrity and survival in various neuropathologies. It has been used to study the p.D163E PRDX3 mutation and to rigorously investigate the cell-autonomous role of Nrg1 in neurite outgrowth.
2. We showed that Nrg1 is necessary for the development of interhemispheric connections *in vivo*. The deletion of Nrg1 impaired the development of callosal axons, indicating that Nrg1 is essential for the proper formation of the corpus callosum, the major interhemispheric connection formed by cortical excitatory neurons.
3. We found that the activation of Nrg1 intracellular signaling is sufficient to promote axonal growth *in vitro* and *in vivo*. Specifically, Nrg1 cell-autonomously promoted the growth of callosal axons *in vivo*. These findings provided a novel perspective on the role of Nrg1 and its intracellular signaling in schizophrenia.
4. This study identified GAP43 as a critical downstream effector of Nrg1 signaling in axonal development. Nrg1 KO mice displayed a strong depletion of GAP43 protein. Furthermore, GAP43 expression rescued the impaired axonal development observed in Nrg1-deficient neurons.
5. We assessed the potential role of Nrg1 in the context of reprogramming astrocytes to neurons as a novel therapeutic approach following brain injury. We found that Nrg1 expression in reprogrammed neurons do not enhance reprogramming efficiency but could promote neurites development in reprogrammed neurons.

Supplementary Material S1. **Media composition used for primary neuronal cultures.**

Plating Medium Composition	**ml**
Minimum Essential Medium (MEM)	42.5
10% Horse serum or FBS	5
30% glucose (0.6% final concentration)	1
PS (Penicillin 1000 U/ml; Streptomycin 10mg/ml)	0.5

Neuronal Medium Composition	**ml**
Neurobasal medium	48.5
B-27	1
Glutamax 200mM	0.125
PS (Penicillin 1000 U/ml; Streptomycin 10mg/ml)	0.5

Supplementary Material S2. **Electroporation conditions for cell transfection by using the NEPA21 electroporation system.**

Poring Pulse					
Length	V	Interval	N	D. Rate	Polarity
2 ms	175	50	2	10	plus
Transfer Pulse					
Length	V	Interval	N	D. Rate	Polarity
50	20	50	5	40	plus/minus

Supplementary Material S3. **Fiji code for image analysis pre-processing.** Signal normalization in the GAP43 axonal length rescue experiments.

```
run("Split Channels");
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel global");
run("Subtract Background...", "rolling=25");
run("Fire");
//run("Brightness/Contrast...");
run("Enhance Contrast", "saturated=0.45");
run("Apply LUT");
run("8-bit");
```

Supplementary Material S4**. Fiji code for image pre-processing and analysis of the astrocyte-to-neuron reprogramming experiments.**

Channels threshold normalization.

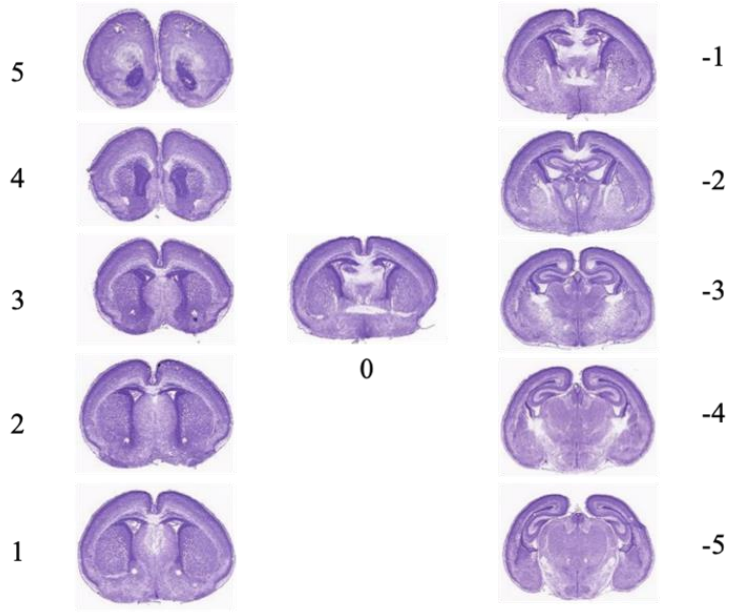
```
// DAPI
Stack.setActiveChannels("1000");
setMinAndMax(2000, 3000);
// 2nd ch GFP (Nrg1)
Stack.setActiveChannels("0100");
setMinAndMax(450, 4000);
// 3rd ch Transcr Factor
Stack.setActiveChannels("0010");
setMinAndMax(900, 6000);
// 4th Tubulin
Stack.setActiveChannels("0001");
setMinAndMax(0, 3000);
```

Images scale down and split.

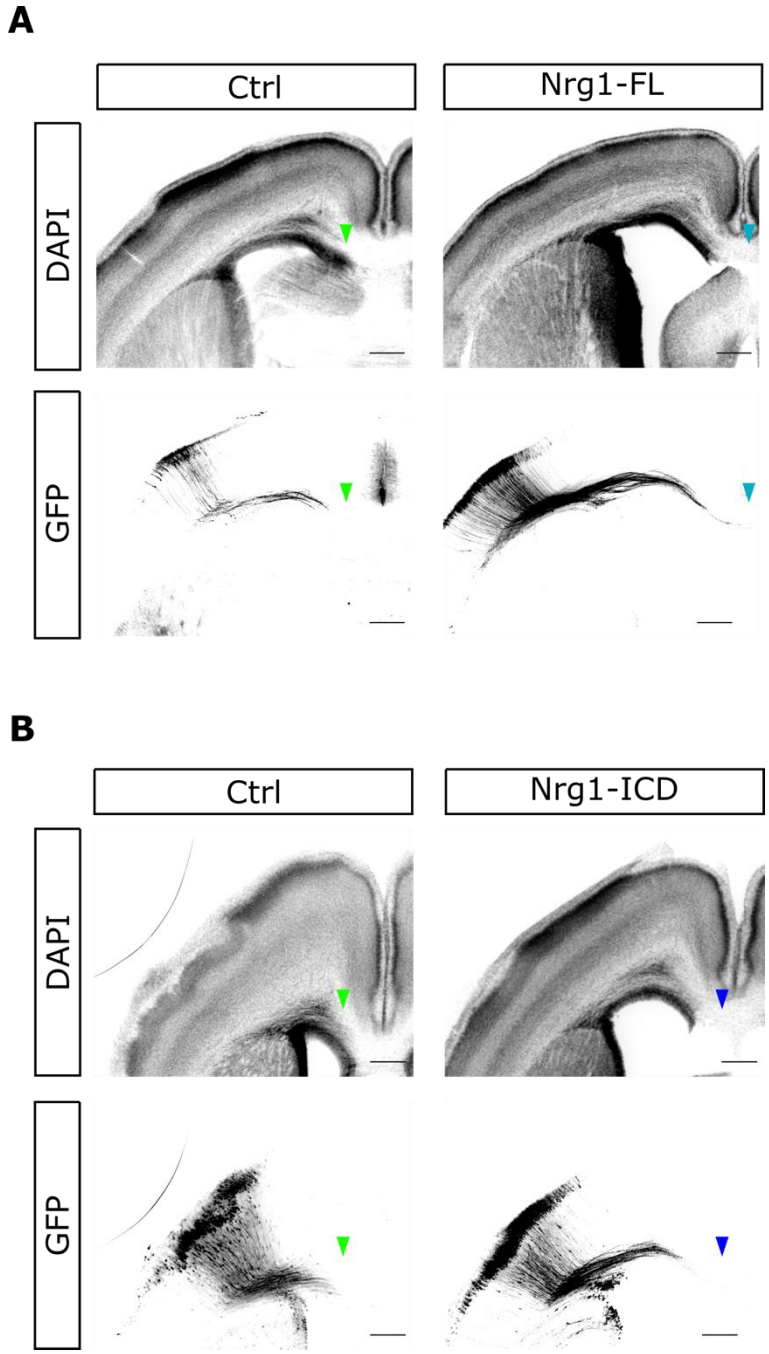
```
sd=getTitle()
print( sd );
run("Scale...", "x=0.4 y=0.4 z=1.0 width=4104 height=3294 depth=4 interpolation=Bilinear average create");
name=sd + "_DN_.tif";
rename(name)
saveAs("Tiff");
close();
//
sd=getTitle()
print( sd );
dir = getDir("file")
rename("s")
run("Split Channels");
// first channel
selectWindow("C1-s");
rename("C1-" + sd)
sc1 = getTitle()
saveAs("Tiff", dir + sc1);
close();
// second
selectWindow("C2-s");
rename("C2-" + sd)
sc1 = getTitle()
saveAs("Tiff", dir + sc1);
```

```
close();
// third
selectWindow("C3-s");
rename("C3-" + sd)
sc1 = getTitle()
saveAs("Tiff", dir + sc1);
close();
// 4th
selectWindow("C4-s");
rename("C4-" + sd)
sc1 = getTitle()
saveAs("Tiff", dir + sc1);
close();
```

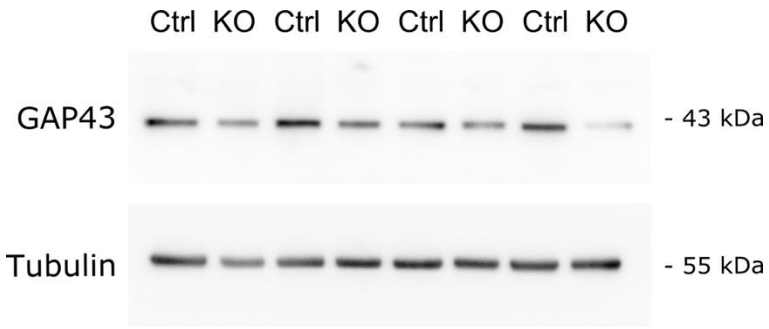
Supplementary Material S5. **P1 mice sections manually classified by using the open source Allen Brain Atlas: Developing Mouse Brain P1.** The position given in the manually created atlas is shown next to each reference section. We used the Allen Brain Atlas at P1, Nissl, coronal sections, section #110 as the 0 reference (http://developingmouse.brain-map.org/experiment/siv?id=100102322&imageId=101634745&initImage=nissl).



Supplementary Figure S6. **Low magnification of the representative images Nrg1-FL, Nrg1-ICD and their respective control littermates presented in the Figure 18 (Chapter 2).** (A) (B) The pictures show the GFP labeling and the DAPI counterstaining. The DAPI staining helps to identify and classify the slice on the rostro-caudal axis, according to the Allen Brain Atlas: Developing Mouse Brain P1. The callosal projections from electroporated L2/3 neurons approaching the midline are indicated with arrowheads. Scale bar, 300 µm.



Supplementary Figure S7. **GAP43 protein expression is highly reduced in Nrg1 KO mice.** Raw (uncropped and unmodified) images of Western blot analysis showing the expression levels of GAP43 protein in control (Ctrl) and Nrg1-deficient neurons (KO) at DIV4. Tubulin levels are shown as loading control.



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