UNIVERSITAT POLITÈCNICA DE VALÈNCIA

OPTOGENETIC STIMULATION OF NEURAL PROGENITOR CELLS AS NEUROREGENERATIVE IMPROVEMENT IN THE TREATMENT OF SPINAL CORD INJURY

Bachelor's thesis

Bachelor's Degree in Biotechnology

Academic year 2019/2020

Universitat Politècnica de València

Escola Tècnica Superior d'Enginyeria Agronòmica i del Medi Natural

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València, July 2020

TITLE: OPTOGENETIC STIMULATION OF NEURAL PROGENITOR CELLS AS NEUROREGENERATIVE IMPROVEMENT IN THE TREATMENT OF SPINAL CORD INJURY.

TÍTULO: ESTIMULACIÓN OPTOGENÉTICA DE CÉLULAS PROGENITORAS NEURALES COMO MEJORA NEURORREGENERADORA EN EL TRATAMIENTO DE LA LESIÓN MEDULAR.

TÍTOL: ESTIMULACIÓ OPTOGENÈTICA DE CÈL·LULES PROGENITORES NEURALS COM A MILLORA NEUROREGENERADORA EN EL TRACTAMENT DE LA LESIÓ MEDUL·LAR.

ABSTRACT:

Spinal cord injury (SCI) implies remarkable health, social and psychological consequences, since damage to the spinal cord can result in permanent motor, sensory and/or autonomic dysfunction. However, still no effective therapy that allows considerable functional recovery of SCI patients exists. Currently, one of the most promising therapeutic approaches under investigation is cell therapy, where transplantation of ependymal neural progenitor cells (NPCs) lining the central canal of the spinal cord is showing encouraging results. NPC transplantation represents a potential therapy aimed at replacing the lost neural tissue and modulating the lesion microenvironment reducing the barriers to neuroregeneration and/or promoting it. Nonetheless, still much work is needed to overcome its limitations (such as poor survival or reduced functional integration), which make evident the need for combination with additional strategies.

In this work, we propose optogenetics as a candidate to improve cell therapy outcomes. Optogenetics represents an innovative approach combining optical and genetic methods. Through the ectopic expression of photosensitive proteins, this technique allows for a precise temporal and spatial control of specific cell populations upon photostimulation. The most commonly used photosensitive proteins are microbial opsins, which are light-gated ion channels that enable the control of cell membrane ion flux and the generation of action potentials in neuronal circuits. Based on previous studies, we hypothesised that optogenetic stimulation of NPCs *in vitro* before transplant or *in vivo* after transplant might increase their neuroregenerative capabilities, namely promoting survival and proliferation, improving neurite growth and functional integration, increasing the secretion of neurotrophic factors and enhancing NPC differentiation into neurons and oligodendrocytes.

Based on this hypothesis, the aim of this work was to study the effect of *in vitro* optogenetic stimulation on ependymal NPCs. Primary cultured NPCs were transduced using an adeno-associated viral vector to ectopically express the excitatory opsin channelrhodopsin-2 (ChR2). Flow cytometry analysis revealed successful mCherry-tagged ChR2 expression, although transduction efficiency was low. Viability assays showed that ectopic ChR2-mCherry expression was not affecting NPC viability. *In vitro* optogenetic stimulation of NPCs under proliferation conditions promoted their capability to grow neurites, although it did not increase their proliferation and viability. Furthermore, *in vitro* optogenetic stimulation of NPCs under differentiation conditions enhanced their differentiation into oligodendrocytes and neurons, which moreover displayed an increased axon length and branching. Also, the predominant morphology of NPC-derived astrocytes changed from a protoplasmic to a fibrous morphology. To our knowledge, this is the first time that the application of optogenetics is studied in the context of cell therapies to treat SCI. Our results highlight its great potential as a neuroregenerative improvement in the treatment of SCI.

RESUMEN:

La lesión medular implica consecuencias sanitarias, sociales y psicológicas considerables, ya que el daño a la médula espinal puede resultar en disfunción motora, sensorial y/o autonómica permanente. Sin embargo, aún no existe ninguna terapia efectiva que permita una recuperación funcional considerable de los pacientes con lesión medular. Actualmente, una de las aproximaciones terapéuticas más prometedoras bajo investigación es la terapia celular, en la cual el trasplante de células progenitoras neurales (NPCs) ependimarias que recubren el canal central de la médula espinal está mostrando resultados esperanzadores. El trasplante de NPCs representa una potencial terapia dirigida a reemplazar el tejido neural perdido y modular el microambiente de la lesión reduciendo las barreras a la neurorregeneración y/o promoviéndola. No obstante, aún se requiere mucho trabajo para superar sus limitaciones (como una supervivencia pobre o una integración funcional reducida), las cuales evidencian la necesidad de combinación con estrategias adicionales.

En este trabajo, proponemos la optogenética como candidata para mejorar los resultados de la terapia celular. La optogenética representa una aproximación innovadora que combina métodos ópticos y genéticos. Mediante la expresión ectópica de proteínas fotosensibles, esta técnica permite un control temporal y espacial preciso de poblaciones celulares específicas al ser fotoestimuladas. Las proteínas fotosensibles más comúnmente empleadas son las opsinas microbianas, que son canales iónicos regulados por luz que posibilitan el control del flujo iónico a través de la membrana celular y la generación de potenciales de acción en circuitos neuronales. Basándonos en estudios previos, hipotetizamos que la estimulación optogenética de NPCs *in vitro* antes del trasplante o *in vivo* tras el trasplante podría aumentar sus capacidades neurorregeneradoras, concretamente promoviendo la supervivencia y proliferación, mejorando el crecimiento de neuritas y la integración funcional, aumentando la secreción de factores neurotróficos y fomentando la diferenciación de NPCs a neuronas y oligodendrocitos.

En base a esta hipótesis, el objetivo de este trabajo era estudiar el efecto de la estimulación optogenética *in vitro* sobre las NPCs ependimarias. NPCs procedentes de cultivo primario fueron transducidas empleando un vector viral adeno-asociado para expresar ectópicamente la opsina excitatoria canalrodopsina-2 (ChR2). El análisis por citometría de flujo reveló la expresión exitosa de ChR2 marcada con mCherry, aunque la eficiencia de transducción fue baja. Los ensayos de viabilidad demostraron que la expresión ectópica de ChR2-mCherry no estaba afectando a la viabilidad de las NPCs. La estimulación optogenética *in vitro* de NPCs en condiciones de proliferación promovió su capacidad para formar neuritas, aunque no aumentó su proliferación y viabilidad. Asimismo, la estimulación optogenética *in vitro* de NPCs en condiciones de diferenciación fomentó su diferenciación a oligodendrocitos y neuronas, que además mostraron una longitud y ramificación axonales aumentadas. Del mismo modo, la morfología predominante de los astrocitos derivados de NPCs cambió de una morfología protoplásmica a una fibrosa. Hasta donde sabemos, esta es la primera vez que se estudia la aplicación de la optogenética en el contexto de las terapias celulares para tratar la lesión medular. Nuestros resultados ponen de manifiesto su gran potencial como mejora neurorregeneradora en el tratamiento de la lesión medular.

KEY WORDS: spinal cord injury; neural progenitor cells; optogenetics; channelrhodopsin-2; adeno-associated virus; cell therapy; neuroregeneration.

PALABRAS CLAVE: lesión medular; células progenitoras neurales; optogenética; canalrodopsina-2; virus adeno-asociado; terapia celular; neurorregeneración.

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This work was elaborated at the Príncipe Felipe Research Centre (CIPF), in the Neuronal and Tissue Regeneration laboratory.

València, July 2020

ACKNOWLEDGEMENTS/AGRADECIMIENTOS

Este trabajo, de algún modo, marca el final de una etapa. Mis estudios de grado en biotecnología llegan a su fin tras cuatro años y llegados a este punto quiero echar la vista atrás y dedicar este espacio para dar las gracias a todas aquellas personas que me han acompañado en este tiempo.

En primer lugar, quiero agradecer a mis padres toda su dedicación a mi educación y a la de mi hermana, porque han volcado todos sus recursos y esfuerzos en proporcionarnos la mejor formación posible. Mamá, papá, todo lo que he logrado en esta vida ha sido gracias a vosotros, por anteponer mi bienestar al vuestro, por el tiempo que me habéis dedicado, por el apoyo que me habéis brindado, por los buenos valores que me habéis inculcado, por darme fuerzas para seguir adelante y alcanzar mis metas, por la libertad que me habéis proporcionado en toda y cada una de mis decisiones. Gracias a mi hermana Sara, por todo lo que hemos vivido juntos y por ayudarme en los momentos difíciles, así como a mi cuñado y a mis sobrinos. Sara, de ti he aprendido que en esta vida hay que ser valiente y que ninguna adversidad debe hacerme perder la alegría. Gracias a mis abuelos y yayos, por apoyarme durante toda esta etapa de mi formación académica. Gracias también a mis tíos y tías y, en general, a toda mi familia porque siempre han estado ahí, por los momentos felices que he pasado con ellos y por los que vendrán. Gracias.

También quiero dar las gracias a mi tutora Esther, quien me ha guiado en mis inicios en la investigación. Gracias, por todo lo que he aprendido contigo y por lo mucho que me has apoyado de cara a mis proyectos futuros. Gracias, Vicky, por permitirme formar parte del grupo I-44, donde me he sentido muy bien acogido desde el primer momento. Gracias, Mara, por todo lo que me enseñaste en mis primeras semanas en el laboratorio (incluidos los secretos del western blot…) y porque con esa jovialidad que irradias le alegras el día a cualquiera. Gracias, Marina, por descubrirme trucos imprescindibles en el día a día del laboratorio (incluida la legendaria técnica del pipeteo inverso). Gracias, Sonia, por esas charlas que tanto me ayudaban en momentos de estrés. Gracias, Eric, por ilustrarme en el complejo mundo de la microscopía y porque los momentos de trabajo en el ordenador no hubiesen sido lo mismo sin ti en el puesto de al lado. Gracias, Bea, por tu amabilidad y buen trato conmigo. Gracias, Ana, por ayudarme cuando lo necesitaba. Gracias, Pablo, por esas conversaciones espontáneas sobre el mundo de la ciencia y la investigación. En resumen, gracias a todos los miembros del I-44, tanto los actuales como tantos otros con los que también compartí mis días de laboratorio, por hacerme sentir como uno más, por el compañerismo y por las risas que siempre estaban presentes.

En estos agradecimientos no podían faltar mis amigos y amigas. A todas mis amistades, tanto a aquellas más antiguas como a las más recientes, gracias, porque formáis una parte esencial de mi vida. En especial, gracias a mis compañeros y compañeras de la carrera, porque juntos hemos formado, sin lugar a duda, una gran piña. De esta carrera me llevo amistades muy especiales con las que he compartido momentos felices y tristes, noches de estrés antes de los exámenes y noches de fiesta tras ellos, viajes y excursiones inolvidables, días de verano fantásticos, comidas y cenas entre risas… Gracias, porque habéis hecho que esta carrera sea mucho más que una experiencia académica.

Por último, quería mostrar mi gratitud hacia aquellos profesores y profesoras que, a lo largo de mis estudios, desde el colegio hasta la universidad, destacaron y fueron importantes para mí por su pasión por la enseñanza y por su especial capacidad de transmitir ese interés a los alumnos. La docencia, algo tan esencial para el futuro de la sociedad, no sería lo mismo sin ellos y sin ellas. En especial, quiero dar las gracias a aquellos que me aconsejaron en mis decisiones académicas y me prestaron su apoyo en la persecución de mis metas.

En definitiva, gracias a todos vosotros por haber formado parte de esta etapa de mi vida tan satisfactoria y que culmino con este trabajo.

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AAV	Adeno-associated virus
AAV9	Adeno-associated virus serotype 9
AKT	Protein kinase B
APC	Adenomatous-polyposis-coli
BDNF	Brain-derived neurotrophic factor
BL	Blue light
BSA	Bovine serum albumin
BSCB	Blood-spinal cord barrier
bFGF	Basic fibroblast growth factor
CaM	Calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
ChR2	Channelrhodopsin-2
CNS	Central nervous system
CREB	cAMP-responsive element-binding protein
CSPG	Chondroitin sulphate proteoglycan
DAG	Diacylglycerol
DAMP	Danger-associated molecular pattern
DAPI	4’,6-diamidino-2-phenylindole
DLK	Dual leucine zipper-bearing kinase
DMEM/F-12	Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12
DPBS	Dulbecco’s phosphate-buffered saline
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPAC2	Exchange protein directly activated by cAMP
ERK	Extracellular-signal-related kinase
FBS	Foetal bovine serum
GAP43	Growth-associated protein 43
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthase kinase 3β
HDAC5	Histone deacetylase 5
hESC	Human embryonic stem cell
hChR2	Humanised channelrhodopsin-2
Ip3	Inositol trisphosphate
iPSC	Induced pluripotent stem cell

JNK	JUN N-terminal kinase
LED	Light emitting diode
MEK	MAPK/ERK kinase
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NFAT	Nuclear factor of activated T cells
NGF	Nerve growth factor
NGS	Normal goat serum
NPC	Neural progenitor cell
NT-3	Neurotrophin-3
Olig2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte progenitor cell
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PMS	Phenazine methosulfate
PNS	Peripheral nervous system
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
Ras-GEF	Ras-Guanine nucleotide exchange factor
SCI	Spinal cord injury
ULA	Ultra-Low Attachment
VGCC	Voltage-gated calcium channel

1. INTRODUCTION

1.1. SPINAL CORD INJURY.

Spinal cord injury (SCI) arises from damage inflicted to the spinal cord and it can result in permanent motor, sensory and/or autonomic disfunction below the level of the injury, as well as in secondary debilitating or even life-threatening conditions (vein thrombosis, chronic pain, osteoporosis, etc.). This damage results from trauma in about 90% of cases (mainly traffic crashes, falls and violence). Traumatic SCI will be the main focus of the following subsection, but it is important to remark that SCI can also arise from degeneration or disease (e.g. cancer). Functional implications vary depending on the anatomical level (being cervical SCI the most severe one, with functional impairments such as respiratory insufficiency and tetraplegia), and on the size of the lesion (distinguishing complete and incomplete lesions, the latter sparing some neural connectivity) (Alilain *et al.*, 2008; Assinck *et al.*, 2017; Dulin & Lu, 2014; Sofroniew, 2018; WHO, 2013). The estimated global annual incidence is between 250 000 and 500 000 cases. It carries enormous social and psychological consequences, as well as great healthcare costs associated with the management of SCI patients (Dalamagkas *et al.*, 2018; WHO, 2013).

1.1.1. PATHOPHYSIOLOGY OF SPINAL CORD INJURY.

An acute traumatic insult to the spinal cord triggers a series of complex multicellular and molecular responses that can be divided into three main phases: (1) physical trauma and primary injury, (2) inflammation and secondary injury, and (3) maturation of the glial scar (Tran *et al.*, 2018). These events have been summarised in Fig. 1.

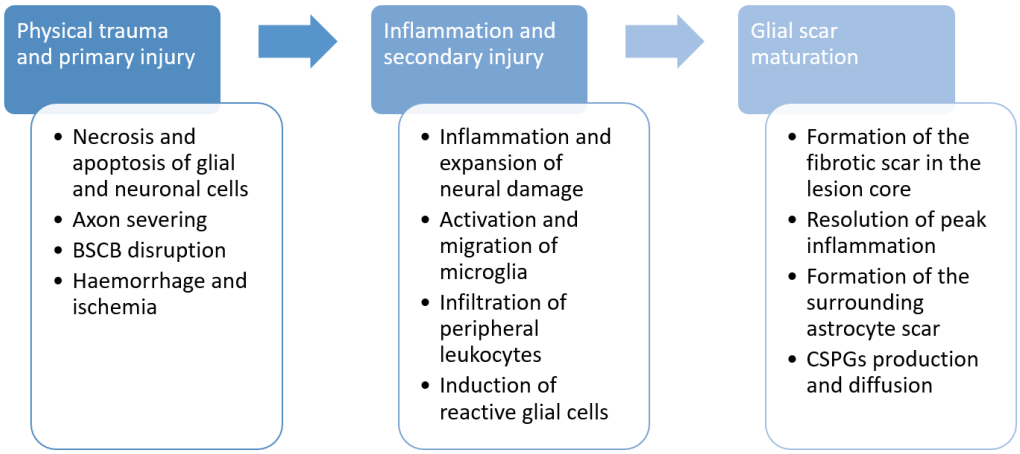

Figure 1. Main phases in the pathophysiology of spinal cord injury. *Spinal cord injury can be divided into three main phases including physical trauma and primary injury, secondary injury, and glial scar maturation. BSCB: Blood-spinal cord barrier; CSPG: Chondroitin sulphate proteoglycan.*

Physical trauma and primary injury

The physical trauma that causes SCI leads to a massive cell death of neurons and glial cells accompanied by local changes in ion levels and the release of neurotransmitters by the surviving neurons resulting in neuronal excitotoxicity. At the same time, physical trauma also causes severing of ascending and descending axons (axotomy), where the distal portion of the axon separated from the neuronal soma undergoes the so-called Wallerian degeneration, as well as degradation of the myelin sheaths (Burda & Sofroniew, 2014; Tran *et al.*, 2018).

The concomitant vascular disruption causes the rupture of the protective blood-spinal cord barrier (BSCB), haemorrhage and oedema, inducing coagulation, vasospasm and vasoconstriction. Resulting ischemia and hypoxia leads to further necrosis within and near the lesion site (Burda & Sofroniew, 2014; Tran *et al.*, 2018).

Inflammation and secondary injury

These early events in the first hours post-trauma unleash a secondary injury cascade, which can last for weeks and which is characterised by inflammation and expansion of neural damage to adjacent tissue, including extensive oligodendrocyte death with the resulting demyelination and axon degeneration. The release of cytokines, chemokines, danger-associated molecular patterns (DAMPs) and other molecules from dead cells and through the disrupted BSCB trigger inflammation at the injury site, with the activation of central nervous system (CNS)-intrinsic microglia and recruitment of peripheral immune cells, such as neutrophils and monocytes-macrophages. Macrophages and microglia are pushed towards a pro-inflammatory M1 phenotype, potentiating inflammation via release of pro-inflammatory cytokines. This M1 phenotype is thought to impair recovery from SCI, promoting retraction of damaged axons and inducing secondary neurodegeneration (Kigerl *et al.*, 2009; Tran *et al.*, 2018). The inflammatory response also induces a reactive state in astrocytes and oligodendrocyte progenitor cells (OPCs) with a subsequent increase of local proliferation, migration and secretion of pro-inflammatory factors. Astrocytes become hypertrophied and accumulate around the lesion core, and OPCs envelope and stabilise the ends of dystrophic axons to protect them from further dying back due to the potent inflammatory environment (Burda & Sofroniew, 2014; Tran *et al.*, 2018).

Maturation of the glial scar and delimitation of the lesion compartments

Approximately 2 to 10 days after the initial insult, certain cell types locally migrate and proliferate to promote tissue repair and replacement (e.g. endothelial cells for neovascularisation). Perivascular fibroblasts and pericytes proliferate and form the fibrotic component of the glial scar at the lesion core. As peak inflammation resolves and inflammatory cells recede, the fibrotic lesion core matures, usually presenting fluid-filled cysts of variable sizes. Reactive astrocytes form the so-called astrocyte scar that surrounds the damaged tissue thus creating a protective barrier that isolates the non-neural lesion core and prevents the spread of inflammation to the surrounding viable neural tissue. At the same time, however, this scar poses a barrier to axon regeneration, acting as a physical as well as a chemical barrier through the secretion of axon-growth inhibitory extracellular matrix (ECM) molecules, such as those belonging to the family of chondroitin sulphate proteoglycans (CSPGs) (Burda & Sofroniew, 2014; Sofroniew, 2018; Tran *et al.*, 2018). Once the glial scar has matured, the surrounding spared neural tissue has the potential to undergo tissue remodelling and circuit reorganisation, which could end up bringing about some functional recovery (Burda & Sofroniew, 2014). However, this circuit remodelling is subjected to numerous factors that hinder successful neuroregeneration in the CNS, as discussed in the next subsection.

1.1.2. NEUROREGENERATION: MOLECULAR MECHANISMS AND FAILURE IN THE CENTRAL NERVOUS SYSTEM.

The differing regenerative capacity of axons in the CNS as compared to the peripheral nervous system (PNS) has been known for years. After axotomy, axons in the adult PNS are able to regenerate, whereas axons in the CNS fail to re-grow. On the one hand, this phenomenon has been attributed to the inability of CNS neurons to reactivate intrinsic growth programmes after injury. After axotomy of regeneration-competent PNS neurons, an immediate influx of Ca^{2+} at the severed axonal tip is rapidly propagated to the soma by voltage-gated calcium channels (VGCCs), accompanied by Ca^{2+} release from the endoplasmic reticulum (Mahar & Cavalli, 2018; van Niekerk *et al.*, 2016). This initial Ca^{2+} signal is crucial for neuroregeneration (Chierzi *et al.*, 2005), being involved in membrane resealing (Ordaz *et al.*, 2017), formation of the so called growth cone (imperative for axon re-growth) via microtubule reorganisation and reduction of membrane tension (Kamber *et al.*, 2009), and activation of signalling molecules, transcription factors and epigenetic modifiers involved in neuroregeneration. After the initial Ca^{2+} wave, these

injury-activated signalling molecules are retrogradely transported from the axon to the nucleus, activating a pro-regenerative transcription profile in the damaged neuron. Relevant pro-regenerative signalling pathways (which can potentially be activated by the initial Ca^{2+} signal) include cyclic adenosine monophosphate (cAMP)/exchange protein directly activated by cAMP 2 (EPAC2), dual leucine zipper-bearing kinase (DLK)/JUN N-terminal kinase (JNK), calmodulin (CaM)/Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), rat sarcoma (Ras)/extracellular-signal-related kinase (ERK), and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/ mammalian target of rapamycin (mTOR) signalling (Mahar & Cavalli, 2018; van Niekerk *et al.*, 2016; Wei *et al.*, 2016). These pathways promote axon re-growth and activate pro-regenerative transcription factors like cAMP-responsive element-binding protein (CREB), phosphorylation of which can be mediated by Ras/ERK, CaM/CaMKII and cAMP/EPAC2 signalling (Tao *et al.*, 1998; Wei *et al.*, 2016; Wu *et al.*, 2001). Also, pro-regenerative epigenetic changes through increased histone acetylation also occur downstream of the initial Ca^{2+} wave. However, in the case of CNS neurons, activation of some of these intrinsic events fails, thus leading to a reduced capability of these neurons to revert to a growth-competent state (Mahar & Cavalli, 2018).

On the other hand, there are also a series of extrinsic impediments in the CNS, including the glial scar as a possible physical barrier to neuroregeneration and the inhibitory lesion microenvironment containing a wide range of axon-growth inhibitors (ECM inhibitors like CSPGs and CNS myelin-derived inhibitors like Nogo). Binding of these molecules to their corresponding receptors on CNS neurons conveys a downstream signalling that activates Rho GTPases, such as the RhoA/ROCK signalling pathway, which promotes neurite retraction and growth cone collapse via microtubule destabilisation. These inhibitors, however, are absent in the PNS (Fujita & Yamashita, 2014; Sofroniew, 2018; Tran *et al.*, 2018; van Niekerk *et al.*, 2016). Also, another potential mechanism of CNS regeneration failure includes the absence of growth facilitators in the CNS as compared to the PNS, where Schwann cells secrete neurotrophic factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF). These neurotrophins activate some of the above-mentioned pro-regenerative signalling pathways, including the ERK and the PI3K/AKT/mTOR pathways (van Niekerk *et al.*, 2016).

All in all, comparison with the PNS evidences the wide number of intrinsic and extrinsic barriers to neuroregeneration after SCI, which constitute the target of the different therapeutic approaches under investigation over the years.

1.1.3. THERAPEUTIC APPROACHES: CELL THERAPY.

Despite many research efforts with promising outcomes in the last decades, still no effective therapy that allows considerable functional recovery of SCI patients has been translated to the clinic. Current therapeutic strategies under investigation include (1) neuroprotection to diminish secondary damage to neural tissue in the early phases after injury and (2) neuroregeneration allowing axon re-growth and functional recovery by regulating intrinsic factors (promoting the intrinsic capability of neurons to regenerate) and extrinsic factors (replacing the inhibitory microenvironment in the injured spinal cord by a more growth-permissive one) (Alastrue-Agudo *et al.*, 2018; Sofroniew, 2018; van Niekerk *et al.*, 2016).

Both neuroprotection and neuroregeneration can be achieved by cell transplantation, thus converting it in a potential therapy for SCI. In the last years, different cell types have been assayed for this purpose both in preclinical and early-phase clinical studies, including Schwann cells, olfactory ensheathing cells, OPCs, mesenchymal stem cells, umbilical-cord blood-derived mononuclear cells and neural progenitor cells (NPCs) (Dalamagkas *et al.*, 2018; Assink *et al.*, 2017). Among these cell types, transplantation of NPCs shows encouraging results in experimental models with regards to regeneration of lost neural tissue and functional improvements (Hofstetter *et al.*, 2005; Moreno-Manzano *et al.*, 2009). In fact, some ongoing

early-phase clinical trials are applying NPC transplantation in humans. Among these, the INSPIRE study carried out by InVivo Therapeutics using combinations of biomaterials with NPC transplant in 20 acute SCI patients is showing promising results (CLINICALTRIALS.GOV, 2019; Dalamagkas *et al.*, 2018). These NPCs are multipotent progenitors that can differentiate into neurons, astrocytes and oligodendrocytes (Assink *et al.*, 2017).

Ependymal cells lining the central canal of the spinal cord constitute a source of NPCs. Indeed, during postnatal development, these cells divide symmetrically contributing to the elongation of the ependymal canal of the spinal cord. Under physiological conditions in the adult spinal cord, these ependymal NPCs remain quiescent and some symmetric division takes place just for ependymal cell maintenance (Barnabé-Heider *et al.*, 2010; Panayiotou & Malas, 2013; Sabelström *et al.*, 2014). However, after SCI they actively proliferate, and the generated cell progeny migrates to the lesion site, where it preferentially differentiates into astrocytes contributing to scar formation. However, this endogenous response is insufficient, since for instance the replacement of dead oligodendrocytes after injury (responsible for axon demyelination) from endogenous cells (mainly OPCs) is incomplete and not fully efficient (Barnabé-Heider *et al.*, 2010; Panayiotou & Malas, 2013; Sabelström *et al.*, 2014).

Consequently, transplantation of NPCs derived from the ependymal canal offers a promising therapeutic tool, which has been proposed to act by a series of mechanisms that contribute to neuroprotection and neuroregeneration. With regard to neuroprotection, transplanted NPCs can mitigate secondary injury after SCI through the secretion of bioactive molecules including trophic factors and cytokines, which potentially improve host cell survival and angiogenesis, and beneficially modulate inflammation and reactive gliosis (Assink *et al.*, 2017; Hawryluk *et al.*, 2012). Particularly regarding inflammation, NPC transplantation has been demonstrated to dampen its neurodegenerative effects by modulating the inflammatory cell infiltrate, e.g. reducing the proportion of pro-inflammatory M1 macrophages (Cusimano *et al.*, 2012). Concerning neuroregeneration, NPC transplantation has been proposed to promote axon regeneration by two main mechanisms: bridge formation and relay formation (Fig. 2).

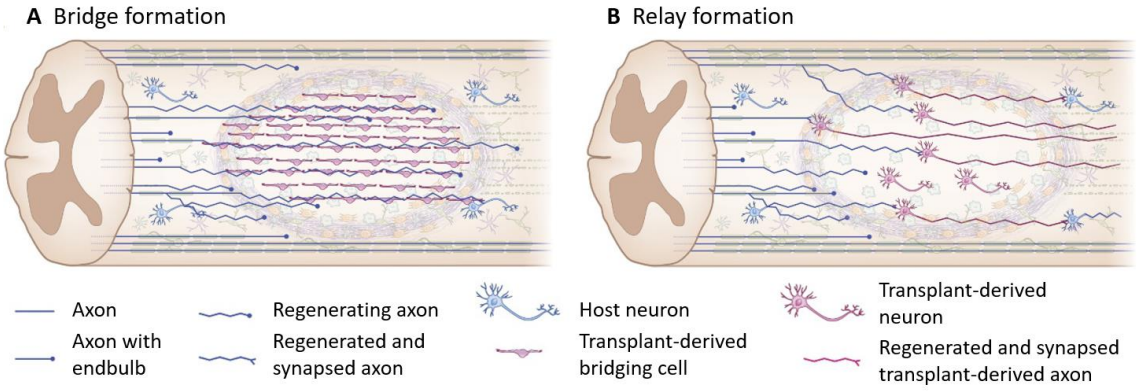

Figure 2. Mechanisms of neuroregenerative improvement by NPC transplantation in SCI. *(A) Transplanted NPCs support endogenous axon growth forming a "bridge" across the lesion. (B) Transplanted NPC-derived neurons form relays by establishing synaptic connections with host neurons. Adapted from Assink et al. (2017).*

In the first case (Fig. 2A), NPCs form multicellular structures that act as a "bridge" crossing the glial scar and provide a growth-permissive substrate (thanks to transplanted NPC-secreted molecules such as laminin) onto which endogenous axons can grow (Assink *et al.*, 2017; Dulin & Lu, 2014). Moreover, these growth-permissive conditions generated by transplanted NPCs have recently been demonstrated to sustain the activation of the pro-regenerative transcriptome in neurons after SCI, which would otherwise be downregulated as soon as two weeks post-injury (Poplawski *et al.*, 2020).

In the case of the formation of neuronal relays (Fig. 2B), this approach relies on the capability of transplanted NPCs to differentiate into neurons, such that these NPC-derived neurons can grow neurites, extend axons that project rostrally or caudally to the injury and stablish synaptic connections with host neurons, thus forming functional relay circuits (Assink *et al.*, 2017; Dulin & Lu, 2014; Lu *et al.*, 2012). An additional neuroregenerative mechanism that has also been attributed to NPCs includes myelin regeneration because of their ability to differentiate into oligodendrocytes able to re-myelinate spared demyelinated axons, improving conduction and reducing neurodegeneration (Assink *et al.*, 2017; Karimi-Abdolrezaee, 2006).

Despite the promising results reported, these cell therapies also come with certain problems which can limit functional recovery after transplant. First, poor cell survival after transplantation is one of the main obstacles to cell therapy, leading to a reduction of the above-mentioned benefits due to a lower number of live NPCs at the lesion site. This is thought to be due to the hostile cytotoxic microenvironment in acute SCI and insufficient vascularisation in the injured area (Lu *et al.*, 2014). Additionally, a very important point for cell therapy to be functionally beneficial is that transplanted cells functionally integrate in the spinal cord circuitry (Assink *et al.*, 2017). Indeed, some experimental evidence has proven that axon growth in transplanted NPC-derived neurons is not always beneficial, for instance, aberrant axon sprouting after NPC transplant has been associated with undesired side effects such as allodynia (pain caused by a normally non-painful stimulus) (Hofstetter *et al.*, 2005). Another drawback found when transplanting NPCs is that despite their multipotency, transplanted NPCs preferentially differentiate into astrocytes, rather than neurons or oligodendrocytes (Barnabé-Heider *et al.*, 2010; Lu *et al.*, 2014; Panayiotou & Malas, 2013). Moreover, the growth of endogenous axons onto NPCs bridges has shown varying results depending on the axon population, with very limited outcomes in the case of corticospinal tracts, which are the ones involved in voluntary movements and which however show the lowest intrinsic growth capacity (Assink *et al.*, 2017).

The limitations seen in the sole application of NPC transplants evidence the need to combine cell therapy with complementary therapeutic strategies (pharmacological approaches, neurotrophin and/or growth factor delivery, biocompatible scaffolds, etc.). For instance, complementary infusion of combinations of growth factors, along with NPC embedding in a fibrin matrix, have been proven to enhance NPC survival and proliferation after transplant (Lu *et al.*, 2014; Panayiotou & Malas, 2013). At this point, it is also relevant to mention the use of biomaterials, such as fibrin matrices, in combination with cell transplant to limit NPC migration to other areas of the CNS, which may carry undesired side effects, such as abnormal activity or tissue compression (Assink *et al.*, 2017). Regarding the need for functional integration of the transplanted cells, approaches such as genetic manipulation could help solving this problem (Hofstetter *et al.*, 2005). In any case, directing functional integration has been proven to be complex, and this may require the use of activity-based approaches that promote neuronal maturation and functional synapse formation with host circuits, thus avoiding aberrant or non-functional connections (Assink *et al.*, 2017). Apart from survival, proliferation and functional integration, combinatorial approaches can also be implemented to manipulate NPC differentiation and favour neuron and oligodendrocyte generation, as it has been assayed using growth factors and genetic manipulation approaches (Lu *et al.*, 2014; Panayiotou & Malas, 2013). Ultimately, neurotrophin delivery (e.g. BDNF) or genetic manipulation of growth-associated genes can also be used to improve the intrinsic regenerative capacity either of the injured endogenous CNS axons or of the transplanted NPCs (Dulin & Lu, 2014; Lu *et al.*, 2012).

Taking into account all the above-mentioned limitations of cell therapy in SCI and how using additional strategies can help improving its outcomes, new approaches directed at manipulating cell biology can be studied to apply them in the context of cell therapy for the treatment of SCI. In this sense, the application of the innovative technique of optogenetics will be explored in the present work, and the rationale behind discussed in the following section.

1.2. APPLICATION OF OPTOGENETICS IN THE TREATMENT OF SPINAL CORD INJURY.

1.2.1. OPTOGENETICS AND ITS MAJOR COMPONENTS.

Optogenetics is an innovative technology combining optical and genetic methods. Since its first application in neuronal cultures (Boyden *et al.*, 2005), huge advances have been made in this field with the development of a vast array of optogenetic tools. In brief, cells are first genetically engineered to express light-sensitive proteins, which upon exposure to light of a particular wavelength will become active and have a series of effects on the targeted cells, including their activation, inhibition or modulation of specific signalling events (Deisseroth, 2011; Guru *et al.*, 2015; Ordaz *et al.*, 2017). The major advantage that optogenetics provides is a precise spatial and temporal control (at the millisecond scale) of specific cell populations, as opposed to electrical stimulation, which lacks spatial precision activating non-target cells, or pharmacological therapies, with relatively lower temporal precision. The precision of optogenetics is especially useful when working with the complexity of the nervous system, allowing precise neuromodulation both for the study of neural circuits and for avoidance of off-target effects in clinical applications (Montgomery *et al.*, 2016; Ordaz *et al.*, 2017). To achieve this precise control of cells, this technology requires three major components: (1) photosensitive proteins, (2) ectopic protein expression systems and (3) light delivery.

Photosensitive proteins

Photosensitive proteins are used as an optogenetic "switch" that modulates the activity of the cell where they are expressed. This modulation will vary in its effects depending on the type of protein chosen. The most studied and widely used proteins are opsins, which are naturally occurring light-sensitive transmembrane proteins found across many different species. Overall, they can be divided in two main classes: microbial or type I opsins, or animal or type II opsins. Type I opsins are 7-transmembrane domain proteins found in bacteria, archaea and algae and work as ion channels or pumps, whereas type II opsins are found in animal cells, where they function in vision and regulation of circadian rhythms. They are G protein-coupled receptors, such that the changes they induce in cells are generally slower as compared to type I opsins, being this the main reason why microbial opsins are more widely employed in neuroscience (Deisseroth, 2011; Guru *et al.*, 2015; Yizhar *et al.*, 2011). The mechanism by which these proteins can become activated upon light exposure involves a molecule called retinal, which is a form of vitamin A. For opsins to be light-sensitive, they require association with retinal. When a photon is absorbed by the retinal molecule, it induces its isomerisation, also called photoisomerisation, which induces a conformational change in the opsin that activates it (Guru *et al.*, 2015).

The optogenetic toolbox has greatly expanded over the years, including opsins directly employed as they are found in nature or synthetic opsins that have been engineered to optimise their properties, including expression, membrane trafficking, photocurrent (i.e. ion current when exposed to light), kinetics, excitation spectrum or light sensitivity (Deisseroth, 2011; Guru *et al.*, 2015; Ordaz *et al.*, 2017). Based on their effect on the cell, opsins can be mainly divided into three types: excitatory opsins, inhibitory opsins and opsins for optogenetic control of intracellular signalling (Fig. 3). Excitatory opsins like channelrhodopsins are cation channels that induce cell depolarisation upon light activation, whereas inhibitory opsins include chloride channels (iC1C2), chloride pumps (eNpHR) and proton pumps (eBR) (mainly belonging to or being derived from the families of halorhodopsins, archaeorhodopsins or bacteriorhodopsins) and induce cell hyperpolarisation upon light activation (Guru *et al.*, 2015; Habibey *et al.*, 2020; Ordaz *et al.*, 2017). Finally, opsins like mammalian rhodopsin-derived optoXRs allow for the direct control of intracellular signalling by modulating different second messengers, e.g. cAMP, inositol trisphosphate (Ip3) or diacylglycerol (DAG) (Airan, 2009). Here other photosensitive proteins can also be used, such as plant cryptochromes and phytochromes, enabling the design

of fusion proteins with light-responsive domains, allowing for specific light-mediated control of protein activity, localisation, clustering, gene expression, etc. (Tischer & Weiner, 2014).

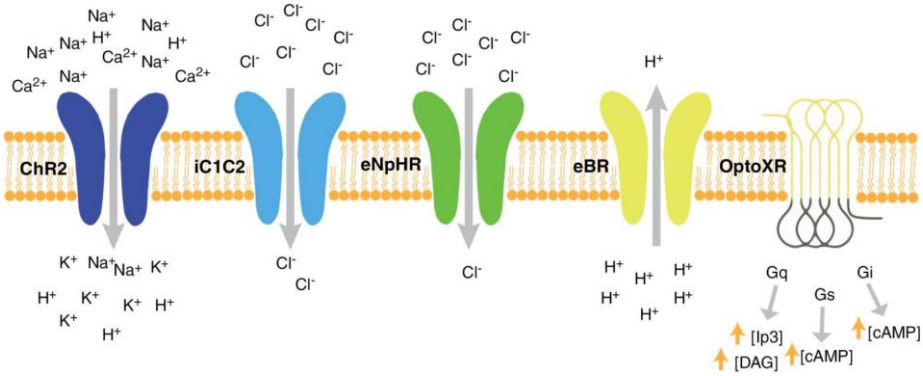

Figure 3. Main opsin types used in optogenetics. *Excitatory opsins such as channelrhodopsin-2 (ChR2) induce cell depolarisation allowing cation diffusion into the cell by electrochemical gradient upon activation. Inhibitory opsins include chloride channels (iC1C2), chloride pumps (eNpHR) and proton pumps (eBR), which induce cell hyperpolarisation upon activation. Mammalian rhodopsin-derived optoXRs allow for the direct control of intracellular signalling by modulating different second messengers, such as cAMP, inositol trisphosphate (Ip3) or diacylglycerol (DAG). Retrieved from Guru et al. (2015).*

The opsin chosen for the present project is the excitatory opsin channelrhodopsin-2 (ChR2), which is a light-gated non-specific cation channel discovered in the unicellular green alga *Chlamydomonas reinhardtii* that opens upon blue light (approx. 460 nm) exposure, allowing the rapid passage of cations (Na^+, Ca^{2+}, H^+) into the cell by electrochemical gradient, and the subsequent cell depolarisation (Fig. 3) (Boyden *et al.*, 2005; Habibey *et al.*, 2020; Guru *et al.*, 2015; Ordaz *et al.*, 2017). More specifically, we use humanised ChR2 (hChR2), which has been codon-optimised for a better expression in mammalian systems (Beyeler *et al.*, 2014). Moreover, this version carries the H134R missense mutation, which induces larger photocurrents upon activation as compared to wild-type ChR2 (Nagel *et al.*, 2005).

Ectopic protein expression systems

The efficiency of optogenetic modulation depends on the sufficiency and specificity of photosensitive protein (e.g. opsin) expression. The most widely used system for opsin delivery is cell transduction using replication-deficient viral vectors (used in gene therapy) packaged with the opsin gene, which is usually combined with a fluorescent tag such as mCherry to detect transduced cells, as it is the case in this work. This method can potentially provide spatial specificity via the site of viral injection, the tissue tropism of the virus (natural or artificially engineered) and the use of cell type specific promoters (Guru *et al.*, 2015; Ordaz *et al.*, 2017, Yizhar *et al.*, 2011). Since in our case we want to express the opsin in *in vitro* cultured NPCs, we do not require this spatial specificity, thus the ubiquitous CAG promoter will be used, which is a synthetic strong promoter obtained by the fusion of the cytomegalovirus enhancer with the chicken beta-actin promoter (Hitoshi, 1991). Among the different viral vectors available, we chose adeno-associated viruses (AAVs), which are useful for clinical applications because they display low immunogenicity and do not have any known associated pathologies, provide a wide tissue tropism from 13 serotypes, and are safer than lentiviruses because they do not normally integrate into the host genome (only at low frequencies), reducing the risk of insertional mutagenesis. One concern, however, is their small packaging capacity (4.7 kb), limiting the use of large cell-type specific promoters (Ambrosi *et al.*, 2019; Kantor *et al.*, 2014; Yizhar *et al.*, 2011). In particular, we chose serotype 9 (AAV9) for this project since it has been found to have the highest tropism for the CNS (Kantor *et al.*, 2014). Another opsin expression system used in optogenetics (although not translatable to humans) is transgenesis in animal models, which can be combined with specific promoters or conditional expression systems such as Cre-loxP for spatial and/or temporal specificity of opsin expression (Guru *et al.*, 2015; Ordaz *et al.*, 2017).

Finally, it is important to remark that cell transplantation is an attractive approach for optogenetic manipulation, especially in pathologies such as SCI, where cell therapy is a promising therapeutic strategy under investigation. By transplanting opsin-expressing cells (e.g. previously transduced *in vitro*), cell specificity is guaranteed, and immunogenic consequences found in the case of direct viral injection to the host could be minimised using progenitor cells from human origin, or even autologous cell transplants, as recently enabled by the induced pluripotent stem cell (iPSC) technology (Montgomery *et al.*, 2016). This strategy allows to optogenetically stimulate transplanted cells in a very specific way (Habibey *et al.*, 2020).

Light delivery

In the case of *in vitro* studies like ours, light delivery is easy, and can be implemented using a laser or light emitting diodes (LEDs) coupled to a microscope or specifically designed devices. *In vivo* light delivery is certainly more complex. For optogenetic stimulation of superficial tissues (e.g. superficial layers of the cortex), LED bulbs can be implanted over the target region. However, for stimulation of deeper tissues, small-diameter optical fibres coupled to LEDs or laser systems are used (Guru *et al.*, 2015; Ordaz *et al.*, 2017). Also, recording systems coupled to these stimulation tools allow for simultaneous stimulation and recording of neural activity (e.g. "optrodes", which combine an optical fibre with a recording electrode) (Habibey *et al.*, 2020; Ordaz *et al.*, 2017). Light delivery to the spinal cord is certainly a challenge because of the relative motion of this organ when the organism moves. However, applying optogenetics to SCI has been enabled by the recent development of small, wireless, fully internal implants for light delivery in animal models, which can be minimally invasive by implanting it dorsally over the dura matter surrounding the spinal cord (Montgomery *et al.*, 2015; Samineni *et al.*, 2017).

All in all, optogenetics is a powerful tool especially in the field of neuroscience. Since its first experimental implementation in 2005 by Boyden *et al.*, this technology has been widely used for studies relating to neural circuitry, neurophysiology and pathological processes of neurological diseases (Ordaz *et al.*, 2017). For instance, its use has been essential in understanding complex processes including sleep (Adamantidis *et al.*, 2007), learning (Schroll *et al.*, 2006) or addiction (Witten *et al.*, 2010). However, there are few studies of its application to the treatment of neurological diseases, such as SCI, but the results obtained so far highlight its high potential to be used in the treatment of SCI, as discussed in the next subsection.

1.2.2. POTENTIAL BENEFITS OF APPLYING OPTOGENETICS IN THE TREATMENT OF SPINAL CORD INJURY.

As indicated previously, application of optogenetics to treatment of SCI is very recent. Two main approaches can be used, which differ mainly in the cells targeted for optogenetic modulation. They include (1) optogenetic modulation of host circuits of the spinal cord or (2) optogenetic modulation of cell transplants in the context of cell therapy, either *in vitro* before transplant as a preconditioning of the cells to be transplanted or *in vivo* after transplant.

Optogenetic modulation of host circuits of the spinal cord

Some studies within the field of SCI have used optogenetics to interrogate functional synapse formation, thus applying it as a tool to assess the effectivity of specific therapeutic strategies, such as genetic manipulation (Dias *et al.*, 2018; Jayaprakash *et al.*, 2016). Nonetheless, in these studies optogenetics just constitutes a validation step and is not applied as a direct therapeutic improvement. In any case, optogenetic modulation of host circuits has been already applied as a direct therapeutic intervention in a cervical 2 hemisection rat model of cervical SCI, where descending bulbospinal inputs to respiratory motor neurons in the ipsilateral phrenic nucleus of the spinal cord are interrupted, thus leading to unilateral hemidiaphragm paralysis and respiratory insufficiency (Alilain *et al.*, 2008). Phrenic motor

neurons were transduced after hemisection, with subsequent expression of ChR2. Intermittent optogenetic stimulation of the denervated phrenic motor neurons led to recovery of normal hemidiaphragmatic activity in synchrony with the non-lesioned side. Moreover, long periods of intermittent optogenetic stimulation resulted in long-lasting recovery of rhythmic diaphragm activity even after the termination of photostimulation, indicating that optogenetic stimulation was inducing neuroplasticity leading to long-lasting functional recovery (Alilain *et al.*, 2008). Likewise, optogenetics has been studied in the context of stroke, a similar CNS injury also displaying neuronal death and scar formation. Neuronal optogenetic stimulation in stroke models has been shown to result in significant improvement in cerebral blood flow, increase in the expression of the axon growth and synaptic plasticity marker growth-associated protein 43 (GAP43), increased expression of neurotrophins (BDNF, NGF, NT-3), and an improved functional recovery (Cheng *et al.*, 2014; Shah *et al.*, 2017). Interestingly, another work showed that optogenetically-increased neuronal activity promotes oligodendrogenesis and myelination (key for proper conduction speed) and improves motor behaviour (Gibson *et al.*, 2014). Even if this was evaluated under physiological conditions and not in the context of SCI, these outcomes might also arise from neuronal optogenetic stimulation after SCI, favouring functional recovery.

Optogenetic modulation of cell transplants

One of the first studies combining optogenetics with cell transplantation was performed by Weick *et al.* (2010), who used optogenetic stimulation to demonstrate successful *in vivo* synaptic integration of transplanted human embryonic stem cell (hESC)-derived neurons expressing ChR2. This study was followed by others also applying optogenetics in the context of cell therapy to assess if transplanted cells were functionally integrated into host circuitry and whether this integration was functionally beneficial (Habibey *et al.*, 2020; Steinbeck *et al.*, 2015). However, these studies just apply optogenetics as a tool to interrogate functional integration of transplanted cells, rather than applying it as a therapeutic improvement, which is the subject of this work. In fact, to our knowledge there are no previously published studies implementing optogenetics as a neuroregenerative improvement to cell therapies for the treatment of SCI. Nevertheless, some examples exist in the context of stroke, where *in vivo* optogenetic stimulation of NPC transplants in animal stroke models led to pro-regenerative improvements, including increased long-term survival of transplanted cells, enhanced axon sprouting and growth, improved synaptic plasticity and connectivity, increased axon myelination and activity-dependent neuronal maturation, and ultimately better functional recovery (Daadi *et al.*, 2016; Yu *et al.*, 2019). Also, *in vitro* optogenetic stimulation resulted in increased expression of synaptic proteins and pro-regenerative BDNF, and promoted neurite growth (Yu *et al.*, 2019). In another study, optogenetic stimulation of ESCs-derived motor neurons transplanted into a ligated sciatic nerve in mice induced activity in the paralysed muscles (Bryson *et al.*, 2014). However, in this case optogenetics was not applied to improve regeneration in a CNS lesion.

Additionally, optogenetics has a great potential to modulate progenitor differentiation, for example, *in vitro* optogenetic stimulation of bipotent glial progenitor cells increased oligodendrocyte differentiation and reduced astrocyte differentiation, and transplant of these previously stimulated progenitors to mice where demyelination in the spinal cord had been induced resulted in remyelination and a significant motor functional recovery (Ono *et al.*, 2017). Also, optogenetic stimulation of differentiating ChR2-expressing mouse ESCs promoted their commitment to the neural lineage and their final differentiation into neurons (Stroh *et al.*, 2011).

In conclusion, based on all these previous works, we hypothesise that *in vitro* and/or *in vivo* optogenetic stimulation of NPCs used for cell therapy to treat SCI could potentially improve the regenerative capacities of NPCs, namely improved survival and proliferation, increased expression and secretion of growth and neurotrophic factors (e.g. BDNF), enhanced neurite growth and increased differentiation into neurons and oligodendrocytes. To our knowledge, this is the first time that optogenetics is applied in the context of cell therapy for SCI treatment.

2. OBJECTIVES

The hypothesis on which this work is based is that optogenetic stimulation of NPCs *in vitro* before transplant and/or *in vivo* after transplant in the context of cell therapy for the treatment of SCI could improve their neuroregenerative capabilities through the modulation of cellular and molecular events in these cells, including enhancing proliferation, increasing neurite growth and promoting differentiation into neurons and oligodendrocytes, thus potentially improving cell therapy outcomes and subsequent functional recovery.

Accordingly, the main objective of this work is to study the effect of *in vitro* optogenetic stimulation on ependymal NPCs. Particular objectives include:

1. Assessing the efficiency of transduction of NPCs using an adeno-associated viral vector for ectopic ChR2 expression.
2. Assessing the effect of transduction and subsequent ChR2-mCherry expression on NPC viability.
3. Assessing the effect of optogenetic stimulation on NPC proliferation and viability and on NPC neurite growth.
4. Assessing the effect of optogenetic stimulation on NPC spontaneous differentiation into neurons, oligodendrocytes and astrocytes.
5. Assessing the effect of optogenetic stimulation on axon length and branching of NPC-derived neurons.

3. MATERIALS AND METHODS

ETHICAL STATEMENT REGARDING THE USE OF ANIMALS

Sprague Dawley rats were used for the experiments. The maintenance and use of all animals were in accordance with the Spanish National Guide for the Care and Use of Experimental Animals (*Real Decreto* 1201/2005 of the *Ministerio de Presidencia*) and the Animal Care Committee of the Príncipe Felipe Research Centre.

3.1. PRIMARY CULTURE OF NEONATAL RAT NEURAL PROGENITOR CELLS.

NPCs for *in vitro* assays were obtained from neonatal (P4-6) female Sprague Dawley rats. The procedure for NPC primary culture obtention has been summarised in Fig. 4. After complete laminectomy, spinal cords were dissected and meninges and blood vessels were cleared away. The extracted tissue was cut into 1 mm^3 pieces and mechanically homogenized without enzymatic treatment in ice-cold washing medium (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) 1:1 (Gibco) supplemented with penicillin and streptomycin 0.5 X (Sigma)). After centrifugation at 200 x g for 4 min, three phases were observed: washing medium in the upper phase, a yellow phase containing the NPCs in the middle, and a bottom white phase consisting of tissue debris. The upper phase was withdrawn with the vacuum aspirator and the middle yellow phase was collected and mechanically disrupted using a P200 pipette. Next, prewarmed washing medium was added (up to 3/4 ml) and the tube was centrifuged at 250 x g for 4 min. The supernatant was removed with the vacuum aspirator and the resulting cell pellet containing the NPCs was resuspended in 200 µl of prewarmed NPC complete proliferation medium (NeuroCult™ Proliferation Medium (STEMCELL) supplemented with NeuroCult™ Proliferation Supplement (STEMCELL), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 0.7 U/ml heparin (Sigma), 20 ng/ml epidermal growth factor (EGF; Invitrogen) and 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen)). Isolated NPCs were finally seeded in Ultra-Low Attachment (ULA) culture plates with prewarmed complete proliferation medium. These ULA treated surfaces allow for the selection and purification of the NPC population, since this cell type is able to clonally divide in suspension forming the so called neurospheres (Fig. 5A). The obtained primary culture was incubated at 37 °C and 5% CO_2 and monitored daily using an inverted microscope.

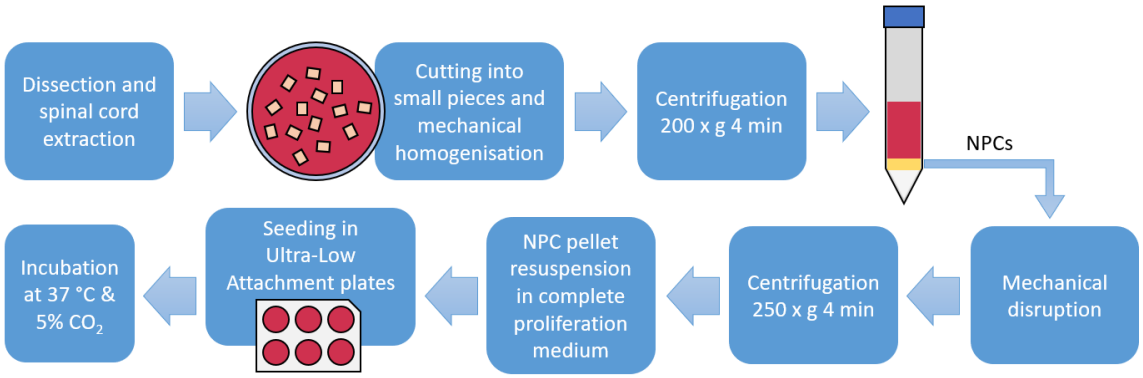

Figure 4. Schematic summary of the experimental procedure to obtain primary cultures of neural progenitor cells (NPCs).

Two to three days later, the primary culture was passaged. Cells were collected and centrifuged at 250 x g for 5 min, the supernatant was withdrawn with the vacuum aspirator and the cell pellet was resuspended in prewarmed complete proliferation medium. The resulting cell suspension was cultured in ULA plates with prewarmed complete proliferation medium, incubated at 37 °C and 5% CO_2 and monitored daily using an inverted microscope.

3.2. CELL CULTURE PASSAGE/DISAGGREGATION.

NPC neurospheres had to be disaggregated when they reached a critical size to avoid death of NPCs located in the centre due to lack of nutrients and gases (approximately every 3 days). This procedure was followed for regular maintenance and passaging of the NPC cultures in ULA plates or when some experiment was going to be performed with the NPCs.

First, cells were collected and centrifuged for 5 min at low speed (200 x g, such that only neurospheres and not individualised cells/cell debris were pelleted). The supernatant was withdrawn with the vacuum aspirator and cells were washed with prewarmed Dulbecco's phosphate-buffered saline (DPBS) 1x (Gibco) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The cell suspension was centrifuged at 300 x g for 5 min, the supernatant was removed and cells were incubated at 37 °C for 5 min with 200-300 µl of prewarmed StemPro Accutase® (Thermo Fisher). Next, approximately 4 ml of prewarmed DPBS supplemented with penicillin and streptomycin were added to dilute the enzymes, and tubes were centrifuged at 400 x g for 5 min. Supernatant was removed and the cell pellet resuspended in 300 µl of prewarmed complete proliferation medium. Neurospheres were finally mechanically disaggregated by pipetting 10-15 times using a P200 pipette. Prewarmed complete proliferation medium was added up to a final cell suspension volume of 1 ml. The number of cells per ml was determined with a Neubauer chamber using Trypan Blue Solution (0.4%; Gibco) to test cell viability. In the case of NPC amplification, approximately 500 000 cells/well were seeded in ULA 6-well plates. For the experiments, NPCs were seeded as a monolayer on Matrigel® (Lonza) coated coverslips (Fig. 5B). First, coverslips were placed in culture plates and incubated with Matrigel® (1:15 in DMEM/F-12) for 30 min at 37 °C. Dissociated NPCs were then seeded on these Matrigel® coated coverslips.

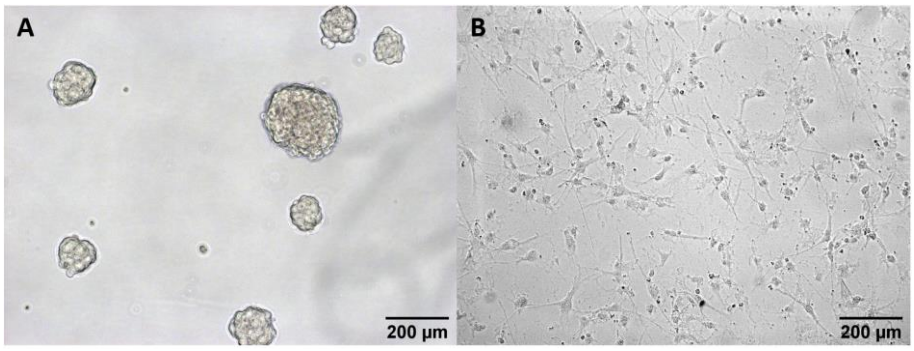

Figure 5. Neural progenitor cells in culture. *Neural progenitors grown (A) as neurospheres in suspension in an Ultra-Low Attachment (ULA) cell culture plate and (B) as a monolayer on Matrigel® coated coverslips.*

3.3. TRANSDUCTION OF NEURAL PROGENITOR CELLS.

Ectopic expression of mCherry-tagged hChR2(H134R) protein for optogenetic manipulation of NPCs was achieved using an AAV9 vector generated from plasmid DNA pAAV.CAG.hChR2(H134R)-mCherry.WPRE.SV40 (Addgene viral prep # 100054-AAV9; http://n2t.net/addgene:100054; RRID:Addgene_100054). In this way, the DNA construct that is delivered to transduced cells carries the codon-optimised gene encoding the blue light (BL)-activated unspecific cation channel ChR2 with the missense mutation H134R, which enables larger photocurrents upon activation compared with wild-type ChR2 (Nagel *et al.*, 2005). In this construct, ChR2 has been fused to a C-terminal mCherry tag with an excitation maximum at 587 nm and an emission maximum at 610 nm (Shaner, 2004), allowing to monitor fusion protein expression using fluorescence microscopy. This expression is driven by a CAG promoter, a synthetic, ubiquitous and strong promoter obtained by the fusion of the cytomegalovirus enhancer with the chicken beta-actin promoter (Hitoshi, 1991).

Neurospheres were first enzymatically and mechanically disaggregated as described in 3.2. and 300 000 cells were seeded in a small final volume of 100 µl of prewarmed complete proliferation medium in a 1.5 ml Eppendorf tube. Two tubes were prepared, one for the cells to be transduced and another for the control cells. From the initial AAV9 stock of $3.3 \cdot 10^{13}$ viral genomes/ml, the pertinent dilutions were performed in sterile phosphate-buffered saline (PBS) 1x. AAV9 vectors were introduced in the tube with the NPCs to be transduced, at the convenient concentration to get the multiplicity of infection (MOI, i.e. the ratio of the number of viral genomes to the number of cells present) which was determined to be suitable (MOI = 10^5 viral genomes/cell) according to the outcome of initial transduction experiments (Fig. 6).

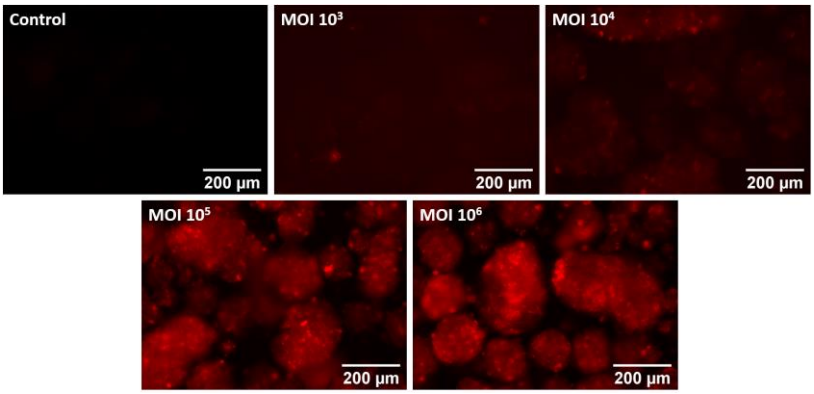

Figure 6. Fluorescence microscopy images of neurospheres transduced at different multiplicities of infection (MOIs) and of control non-transduced neurospheres. *ChR2-mCherry expression was interrogated by excitation of the mCherry tag at a wavelength of 555 nm.*

NPCs were then incubated at 37 °C and 5% CO_2 in these tubes for approximately 3 h with gentle pipetting every 1 h (same with control NPCs). Next, the content of both tubes was transferred to separate wells in ULA plates and prewarmed complete proliferation medium was added. The plate was incubated for 5 days at 37 °C and 5% CO_2 and monitored daily with an inverted fluorescence microscope to check ChR2-mCherry expression. After these 5 days, virus-containing medium was exchanged by fresh virus-free complete proliferation medium. To do so, NPCs were collected (separately) and centrifuged at 300 x g for 5 min, and after supernatant removal the cell pellet was resuspended in the new prewarmed complete proliferation medium and seeded back in the ULA plate (previously washed with washing medium). Cell cultures were incubated at 37 °C and 5% CO_2 for 48 h before they could be taken out of the room for viral manipulation to conduct the next experiments with the transduced and control NPCs.

3.4. TRANSDUCTION EFFICIENCY ASSESSMENT BY FLOW CYTOMETRY.

48h post-transduction, control and transduced NPCs were dissociated from neurospheres, washed with complete proliferation medium and immersed in PBS 1x (pH = 7.4) before quantifying the level of transduction by flow cytometry (CytoFLEX S from Beckman-Coulter). Non-transduced cells were used as a negative control to establish positive gate regions. Positive cells were those in positive gate regions, and the proportion of positive cells as a percentage of live cells was determined. A minimum total of 10 000 live events were acquired for each sample.

3.5. CELL VIABILITY ASSAYS.

Cell metabolic activity was estimated as an indicator of cell viability using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay for studying the effect of both ectopic ChR2-mCherry expression and optogenetic

stimulation on NPC viability (Fig. 7). Neurospheres were dissociated 48 h post-transduction and control and transduced cells were seeded on Matrigel® coated wells in a 96-well plate at a density of 15 000 cells/well and with 150 µl/well of prewarmed complete proliferation medium. The culture plate was incubated at 37 °C and 5% CO_2 for 24 h before the MTS assay was performed to allow live NPCs to settle and adhere to the culture surface. For the MTS assay, the appropriate volumes of the MTS and phenazine methosulfate (PMS) reagents were mixed (to get a 1:20 dilution of the PMS reagent in the MTS solution according to manufacturer guidelines). 10 µl of the MTS/PMS mixture were pipetted into each well (as well as into blank wells containing only complete proliferation medium) and the plate was incubated for 1-2 h at 37 °C and 5% CO_2. Finally, absorbance at 490 nm was read using the spectrophotometer Victor2 PerkinElmer.

Similarly, to check the effect of optogenetic stimulation on NPC viability, dissociated NPCs (both control and transduced) were seeded on Matrigel® coated wells in a 96-well plate at a density of 15 000 cells/well with 150 µl/well of prewarmed complete proliferation medium, and incubated at 37 °C and 5% CO_2 for 24 h. Next, photostimulation was applied to the corresponding transduced NPC culture using Ensight PerkinElmer. The stimulation pattern consisted of 3 BL exposures (100 pulses at 470 nm) separated by 10 s. This was repeated for 3 days. MTS assay was carried out (as described above) after the last photostimulation.

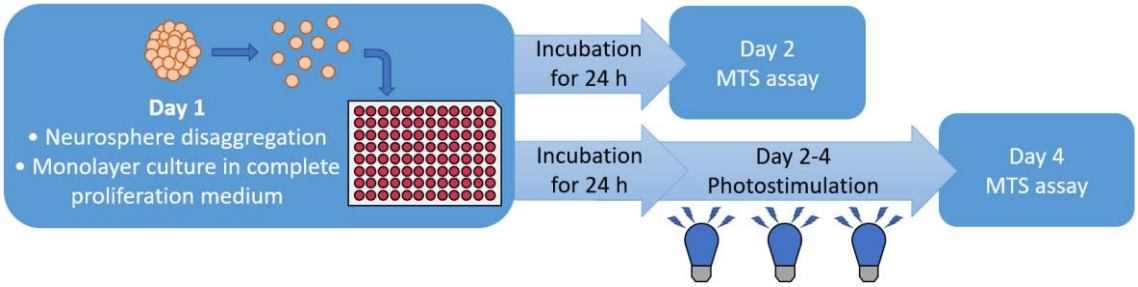

Figure 7. Schematic summary of the experimental procedure for the assessment of the effect of ChR2-mCherry expression and optogenetic stimulation on neural progenitor cell viability. *MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.*

3.6. OPTOGENETIC STIMULATION OF NEURAL PROGENITOR CELLS UNDER PROLIFERATION CONDITIONS.

48 h post-transduction, 80 000 dissociated NPCs/well (both control and transduced) were seeded on Matrigel® coated coverslips in a 24-well plate with 700 µl/well of complete proliferation medium, and incubated at 37 °C and 5% CO_2 for 24 h. Next, photostimulation was applied to the corresponding transduced NPC culture using Ensight PerkinElmer. The stimulation pattern consisted of 3 BL exposures (100 pulses at 470 nm) separated by 10 s. This was repeated for 3 days. After the third and last photostimulation, BL exposed and non-exposed samples were immediately processed for immunocytochemical staining for Ki67 and nestin (Fig. 8).

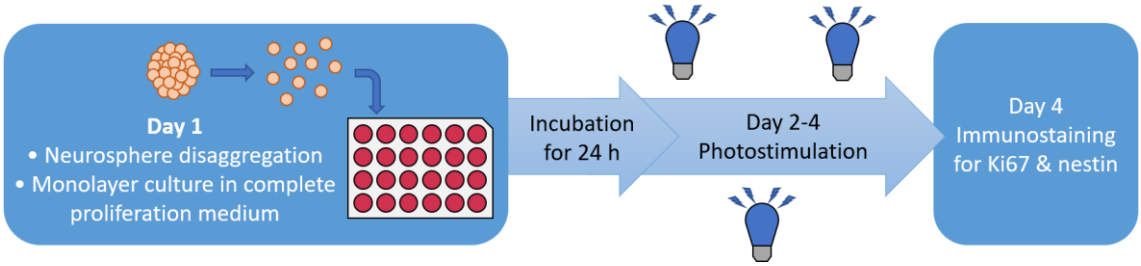

Figure 8. Schematic summary of the experimental procedure for the assessment of the effect of optogenetic stimulation on neural progenitor cell proliferation and neurite growth.

3.7. OPTOGENETIC STIMULATION OF NEURAL PROGENITOR CELLS UNDER DIFFERENTIATION CONDITIONS.

For spontaneous differentiation of NPCs into neurons, oligodendrocytes and astrocytes, 48 h post-transduction, 70 000 dissociated NPCs/well (both control and transduced) were seeded on Matrigel® coated coverslips in a 24-well plate containing a final volume of 700 µl/well of differentiation medium, consisting of DMEM/F-12 supplemented with 0.5% HEPES buffer, 1% $NaHCO_3$, 2% glucose, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine, 0.2% insulin, 0.7 U/ml heparin, 20 ng/ml bFGF and 10% hormone mix (composed by DMEM/F12 supplemented with 0.4% HEPES, 1.2% $NaHCO_3$, 1.6% glucose, 0.008% progesterone, 0.024% sodium selenite, 0.064% apotransferrin and 0.1% putrescin). Cells were incubated at 37 °C and 5% CO_2. On the third day, the medium was carefully withdrawn with the vacuum aspirator and replaced by 700 µl/well of differentiation medium without bFGF and with 2% inactivated foetal bovine serum (FBS; Thermo Fisher). The same day, photostimulation was applied to the corresponding transduced NPC culture using Ensight PerkinElmer. The stimulation pattern consisted of 3 BL exposures (100 pulses at 470 nm) separated by 10 s. This was repeated for 5 days. After the fifth photostimulation, BL exposed and non-exposed samples were immediately processed for immunocytochemical staining for NPC differentiation markers (Fig. 9).

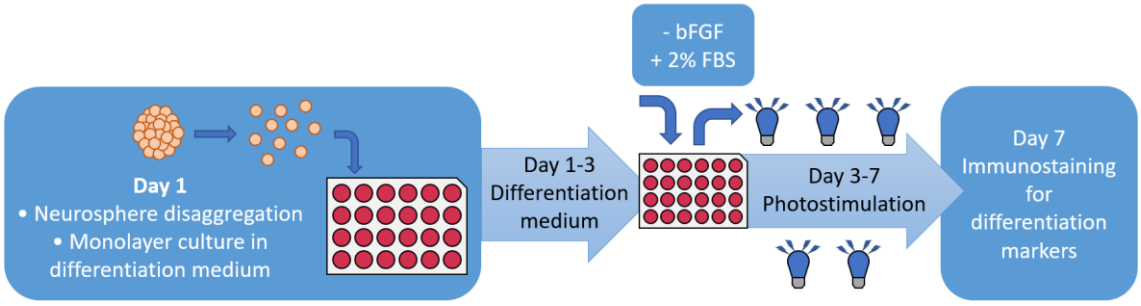

Figure 9. Schematic summary of the experimental procedure for the assessment of the effect of optogenetic stimulation on neural progenitor cells (NPCs) under differentiation conditions. *bFGF: basic fibroblast growth factor; FBS: foetal bovine serum.*

3.8. IMMUNOCYTOCHEMICAL STAINING.

First, NPCs cultured on Matrigel coated coverslips were fixed with ice-cold 4% paraformaldehyde (PFA) for 10 min and rinsed 3 times with PBS 1x. Cell membrane permeabilization and blocking of unspecific antibody binding sites was then performed for 1 h by addition of a blocking solution consisting of PBS 1x with 5% normal goat serum (NGS; Thermo Fisher) and 0.2% Triton X-100 (Sigma). This was followed by overnight incubation at 4 °C with the desired primary antibody (Table 1), which was prepared at the corresponding dilution in antibody solution composed by PBS 1x with 0.2% Triton X-100 and 1% w/v bovine serum albumin (BSA; Sigma). The following day, samples were rinsed 3 times with PBS 1x and the corresponding fluorescent secondary antibodies (Table 1) diluted in antibody solution (1:400) were added for 1 h incubation at room temperature and in the dark. After incubation, samples were rinsed again three times with PBS 1x. Next, they were incubated for 5 min with the DNA stain 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) diluted in PBS 1x (1:1000) at room temperature and in the dark, and then rinsed three times with PBS 1x. Finally, the coverslips were prepared on microscope slides using mounting medium (Fluoromount, F4680, Southern Biotech) and left to dry at room temperature and in the dark overnight. Slides were then stored at 4 °C until image acquisition.

Table 1. Primary and secondary antibodies used for immunocytochemical staining. *Olig2: Oligodendrocyte Transcription Factor 2; GFAP: Glial fibrillary acidic protein.*

Primary antibodies			
Antibody	**Species**	**Dilution**	**Reference**
Anti-Ki67	Rabbit	1:400	ab15580 Abcam
Anti-Nestin	Mouse	1:400	ab6142 Abcam
Anti-βIII-tubulin	Mouse	1:400	MAB1637 Chemicon
Anti-Olig2	Rabbit	1:400	ab33427 Abcam
Anti-GFAP	Chicken	1:1000	PA1-10004 Thermo Fisher
Secondary antibodies			
Antibody	**Species**	**Dilution**	**Reference**
Oregon Green® 488 goat anti-mouse	Goat	1:400	O6380 Invitrogen
Alexa Fluor® 555 goat anti-rabbit	Goat	1:400	A21428 Invitrogen
Alexa Fluor® 647 goat anti-chicken	Goat	1:400	A21449 Invitrogen

3.9. IMAGE ACQUISITION AND ANALYSIS.

Immunostained samples were visualised and images were acquired using a vertical fluorescence microscope (DM6000B, Leica) or ZEISS ApoTome, taking 10 images per sample/condition. Images were then analysed using the ImageJ software. The criteria followed for the analysis of each marker were the following:

- For determination of cell proliferation, the proportion of NPCs positive for Ki67 (nuclear protein expressed in all active cell cycle phases, but absent in G0) over DAPI stained nuclei was calculated for each acquired image using the Cell Counter function of ImageJ.
- To assess neurite growth, nestin staining (intermediate filament protein expressed in NPCs) was used to measure the length of the longest neurite of each NPC using NeuronJ plugin of Image J. Mean and maximum neurite length, and proportion of NPCs with no neurite growth (over the total number of nestin positive NPCs) were determined for each acquired image.
- For cell differentiation analysis, the proportion of NPCs positive for βIII-tubulin (neurons), Olig2 (Oligodendrocyte Transcription Factor 2; oligodendrocytes) and GFAP (glial fibrillary acidic protein; astrocytes) over DAPI stained nuclei was calculated for each acquired image using the Cell Counter function of ImageJ. Also, GFAP staining was used to distinguish and quantify different astrocyte morphologies.
- To study neuronal morphology, axon length (using NeuronJ plugin) and number of axonal branches of βIII-tubulin stained cells was determined.

3.10. STATISTICAL ANALYSIS.

Statistical analyses were performed using GraphPad Prism Version 8.0.2. Normality of the data was checked using Kolmogorov-Smirnov test and homoscedasticity with Brown-Forsythe test. Multiple comparisons were done using one-way ANOVA followed by Tukey's post hoc test for multiple pairwise comparisons in the case of normal and homoscedastic data. In the case of data not following a normal distribution, multiple comparisons were done using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Results were expressed as the mean ± SEM and in all cases, differences were considered significant when p-value < 0.05.

4. RESULTS AND DISCUSSION

4.1. ASSESSMENT OF CELL TRANSDUCTION AND ChR2 EXPRESSION.

4.1.1. TRANSDUCED NPCs EXPRESS ChR2 BUT TRANSDUCTION EFFICIENCY IS LOW.

Transduced NPCs were analysed using flow cytometry. Detection of mCherry-specific fluorescence confirmed successful ChR2-mCherry expression, although it was only detected in 38.54% of live cells in the assayed sample (Fig. 10).

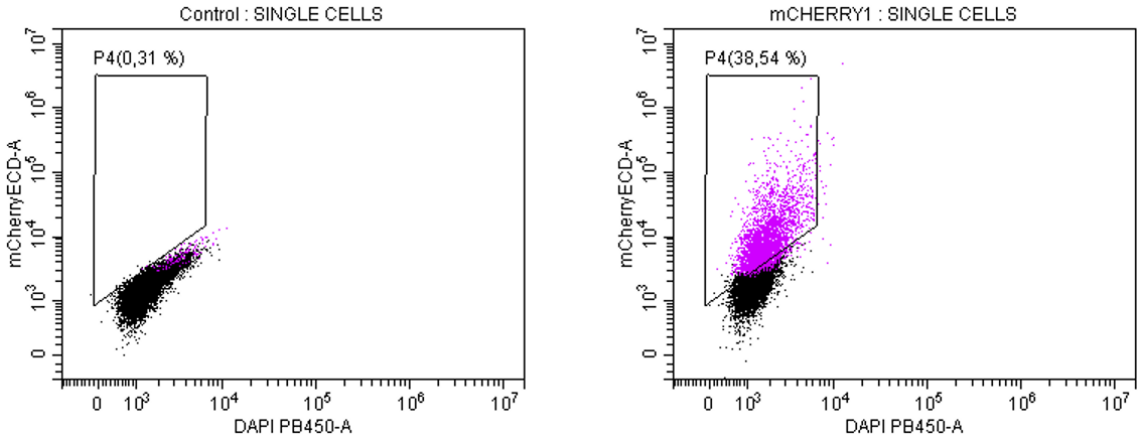

Figure 10. Quantification of neural progenitor cells expressing ChR2-mCherry by flow cytometry*. Non-transduced cells were used as a negative control to establish positive gate regions. Positive cells were those in positive gate regions; the proportion of positive cells as a percentage of live cells is given. A minimum total of 10 000 live events were acquired for each sample.*

This measurement clearly highlights a low NPC transduction efficiency with the AAV9 vectors used. AAV vectors have been proven to be safe candidates for clinical applications because of their low immunogenicity and their lack of pathogenicity, and because they are predominantly episomal and integrate into the host genome at relatively low frequencies, reducing the risk of insertional mutagenesis as compared to integrative viral vectors such as lentiviruses (Kantor *et al.*, 2014; Rapti *et al.*, 2015; Yizhar *et al.*, 2011). Moreover, AAV vectors have been described to allow for long-term expression of the delivered genetic construct (Rapti *et al.*, 2015). Despite these advantages, AAVs have been observed to be inefficient for transduction of several stem cell types (Jang *et al.*, 2011), including NPCs (Hughes *et al.*, 2002). Rapti *et al.* (2015) showed that AAVs are much more efficient for gene delivery to differentiated cells (e.g. cardiomyocytes), whereas in the case of undifferentiated cells (e.g. hESCs and iPSCs) other viral vectors (lentivirus and adenovirus) display higher efficiencies. This lower efficiency in AAV transduction of stem and progenitor cells has been largely attributed to the low presence of appropriate receptors for the given AAV serotype (e.g. heparan sulfate proteoglycans, sialylated glycans, etc.) on the surface of these undifferentiated cell types, leading to low affinity for viral attachment and cellular internalisation (Hughes *et al.*, 2002; Jang *et al.*, 2011).

We chose serotype 9 for this project since it has been found to have the highest tropism for the CNS (Kantor *et al.*, 2014). In the particular case of AAV9, described receptors include the laminin receptor LamR and terminal galactose on cell surface glycoproteins (Ambrosi *et al.*, 2019; Shen *et al.*, 2011). Interestingly, AAV9 has been described to have an especially low transduction efficiency in cell cultures *in vitro* as opposed to *in vivo* delivery. For instance, a study by Ambrosi *et al.* (2019) revealed considerable differences between the *in vitro* and *in vivo* performance of AAV9 in the context of cardiac optogenetics, suggesting the correlation of AAV9 efficiency with robust LamR expression in rat hearts *in vivo*, but absent expression in *in vitro* cultured cardiomyocytes. Similarly, Shen *et al.* (2011) suggested that the low AAV9 transduction efficiency *in vitro* may be explained by the low abundance of non-sialylated glycans with terminal galactose on the surface of *in vitro* cultured cells. In this sense, enzymatic desialylation of cultured cells has been demonstrated to increase AAV9 transduction efficiency *in vitro*,

presumably because removal of terminal sialic acid exposes galactose residues of cell surface glycoproteins, which act as AAV9 receptors (Shen *et al.*, 2011).

Therefore, the low affinity of AAVs for stem and progenitor cells, and in particular the low affinity of AAV9 for *in vitro* cultured cells probably as a consequence of low AAV9 cell receptor availability, may explain the low transduction efficiency observed in our results. Strategies such as the design of recombinant AAVs with higher NPC transduction efficiencies, as performed by Jang *et al.* (2011), or simpler approaches such as enzymatic desialylation of NPCs may be considered for future experiments.

4.1.2. ECTOPIC ChR2-mCherry EXPRESSION DOES NOT AFFECT NPC VIABILITY.

To check whether transduction and subsequent ChR2-mCherry expression was affecting NPC viability, we measured their metabolic activity via MTS assay as an indicator of cell viability, and compared control non-transduced NPCs with transduced NPCs. In the case of transduced NPCs, cell viability was measured under four different conditions or MOIs in order to evaluate if there was any correlation between the MOI used for transduction and cell viability. The results obtained (Fig. 11) show no significant difference in viability between the conditions assayed, thus demonstrating that transduction and ChR2-mCherry expression is not affecting NPC viability.

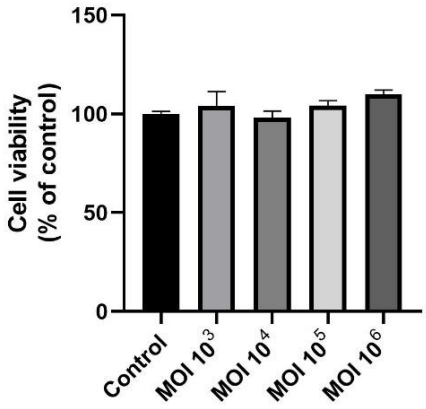

Figure 11. Effect of transduction on neural progenitor cell viability. *Cell viability of neural progenitors transduced using different multiplicities of infection (MOIs) was calculated with respect to control neural progenitors. Values represent the mean ± SEM. No significant differences were found (Kruskal-Wallis test; n = 3).*

Our motivation to check viability of ChR2-expressing NPCs was based on previous observations where AAV transduction considerably reduced the viability of pluripotent stem cells (hESCs and iPSCs) due to the induction of cell cycle arrest and apoptosis (Rapti *et al.*, 2015), or where CAG-driven strong opsin expression led to protein accumulations or structural abnormalities altering cell physiology or even resulting in cytotoxicity (Yizhar *et al.*, 2011). Moreover, fluorescent tags such as mCherry can clump and accumulate in the cell, although this may not necessarily alter cell health (Yizhar *et al.*, 2011). In our case, these potential cytotoxic effects seem to be absent, either because expressed ChR2-mCherry does not accumulate intracellularly or because even if it does, this does not compromise cell viability. Also, even if AAV transduction negatively affects viability of pluripotent stem cells (Rapti *et al.*, 2015), this does not seem to be translatable to NPCs, which are multipotent progenitors, as shown by our results. In any case, complementary assays, e.g. assessment of apoptotic markers, could be carried out to confirm this observation.

4.2. EFFECT OF *IN VITRO* OPTOGENETIC STIMULATION ON NEURAL PROGENITOR CELLS UNDER PROLIFERATION CONDITIONS.

For the assessment of the effects of optogenetic stimulation on NPC biology, the experimental design included three conditions:

- Non-transduced control cells.
- ChR2-expressing cells not exposed to blue light (ChR2) to distinguish any effect which may be solely due to ChR2-mCherry expression and not to optogenetic stimulation.
- Optogenetically stimulated cells (ChR2 + BL), i.e. ChR2-expressing cells exposed to blue light (BL) so as to activate the ChR2 cation channel and induce cell depolarisation.

At this point, it is important to remark that "ChR2" and "ChR2 + BL" conditions do not exclusively contain ChR2 expressing cells, but also non-transduced cells because transduction efficiency was not 100%.

4.2.1. OPTOGENETIC STIMULATION DOES NOT INCREASE NPC PROLIFERATION AND VIABILITY.

We hypothesised that optogenetic stimulation of NPCs could promote their proliferation and consequently the number of viable cells present. This hypothesis was based on the known role of transient elevations of intracellular Ca^{2+} in modulating cell cycle progression and proliferation. Although ChR2 is an unspecific cation channel which triggers the influx of other cations apart from Ca^{2+} when photoactivated (mainly Na^{+}), the membrane depolarisation induced by this cation influx can trigger the activation of voltage-gated calcium channels (VGCCs), which are not only present in mature, differentiated cell types, but also in undifferentiated cells (Stroh *et al.*, 2011), including proliferating NPCs *in vitro* (Louhivuori *et al.*, 2013). Therefore, activated ChR2 could potentially recruit Ca^{2+}-dependent cell processes not only through its Ca^{2+} influx when photoactivated, but also through the induction of VGCC in the cell (Stroh *et al.*, 2011).

Ca^{2+} modulation of cell proliferation appears to be mediated by Ca^{2+}-activated calmodulin, which through its downstream targets calcineurin and CaMKII has been observed to regulate key cell cycle regulatory proteins, such as cyclin D and cdk4 in early/mid G1 phase of the cell cycle, cdk2 in the late G1/S phase, or cdc2 in the G2/M phases (Kahl & Means, 2003). Although some experimental evidence shows that *in vitro* electrical stimulation of foetal NPCs promotes their proliferation (Chang *et al.*, 2011), to our knowledge there is still no experimental evidence studying the effect of optogenetic stimulation on NPC proliferation. To assess this effect, NPCs were grown in proliferation medium with growth factors (EGF and bFGF) and transduced NPCs were subjected to the 3-day photostimulation protocol (ChR2 + BL). The proportion of actively proliferating cells in each condition was determined by calculating the proportion of NPCs displaying positive nuclear immunostaining for the Ki67 proliferation marker (Fig. 12). ANOVA analysis of the results shows no significant differences between the experimental conditions. Furthermore, MTS assay results reveal no significant differences in cell viability among the different conditions (Fig. 13). Thus, these results indicate that optogenetic stimulation of NPCs is not enhancing NPC proliferation and, accordingly, it is not increasing the number of viable cells/the level of metabolic activity in our cell sample.

Cell cycle progression and proliferation is a highly complex and tightly regulated cell process with multiple checkpoints, where intracellular Ca^{2+} transients have been particularly identified during early G1, G1/S transition and mitosis (Kahl & Means, 2003). Thus, a possible explanation for the absence of any effect in our results could be that our applied 3-day photostimulation pattern would not have successfully reproduced or induced the temporal

pattern of intracellular Ca^{2+} waves required at specific timepoints of the cell cycle to promote effective cycle progression and cell division. In any case, this outcome should not be negatively regarded, since if we had observed an excessive induction of NPC proliferation and viability as a result of optogenetic stimulation (which is not the case here), this would have raised concerns about possible tumorigenic potential of NPCs when stimulated.

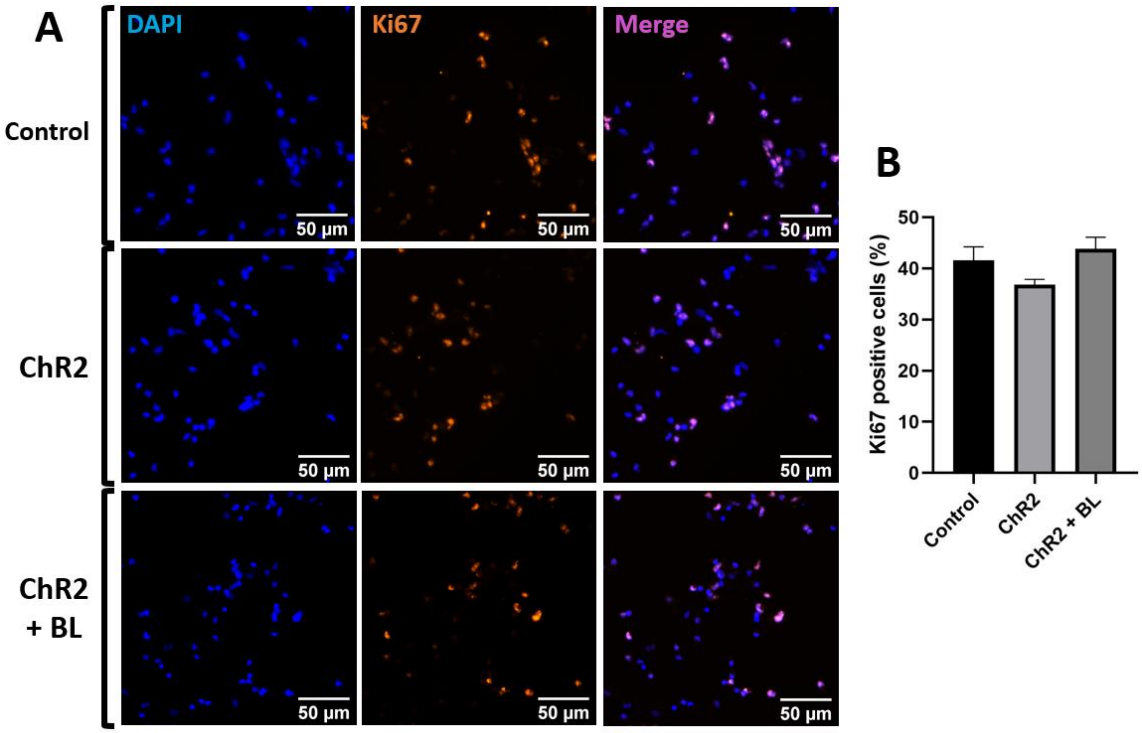

Figure 12. Effect of optogenetic stimulation on neural progenitor cell (NPC) proliferation. *(A) Fluorescence microscopy images of NPCs immunostained for the cell proliferation marker Ki67. (B) Quantification of the percentage of Ki67 positive NPCs over DAPI. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. No significant differences were found (ANOVA; n = 3).*

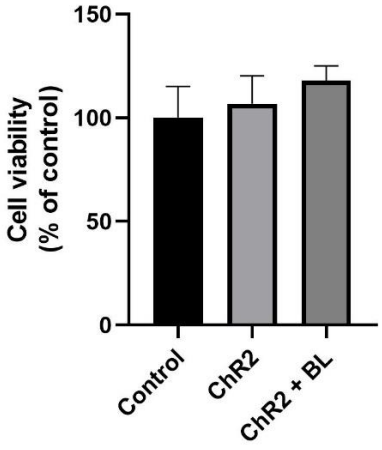

Figure 13. Effect of optogenetic stimulation on neural progenitor cell viability. *Cell viability of transduced (ChR2) and transduced and blue light exposed (ChR2 + BL) neural progenitor cells was calculated with respect to control cells. Values represent the mean ± SEM. No significant differences were found (Kruskal-Wallis test; n = 3).*

4.2.2. OPTOGENETIC STIMULATION ENHANCES NPC NEURITE GROWTH VIA INCREASED NEURITOGENESIS.

After photostimulation of the corresponding transduced cells (ChR2 + BL), NPCs grown in complete proliferation medium were immunostained for the NPC marker nestin to evaluate their morphology and assess neurite growth. After measuring the longest neurite for each NPC (where an NPC with no neurites was quantified as a length of 0 µm), the mean neurite length was calculated for each condition, as represented in Fig. 14B. The results show that optogenetic stimulation significantly increases the mean neurite length as compared to rest of conditions.

With the aim of looking into this phenomenon in more detail, maximum neurite length and proportion of NPCs with no neurite growth were quantified. In the case of the maximum neurite length (Fig. 14C), even if optogenetically stimulated NPCs (ChR2 + BL) display a higher maximum neurite length compared with the rest of conditions, no statistically significant differences are found. In the case of the proportion of NPCs with no neurite growth (Fig. 14D), however, optogenetic stimulation significantly reduces the proportion of NPCs with no neurites as compared to the control and ChR2 NPCs without BL exposure. These results indicate that *in vitro* optogenetic stimulation of NPCs under proliferation conditions enhances neurite growth, and this increase can be attributed to an improved neuritogenesis or neurite sprouting (i.e. more NPCs are stimulated to form neurites) rather than to an increase in the maximum neurite length.

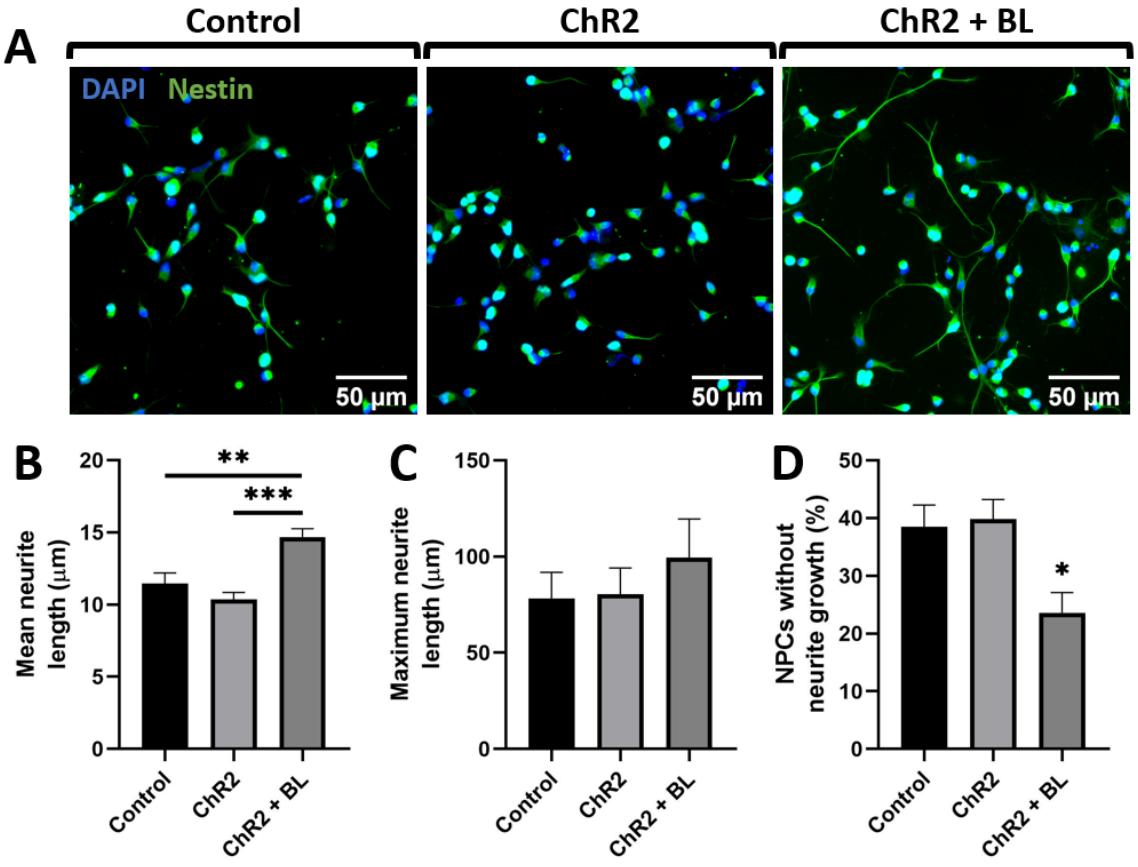

Figure 14. Effect of optogenetic stimulation on neural progenitor cell (NPC) neurite growth. *(A) Fluorescence microscopy images of NPCs immunostained for the NPC marker nestin to assess cell morphology. (B) Mean length of the longest neurite of each NPC. (C) Maximum neurite length. (D) Quantification of the percentage of NPCs with no neurites over total nestin positive cells. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. For B: **p-value<0.01, ***p-value<0.001; for C: no significant differences were found; for D: *p-value<0.05 compared with control and ChR2 (ANOVA; n = 3).*

These results suggest that *in vitro* optogenetic stimulation of NPCs before transplant could serve as a preconditioning approach to improve their regenerative capabilities. A successful NPC transplant should be able to grow neurites that extend into the host spinal cord tissue and display connectivity and integration with the CNS circuitry, especially in the case of neurons (Dulin & Lu, 2014). Thus, optogenetic stimulation prior to transplant could activate NPC intrinsic mechanisms contributing to an enhanced neurite growth and consequent connectivity in the host spinal cord. Intrinsic mechanisms potentially activated by Ca^{2+} increases due to optogenetic stimulation include the activation of Ca^{2+}-dependent adenylyl cyclases resulting in the formation of cAMP (Mahar & Cavalli, 2018). Subsequent activation of cAMP/EPAC2 signalling has been associated with increased neurite outgrowth through the induction of the pro-regenerative transcription factor CREB (Wei *et al.*, 2016). Additionally, increased intracellular Ca^{2+} levels have been described to activate Ras via different mechanisms, including via CAMKII-dependent disinhibition of Ras through inactivation of the Ras inhibitor synGAP (Chen *et al.*, 1998; Wu *et al.*, 2001), or via activation of Ca^{2+}-sensitive Ras-Guanine nucleotide exchange factors (Ras-GEFs) (Schöneborn *et al.*, 2018). One of the signalling pathways induced by active Ras is PI3K/AKT/mTOR signalling, where mTOR activation is crucial for local protein synthesis and growth cone formation, which enables sprouting and growth of new neurites (Park *et al.*, 2008; van Niekerk *et al.*, 2016). Moreover, active AKT inactivates glycogen synthase kinase 3β (GSK3β) and consequently its downstream target adenomatous-polyposis-coli (APC) is not phosphorylated and increases microtubule stability at the growth cone binding to their plus-ends (Schöneborn *et al.*, 2018).

Additionally, an indirect mechanism potentially induced by optogenetic stimulation that could also be contributing to the enhanced neurite growth observed in NPCs is an increased secretion of neurotrophic factors by the stimulated NPCs, such as NT-3, NGF or BDNF, which have been associated with enhanced neurite sprouting and extension (Yu *et al.*, 2019). For instance, PI3K/AKT/mTOR signalling is thought to be activated by neurotrophins such as NGF (van Niekerk *et al.*, 2016). This increased expression and secretion of neurotrophins could be potentially mediated by Ca^{2+}-induced CREB activation, which is responsible for transcriptional activation of neurotrophic factors such as BDNF (Tao *et al.*, 1998; Wu *et al.*, 2001). Moreover, depolarisation-induced neurotrophin secretion is a physiological process of activity-dependent synaptic plasticity, which has been demonstrated to be mediated by CaMKII signalling (Kolarow *et al.*, 2007). Indeed, this Ca^{2+}-dependent increased expression and secretion of neurotrophins as a result of optogenetic stimulation has already been demonstrated both *in vitro* (Yu *et al.*, 2019) and *in vivo* (Cheng *et al.*, 2014), which leads us to think that this increased secretion could also have been induced in our optogenetically stimulated NPC cultures, thus indirectly contributing to enhanced neuritogenesis. Therefore, it may be interesting for future experiments to determine the levels of secreted neurotrophins in our NPC culture media for the different conditions.

Finally, it is important to point out that previous studies assessing the effect of optogenetic stimulation on neurite growth do so on differentiated NPC-derived neurons and will be therefore discussed in the corresponding section dealing with morphology of NPC-derived neurons. In any case, Kobelt *et al.* (2014) did show an increased neurite outgrowth in NPCs cultured in complete proliferation medium with EGF and FGF, as it is the case here, but in this study intracellular calcium elevations were achieved by *in vitro* electrical stimulation, and not by optogenetic stimulation.

4.3. EFFECT OF *IN VITRO* OPTOGENETIC STIMULATION ON NEURAL PROGENITOR CELLS UNDER DIFFERENTIATION CONDITIONS.

4.3.1. OPTOGENETIC STIMULATION ENHANCES NPC DIFFERENTIATION INTO NEURONS AND OLIGODENDROCYTES.

The effect of optogenetic stimulation on spontaneous differentiation of NPCs cultured under spontaneous differentiation conditions and photostimulated in the case of the corresponding transduced NPCs (ChR2 + BL) was quantified through immunostaining for the neuronal marker βIII-tubulin, the oligodendrocyte marker Olig2 and the astrocyte marker GFAP.

In the case of βIII-tubulin immunostaining, results show a significantly higher percentage of βIII-tubulin positive cells in the optogenetically stimulated condition (ChR2 + BL) (Fig. 15) compared with control and ChR2 cells, thus indicating improvement in neuronal differentiation as a result of optogenetic stimulation.

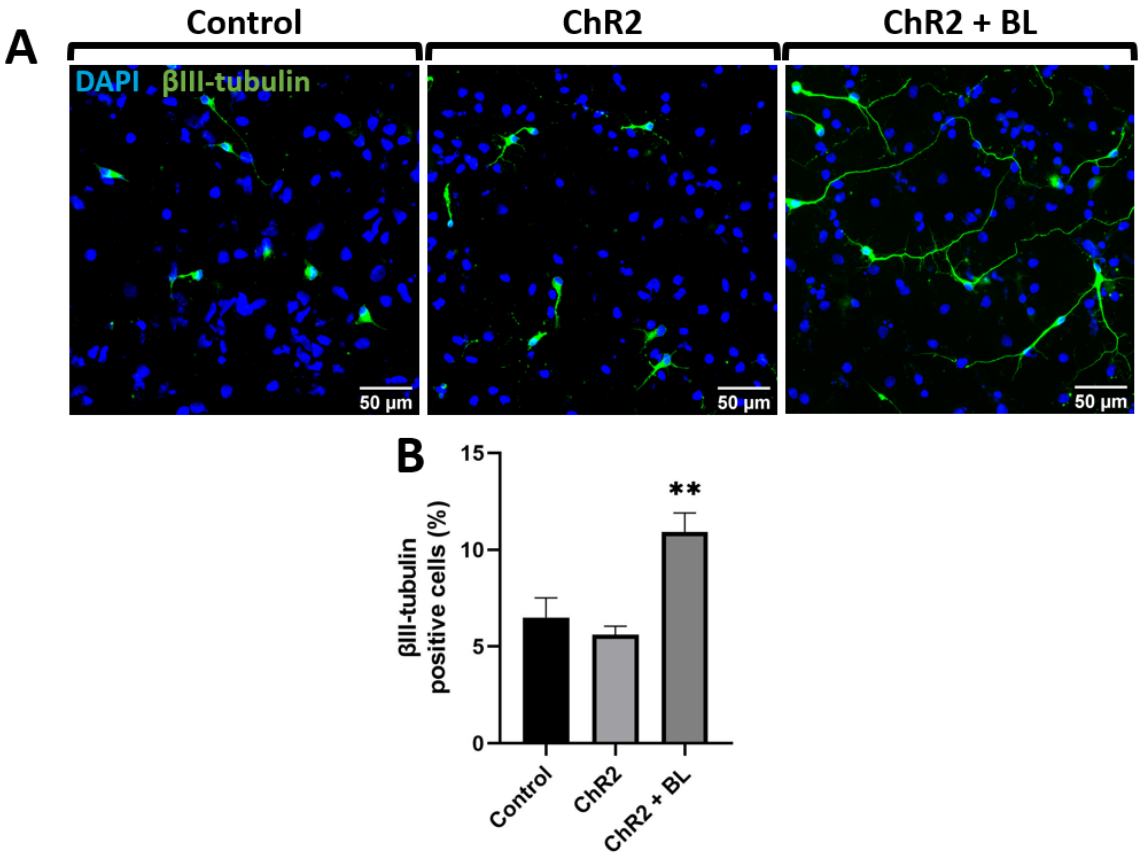

Figure 15. Effect of optogenetic stimulation on neuronal differentiation. *(A) Fluorescence microscopy images of NPC-derived cells immunostained for the neuronal marker βIII-tubulin. (B) Quantification of the percentage of βIII-tubulin positive cells over DAPI. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. **p-value<0.01 compared with control and ChR2 (Kruskal-Wallis test; n = 3).*

Our results seem consistent with previous experimental observations. Stroh *et al.* (2011) proved that optogenetic stimulation of differentiating ChR2-expressing ESCs leads to a higher percentage of βIII-tubulin positive cells and indicated the role of Ca^{2+} as an important mediator of neuronal differentiation and maturation. Also, electrical stimulation of NPCs has been observed to increase neuronal differentiation and maturation (Chang *et al.*, 2011; Kobelt *et al.*, 2014). Indeed, it has been shown that excitation of NPCs, e.g. applying depolarising levels of extracellular potassium or by glutamate addition, increases the proportion of NPCs that differentiate into neurons both *in vitro* and *in vivo* (Deisseroth *et al.*, 2004). This "excitation-

neurogenesis coupling" is mediated by Ca^{2+} influx through VGCCs and NMDA receptors, resulting in a rapid activation of pro-neuronal genes including *HES1*, *Id2* and *NeuroD* (Deisseroth *et al.*, 2004). Furthermore, Lepski *et al.* (2013) proved that increased levels of cAMP promote neuronal differentiation of NPCs, potentially via cAMP-mediated upregulation of VGCC. Thus, membrane depolarisation, electrical excitability and Ca^{2+} currents have been demonstrated to be crucial for neuronal differentiation from NPCs. In this sense, ChR2-mediated depolarisation of differentiating NPCs, along with the potential subsequent activation of VGCCs, could be leading to an increase in intracellular Ca^{2+} concentrations that could be promoting cAMP production, upregulation of further VGCCs leading to an increased excitability and upregulation of pro-neuronal genes, ultimately resulting in an increased NPC differentiation into neurons.

This enhanced neuronal differentiation may constitute a potential therapeutic improvement in the context of cell therapies for SCI treatment, taking into account that one of the problems encountered in NPC transplants is that in spite of the multipotency of NPCs, the lesion microenvironment promotes their preferential differentiation into astrocytes after transplantation, thus preventing their effective differentiation into neurons (Barnabé-Heider *et al.*, 2010; Lu *et al.*, 2014; Panayiotou & Malas, 2013). Thus, the increased neuronal differentiation observed in our results could promote functional recovery through an increased number of neurons at the injury site with the potential of forming neuronal relay circuits with the spared host neurons and recover spinal cord connectivity.

Regarding Olig2 immunostaining, quantification results clearly display a statistically significant increase in the percentage of Olig2 positive cells in the optogenetically stimulated condition as compared to the rest of conditions (Fig. 16), thus indicating that optogenetic stimulation is promoting NPC differentiation into oligodendrocytes.

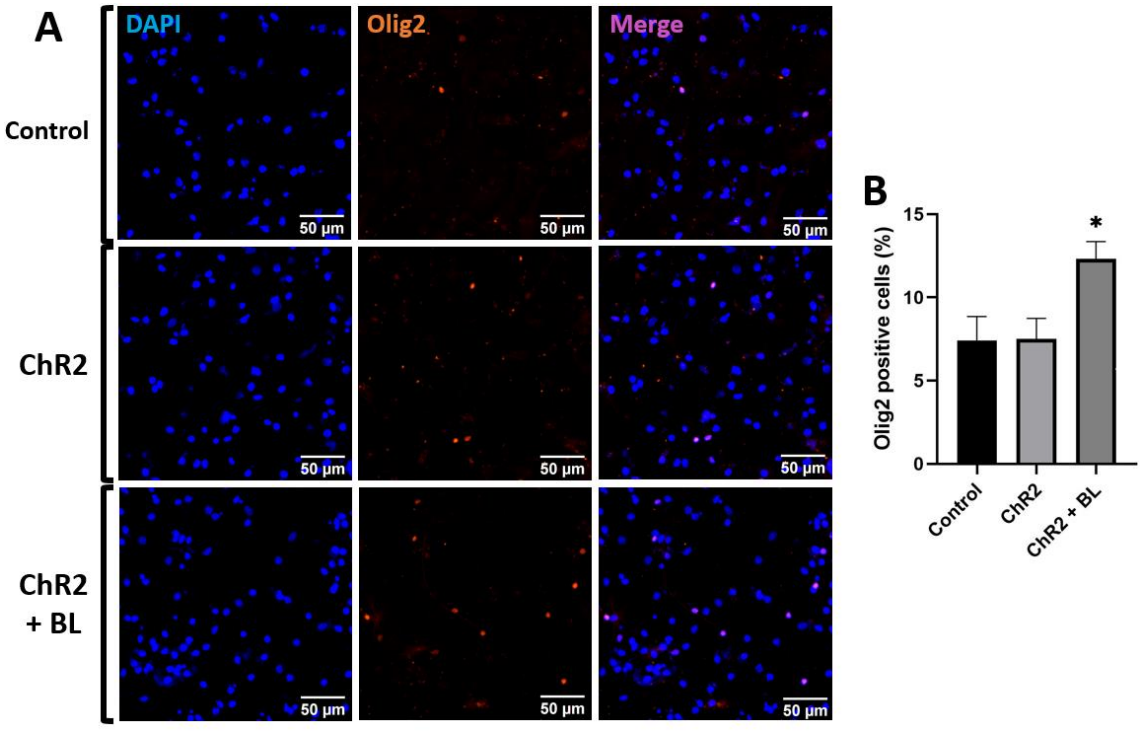

Figure 16. Effect of optogenetic stimulation on oligodendrocyte differentiation. *(A) Fluorescence microscopy images of NPC-derived cells immunostained for the oligodendrocyte marker Oligodendrocyte Transcription Factor 2 (Olig2). (B) Quantification of the percentage of Olig2 positive cells over DAPI. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. *p-value<0.05 compared with control and ChR2 (ANOVA; n = 3).*

Oligodendrocytes are essential glial cells in the CNS, being responsible for the production of the myelin sheath insulating neuronal axons and ensuring adequate neuronal conduction speed. Extensive demyelination is observed after SCI due to oligodendrocyte death, therefore replacing lost oligodendrocytes to improve conduction and protect axons is one of the objectives of NPC transplantation. Nonetheless, as previously stated, the lesion microenvironment strongly directs NPC differentiation to an astrocytic fate, thus strategies trying to promote oligodendrocyte differentiation (e.g. genetic manipulation) have been assayed, yielding increased remyelination and functional recovery (Assink *et al.*, 2017; Barnabé-Heider *et al.*, 2010; Panayiotou & Malas, 2013; Sabelström *et al.*, 2014). Here we demonstrate that optogenetic stimulation may be a potential approach to increase this oligodendrocyte differentiation of NPCs.

Our results agree with a previous study where optogenetic stimulation of a bipotential glial progenitor cell line (OS3) resulted in an increased differentiation into oligodendrocytes. Transplantation of these stimulated OS3 cells led to an increased remyelination and functional recovery (Ono *et al.*, 2017). Our results prove that this enhanced oligodendrocyte differentiation also occurs in the case of optogenetically stimulated multipotent NPCs. This same study also showed that optogenetic activation of these progenitors came with increased intracellular Ca^{2+} concentrations and that these alterations in the intracellular ion environment triggered the activation of the PI3K/AKT/mTOR pathway, which plays a key role in oligodendrocyte differentiation (Ono *et al.*, 2017).

Alterations in the intracellular ion environment are known to be a key contributor to oligodendrocyte differentiation from neural and glial progenitor cells, and in fact different types of Ca^{2+} channels, including VGCC and ligand-gated Ca^{2+} channels, are expressed in cells of the oligodendrocyte lineage (Ono *et al.*, 2017). However, the molecular mechanisms by which Ca^{2+} may regulate oligodendrocyte differentiation are still not fully understood, although some insights have recently appeared. Apart from the already mentioned implication of Ca^{2+}-activated PI3K/AKT/mTOR signalling, another potential molecular mechanism involves nuclear factor of activated T cells (NFAT) transcription factors. The transcription factors Olig2, Sox10 and Nkx2.2 are essential for oligodendrocyte differentiation and myelination, and NFAT has been identified as a downstream target of Sox10 that contributes to this differentiation process (Weider *et al.*, 2018). NFAT activity has been seen to be dependent on Ca^{2+}-activated CaM/calcineurin signalling, thus converting this in a potential molecular mechanism explaining the role of Ca^{2+} in oligodendrocyte differentiation (Weider *et al.*, 2018).

Interestingly, Gibson *et al.* (2014) demonstrated that optogenetically induced neuronal activity enhanced oligodendrogenesis and myelination, and consequently motor behaviour. Although the mechanisms by which neuronal activity may improve oligodendrocyte differentiation and myelination are not very clear, it seems that one potential mechanism could be neurotransmitter-mediated neuron-oligodendrocyte precursor communication, where ligand-gated calcium channels expressed by oligodendrocyte precursors would be involved (Bergles *et al.*, 2000). Moreover, several growth factors and neurotrophins such as NT-3 have been described to induce NPC differentiation into oligodendrocytes (Ono *et al.*, 2017).

On the basis of the above, it can be hypothesised that the optogenetic improvement of oligodendrocyte differentiation seen in our results could have been due to both direct and indirect mechanisms. Direct mechanisms would involve the ChR2-induced increases of intracellular Ca^{2+} leading to activation of described pro-oligodendrocyte differentiation pathways such as PI3K/AKT/mTOR or CaM/calcineurin through NFAT transcription factors. Indirect mechanisms that could promote NPC differentiation into oligodendrocytes (even if that particular NPC itself is not expressing ChR2) would arise from paracrine signalling coming from stimulated ChR2-expressing cells in the same culture well, either in the form of activity-induced

secreted neurotrophic/growth factors, or in the form of neurotransmitters released from NPC-derived neurons when stimulated.

Finally, in the case of GFAP immunostaining, ANOVA analysis of our results revealed no significant differences between the different conditions (Fig. 17). To our knowledge, there are no studies showing that optogenetic stimulation could promote astrocyte differentiation. In fact, Ono *et al.* (2017) described a reduction of the astrocytic fraction arising from optogenetically stimulated glial progenitors, which preferentially differentiated into oligodendrocytes. However, this is not the case in our experiments, where astrocytic differentiation remains unaffected even if neuronal and oligodendrocyte differentiation increase, suggesting that optogenetic stimulation is increasing the degree of differentiation in our NPC cultures rather than diverting their differentiation from one fate to others.

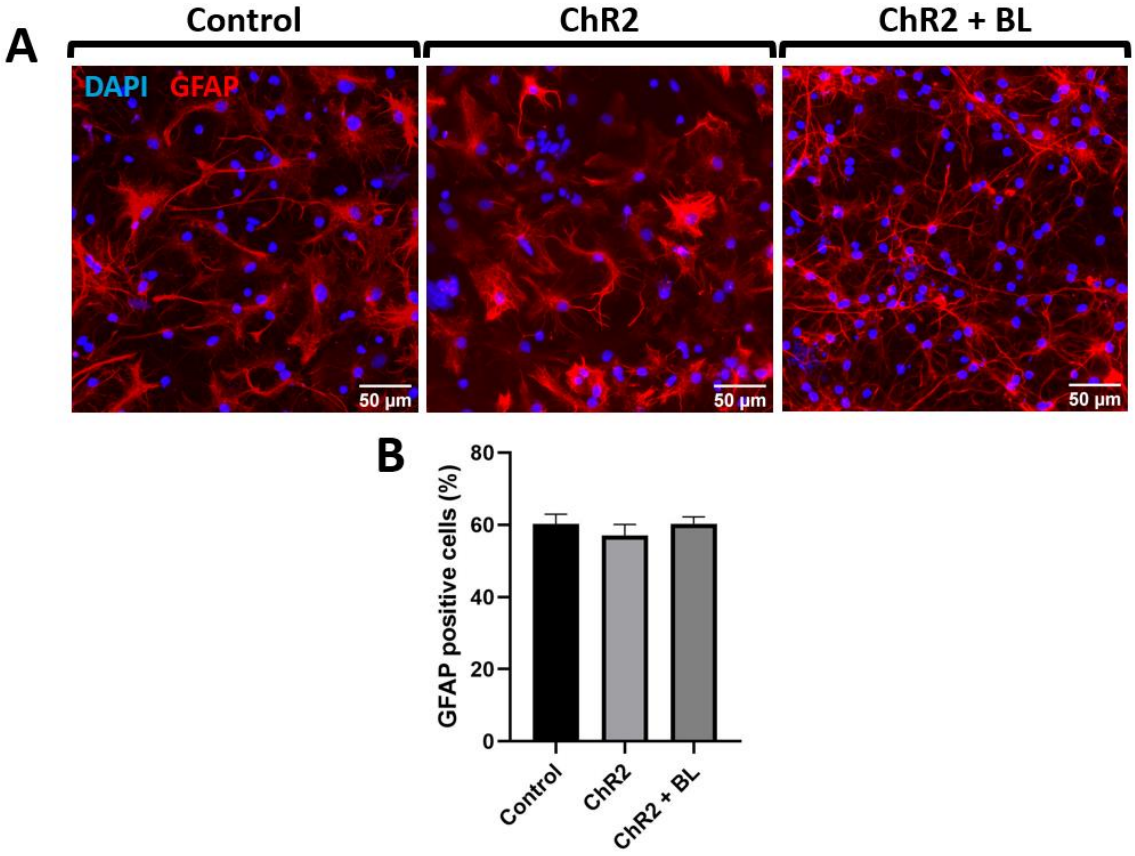

Figure 17. Effect of optogenetic stimulation on astrocyte differentiation. *(A) Fluorescence microscopy images of NPC-derived cells immunostained for the astrocytic marker glial fibrillary acidic protein (GFAP). (B) Quantification of the percentage of GFAP positive cells over DAPI. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. No significant differences were found (ANOVA; n = 3).*

4.3.2. OPTOGENETIC STIMULATION AFFECTS ASTROCYTE MATURATION TO ITS DIFFERENT MORPHOLOGICAL SUBTYPES.

Even if optogenetic stimulation does not affect the proportion of NPCs that differentiate into astrocytes, we noticed that it was considerably affecting astrocyte morphology. Therefore, on the basis of GFAP immunostaining, we quantified the number of GFAP positive cells corresponding to each of two distinct and well-described astrocyte morphologies: fibrous and protoplasmic astrocytes. Protoplasmic astrocytes (Fig. 18D) have highly branched, densely packed, bushy processes, whereas fibrous astrocytes (Fig. 18A) display long, straight and less

densely packed processes (Tabata, 2015). According to the quantification of the distinct morphological subtypes, optogenetically stimulated cells show a highly significant larger proportion of fibrous astrocytes (Fig. 18B-C) and a highly significant lower proportion of protoplasmic astrocytes (Fig. 18E-F) as compared to rest of conditions. Thus, optogenetic stimulation diverts astrocyte morphological maturation from a protoplasmic to a fibrous morphology.

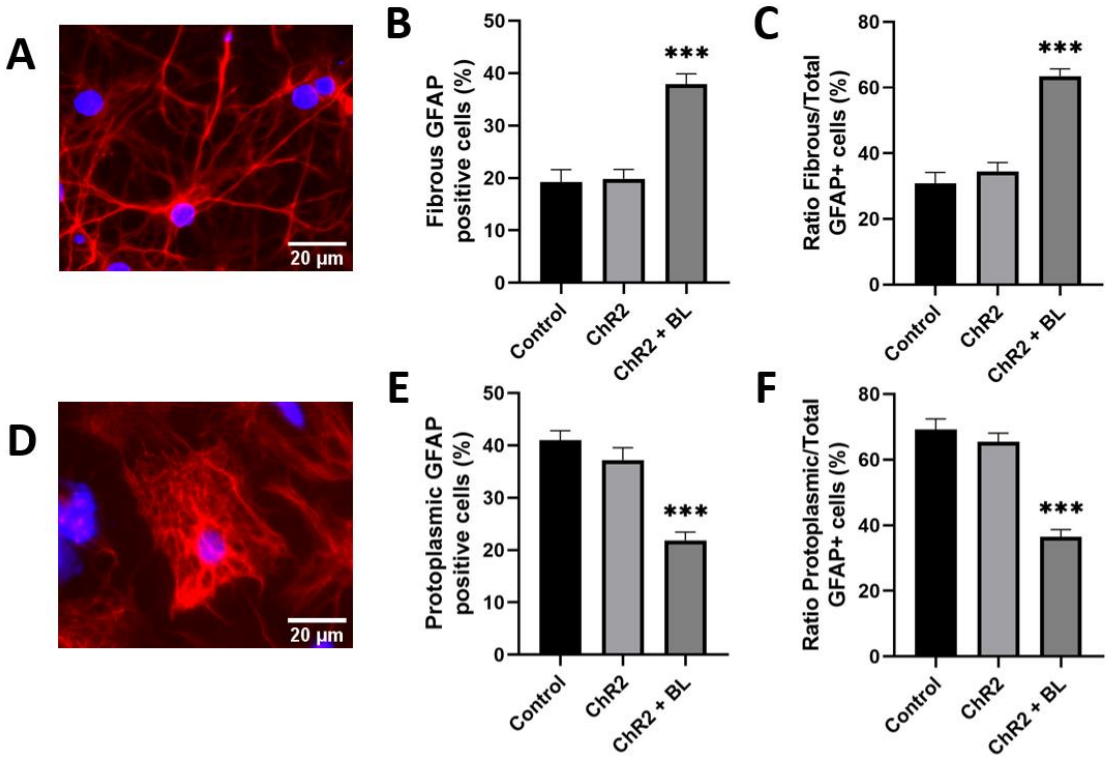

Figure 18. Effect of optogenetic stimulation on astrocyte morphological maturation. *(A, D) Fluorescence microscopy image of an astrocyte with (A) fibrous and (D) protoplasmic morphology. (B, E) Quantification of the percentage of (B) fibrous and (E) protoplasmic GFAP positive cells over DAPI. (C, F) Quantification of the percentage of (C) fibrous and (F) protoplasmic GFAP positive cells over total GFAP positive cells. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. ***p-value<0.001 compared with control and ChR2 (ANOVA for B, C and F, Kruskal-Wallis test for E; n = 3).*

This classification of astrocyte subtypes was established in the late 19th century based on differences in their morphology and anatomical location, since protoplasmic astrocytes are mainly located in the grey matter and fibrous astrocytes in the white matter (Bylicky *et al.*, 2018; Sun & Jakobs, 2012; Tabata, 2015). Since then, it has been realised that these astrocyte subtypes are functionally and biochemically distinct, having distinct profiles of gene expression (Bylicky *et al.*, 2018). It is thought that during development, glial progenitors migrate either to the white or grey matter, where they differentiate into fibrous or protoplasmic astrocytes, respectively (Tabata, 2015). Thus, astrocyte differentiation into its different subtypes seems to be mediated by the microenvironment in these compartments. There are some insights into their differentiation that highlight that the cellular and molecular mechanisms that govern the maturation to one subtype or the other are clearly distinct (Tabata, 2015); however, how this maturation is specifically regulated at the molecular level is still not clear, since recent studies have rather focused on the functional and physiological role of these subtypes. Thus, it is difficult to tell how ChR2 activation might have specifically induced the preferential maturation to a fibrous morphology, although some evidence indicating a role of Ca^{2+} in morphological differentiation of astrocytes exists. For instance, it has been demonstrated that the Ca^{2+}-binding protein S100B regulates astrocyte morphology and migration in a Ca^{2+}-dependent manner

(Brozzi *et al.*, 2009). Also, as indicated in the above-mentioned experiments, we think that there might be some sort of paracrine effect in our cultures, taking into account that the anatomical location and microenvironment seem to play an essential role in astrocyte maturation and that we are observing very marked effects of optogenetic stimulation even if just a part of the cell population in the "ChR2 + BL" cultures actually expresses ChR2 and is optogenetically activated. This paracrine effect might occur in different forms, e.g. activity-induced neurotrophic/growth factor secretion that modulate astrocyte maturation, or propagation of ion fluxes between neighbouring cells via gap junctions, which for instance have been seen to couple astrocytes and oligodendrocytes in the nervous system (Orthmann-Murphy, 2008).

Nonetheless, trying to predict the functional implications of the observed effect of optogenetic stimulation on astrocyte maturation in the context of cell therapy for SCI is difficult. Much information about the physiological function of astrocytes comes from studies in the last years, showing their key roles in synapse formation, regulation of the extracellular microenvironment, metabolic support to neurons, etc. (Bylicky *et al.*, 2018; Sun & Jakobs, 2012). Specifically, protoplasmic astrocytes of the grey matter participate in the maintenance of the blood-brain barrier and in the regulation of blood flow and they are closely associated with synapses, potentially modulating synaptic functions (Tabata, 2015). Fibrous astrocytes are also associated with blood vessels and provide support to axons contacting them at the nodes of Ranvier (Tabata, 2015; Sun & Jakobs, 2012). In that sense, having a higher number of fibrous astrocytes might support re-growing axons at the lesion site, but we do not know if the concomitant reduction in the number of protoplasmic astrocytes produced will have deleterious consequences, since for example they have been described to secrete BDNF, which exerts beneficial neuroregenerative effects (Bylicky *et al.*, 2018).

Upon injury, both protoplasmic and fibrous astrocytes become reactive, although they present distinct hypertrophic morphologies (Bylicky *et al.*, 2018; Sun & Jakobs, 2012). Even if the proliferation of fibrous astrocytes seems to be more prominent than in the case of protoplasmic astrocytes upon injury (Tran *et al.*, 2018), the particular contribution of each of them to the beneficial and harmful effects of reactive astrogliosis have not been deciphered, mainly due to the high complexity and heterogeneity of the astrocyte response to SCI (Tran *et al.*, 2018). Also, Ca^{2+} signalling is involved in the induction of the reactive state in astrocytes upon injury (Tran *et al.*, 2018), and indeed Ca^{2+}-dependent S100B protein is thought to participate in this activation process (Brozzi *et al.*, 2009), thus this could be another way in which optogenetic stimulation may influence the activity of NPC-derived astrocytes, although we cannot know for now if this effect could be positive, negative or neutral. In any case, further work and insights into astrocyte biology will be required to assess the possible functional implications of optogenetically promoting fibrous astrocyte maturation.

4.3.3. OPTOGENETIC STIMULATION ENHANCES AXON GROWTH AND BRANCHING IN NEURAL PROGENITOR CELL-DERIVED NEURONS.

Finally, to determine whether optogenetic stimulation of differentiated NPCs is having an effect on axon length and axon branching in NPC-derived neurons, axons (= longest neurite) of βIII-tubulin positive cells were measured and the number of branches per axon counted (Fig. 19). Results show that optogenetic stimulation leads to a highly significant increase in mean axon length (Fig. 19C), maximum axon length (Fig. 19D) and axon branching (Fig. 19E) as compared to the control and transduced cells without BL exposure.

Previous works implementing optogenetic modulation of NPC transplants in the context of stroke agree with our results. These studies showed that *in vivo* optogenetic stimulation of NPC transplants in stroke models enhanced axon sprouting, axon growth and synaptogenesis, increased activity-dependent neuronal maturation and neural network connections, and

improved functional recovery (Daadi *et al.*, 2016; Yu *et al.*, 2019). Moreover, it was also demonstrated that *in vitro* optogenetic stimulation of NPCs led to an improved axon outgrowth and an increased expression of synaptic proteins (Yu *et al.*, 2019). Moreover, *in vitro* and *in vivo* electrical stimulation experiments have also shown improved axon growth as a consequence of cell depolarisation and Ca^{2+} entry (Grumbles et al., 2013; Kobelt et al., 2014).

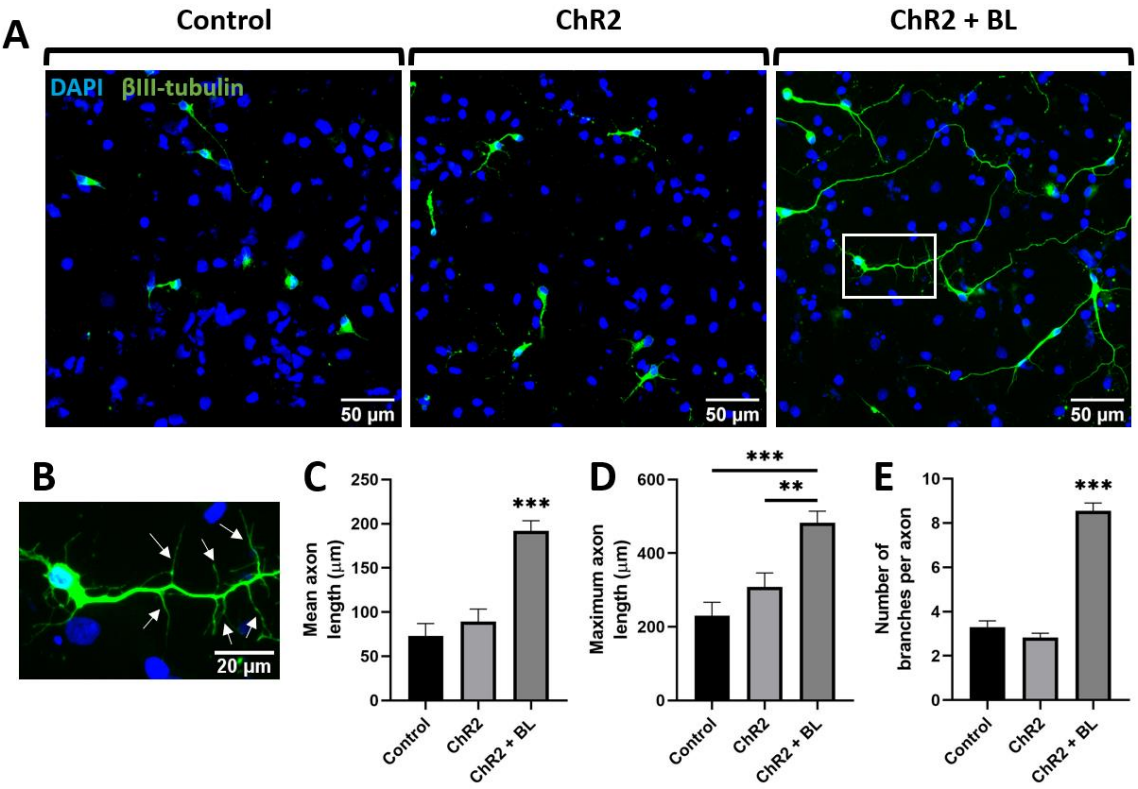

Figure 19. Effect of optogenetic stimulation on neuronal axon growth and branching. *(A) Fluorescence microscopy images of NPC-derived cells immunostained for the neuronal marker βIII-tubulin to assess cell morphology. (B) Zoom of part of a neuron in the optogenetically stimulated condition as marked with a rectangle in A. Arrows point at axon branches. (C) Mean axon length. (D) Maximum axon length. (E) Quantification of the number of branches per axon. Conditions: control cells, transduced cells (ChR2) and blue light exposed transduced cells, i.e. optogenetically stimulated cells (ChR2 + BL). Values represent the mean ± SEM. For C and E: ***p-value<0.001 compared with control and ChR2. For D: **p-value<0.01, ***p-value<0.001 (ANOVA for C and D, Kruskal-Wallis test for E; n = 3).*

These neuroregenerative benefits upon optogenetic stimulation might potentially be due to the increased intracellular Ca^{2+} concentrations, whose essential role in axon growth have been experimentally demonstrated (Chierzi *et al.*, 2005; Kamber *et al.*, 2009; Mahar & Cavalli, 2018). Indeed, after axotomy, the initial Ca^{2+} signals are crucial for growth cone formation through their participation in the required cellular events, including microtubule reorganisation and reduction of membrane tension by activating proteases such as calpains to cleave membrane spectrins (Kamber *et al.*, 2009). However, this is not the only way in which Ca^{2+} can potentially promote axon sprouting, growth and branching. As mentioned previously, Ca^{2+} can activate cAMP/EPAC2 signalling, which promotes neurite outgrowth via CREB induction (Wei *et al.*, 2016), and PI3K/AKT signalling, which is essential for growth cone formation through its downstream targets mTOR, GSK3β and APC (Park *et al.*, 2008; Schöneborn *et al.*, 2018; van Niekerk *et al.*, 2016). Additionally, AKT activation has been closely related to an increased axon branching (Schöneborn *et al.*, 2018). Concomitantly, Ca^{2+}-dependent activation of Ras leads to a downstream induction of the ERK1/2 pathway, which has been demonstrated to be necessary for axon lengthening (Perlson *et al.*, 2005; Schöneborn *et al.*, 2018). Also, CaM/CaMKII signalling is thought to mediate synaptic plasticity via modification of the actin and microtubule

cytoskeleton (McVicker *et al.*, 2015) and to stimulate neuroregeneration via activation of the pro-regenerative transcription factor CREB, which indeed is also activated by Ras/ERK signalling (Wu et al., 2001). In fact, CREB has been shown to be very important for neuronal maturation, neurite growth and synapse formation during neuronal development, and its activation seems to be highly Ca^{2+}-dependent, since blocking Ca^{2+} currents impairs CREB phosphorylation (Lepski *et al.*, 2013). Moreover, Ca^{2+}/cAMP-dependent activation of DLK via protein kinase A (PKA) has also been implicated in neuroregeneration through DLK-mediated induction of the pro-regenerative transcription factor JUN (Mahar & Cavalli, 2018). Apart from activating all the above-mentioned signalling pathways, Ca^{2+} rises can lead to pro-regenerative epigenetic changes through increased histone acetylation via histone deacetylase 5 (HDAC5) nuclear export (Mahar & Cavalli, 2018). As an added effect, exported HDAC5 is activated by Ca^{2+}-dependent protein kinase C and induces tubulin deacetylation at the axon tip, which is essential for growth cone dynamics (Cho & Cavalli, 2012). All in all, the molecular mechanisms through which optogenetic stimulation and subsequent Ca^{2+} entry might potentially have led to the observed results are vast.

These results are of great importance when thinking about implementing *in vivo* optogenetic stimulation of NPC transplants for the treatment of SCI, since through the improvement of axon extension and branching, optogenetics can potentially lead to longer axons that project over longer distances rostrally or caudally to the injury and that stablish a higher number of synaptic connections with host neurons thanks to the increased branching, thus enhancing the potential of NPC transplants to form functional neuronal relay circuits. Moreover, the observed increased length and branching of optogenetically stimulated NPC-derived neurons indicates an enhanced neuronal maturation and synaptogenesis potential, which may be due to the generation of action potentials in these neurons, as previously noticed in electrical stimulation experiments (Kobelt et al., 2014), and here as a result of ChR2-mediated cell depolarisation upon photostimulation. In fact, neuronal activity has been described to be essential for neuronal maturation, synaptogenesis and functional integration, which are crucial for beneficial functional recovery after transplant (Yu *et al.*, 2019; Zhang & Poo, 2001).

Additionally, the strong effect that optogenetic stimulation displays in our results despite the fact that just a part of the cell population expresses ChR2 further supports our hypothesis that there might be a second indirect mechanism responsible for the observed effects, which might rely on an activity-induced secretion of neurotrophic factors by the stimulated ChR2-expressing cells. As mentioned earlier, these neurotrophins enhance axon sprouting and growth through the activation of pro-regenerative pathways such as PI3K/AKT/mTOR (van Niekerk *et al.*, 2016; Yu *et al.*, 2019).

4.4. FINAL REMARKS OF THE DISCUSSION.

We have shown that *in vitro* cultured and transduced NPCs can successfully express ChR2-mCherry even if transduction efficiency was limited, which agrees with previous works reporting a low efficiency of AAV gene delivery to undifferentiated cells (Hughes et al., 2002; Jang et al., 2011; Rapti et al., 2015) and particularly of AAV9 gene delivery to *in vitro* cultured cells (Ambrosi *et al*., 2019; Shen *et al.*, 2011), probably due to the low availability of AAV9 receptors (LamR and terminal galactose) on the surface of these cells. The use of more efficient recombinant AAVs or enzymatic desialylation of NPCs could be tried out in future experiments to achieve higher efficiencies. We have also demonstrated that ectopic ChR2-mCherry expression in the transduced NPCs does not affect NPC viability, making it feasible for implementation in cell therapy.

Light activation of ChR2 expressed in NPCs gives rise to an initial cell depolarisation through the influx of cations, including Ca^{2+}. Probably, the amount of Ca^{2+} ions that is ultimately

incorporated into the cell after ChR2 activation might not be solely due to ChR2 induction, but also to the activation of VGCCs as a consequence of ChR2-induced cell depolarisation. Thus, activated ChR2 could potentially recruit Ca^{2+}-dependent cell processes not only through its activation-dependent Ca^{2+} influx, but also via activation of VGCCs expressed in NPCs and NPC-derived cells (Louhivuori *et al.*, 2013; Stroh *et al.*, 2011). We hypothesise that this increase in cytosolic Ca^{2+} concentration is the main cellular signal responsible for the effects observed in our experiments, namely an improved neurite outgrowth (due to an increased neurite sprouting) in undifferentiated NPCs, an enhanced neuronal and oligodendrocyte differentiation, morphological changes in NPC-derived astrocytes and an increased axon growth and branching. According to the information available in the literature presented in the previous sections, this Ca^{2+} signal could be inducing these cellular events through the activation of signalling pathways with neuroregenerative effects, which could potentially include cAMP/EPAC2, DLK/JNK, CaM/CaMKII, CaM/calcineurin, ERK1/2 and PI3K/AKT/mTOR pathways. The described downstream effects of this Ca^{2+} signal and these signalling pathways, which could explain the results we have obtained, have been schematically summarised in Fig. 20.

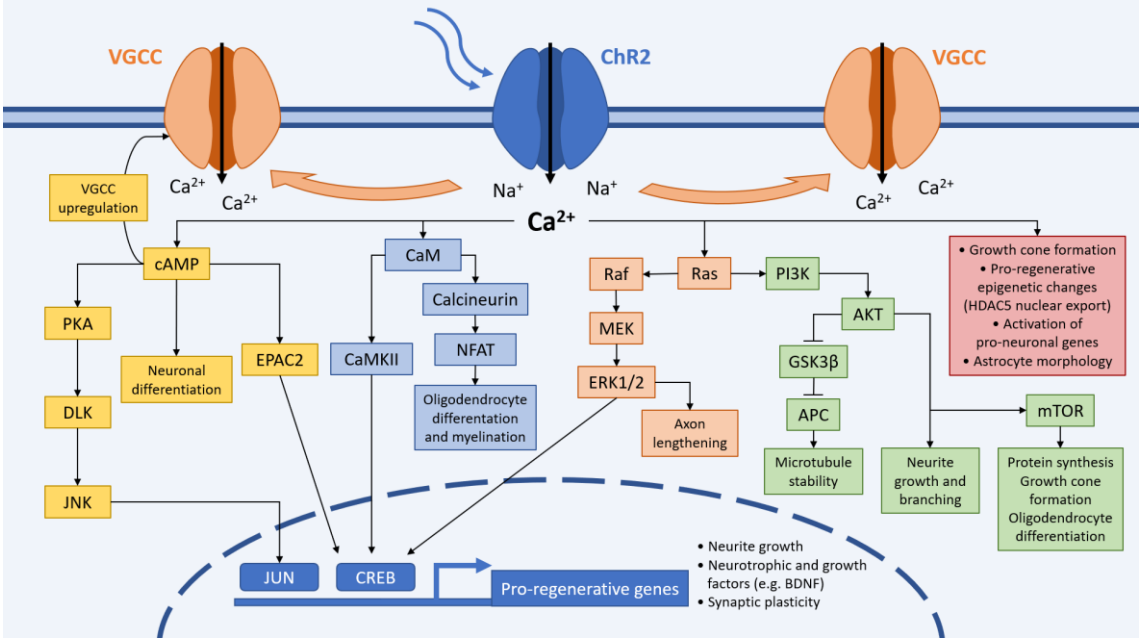

Figure 20. Schematic representation of signalling pathways and cellular events triggered by intracellular Ca^{2+} elevation due to ChR2 photoactivation. *ChR2: channelrhodopsin-2; VGCC: voltage-gated calcium channel; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; DLK: dual leucine zipper-bearing kinase; JNK: JUN N-terminal kinase; EPAC2: exchange protein directly activated by cAMP 2; CaM: calmodulin; CAMKII: Ca^{2+}/calmodulin-dependent protein kinase II; CREB: cAMP-responsive element-binding protein; Ras: rat sarcoma; Raf: rapidly accelerated fibrosarcoma; MEK: MAPK/ERK kinase; ERK: Extracellular-signal-related kinase; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B; GSK3β: glycogen synthase kinase 3β; APC: adenomatous-polyposis-coli; mTOR: mammalian target of rapamycin; HDAC5: histone deacetylase 5; BDNF: brain-derived neurotrophic factor.*

Functional implications of our results reveal a considerable potential of optogenetic stimulation of NPCs to improve cell therapy outcomes. First, it could be implemented as a preconditioning approach in the form of *in vitro* optogenetic stimulation prior to transplant, since our results have demonstrated that stimulated NPCs have an enhanced capability to grow neurites, which would contribute to an improved connectivity and functional integration of transplanted NPCs with the host spinal cord circuitry. Increases in NPC proliferation and viability after optogenetic stimulation have not been observed, which anyway dissipates concerns of possible excessive NPC activation and potential tumorigenesis.

Secondly, our results regarding the effects of optogenetic stimulation on NPC differentiation and maturation reveal the great potential of *in vivo* optogenetic stimulation after transplant, which has been recently enabled by the development of small, wireless, fully internal implants for light delivery (Montgomery *et al.*, 2015; Samineni *et al.*, 2017). When NPCs are transplanted, the lesion microenvironment induces them to largely differentiate into astrocytes, with very little oligodendrocyte and neuronal differentiation (Assink *et al.*, 2017; Barnabé-Heider *et al.*, 2010; Panayiotou & Malas, 2013; Sabelström *et al.*, 2014). Our results show that optogenetic stimulation of NPCs under spontaneous differentiation conditions promotes their differentiation into oligodendrocytes and neurons, potentially translating into increased myelination and formation of neuronal relay circuits, respectively, which are crucial for cell therapy to be effective. A very relevant improvement observed in NPC-derived neurons as a result of optogenetic stimulation is their strongly increased axon growth and branching, indicating an enhanced activity-dependent neuronal maturation, which might potentially lead to an improved capability to form functional relay circuits and synaptic connections with host neurons, leading to proper functional recovery. However, whether all these effects observed *in vitro* will be maintained *in vivo* remains to be confirmed in future experiments with SCI animal models. Also, the proportion of NPC-derived astrocytes remains unchanged, although a strong effect is observed with regard to their morphological maturation, since optogenetic stimulation favours a fibrous morphology over a protoplasmic one. However, we cannot speculate about possible functional implications with the current scientific knowledge about these subtypes and their implications in SCI, thus further work will be needed to address this issue.

Interestingly, even if just a minority of the NPC population incubated with AAV9 ultimately expressed ChR2, making them susceptible to optogenetic stimulation, very marked effects have been observed, for instance in the case of axon growth and branching, strongly suggesting that apart from the effect caused by direct stimulation on ChR2-expressing cells, an indirect mechanism might have also induced changes in the majority of cells not expressing ChR2. This indirect effect might involve paracrine signalling, possibly in the form of stimulation-induced increased secretion of neurotrophins and growth factors by stimulated ChR2-expressing cells, propagation of ion fluxes between neighbouring cells via gap junctions, or in the form of neurotransmitters released from stimulated ChR2-expressing NPC-derived neurons.

Future directions for *in vitro* assays should try to decipher which signalling pathways are implicated in the effects observed as a result of *in vitro* stimulation (e.g. by western blotting), taking into account the potential candidates as deduced from the current literature and summarised in Fig. 20. Moreover, the secretome of optogenetically stimulated cells should be analysed to assess our hypothesis of paracrine signalling. Also, it would be interesting to check if effects such as optogenetically enhanced neurite/axon growth can also be reproduced when incubating the cells with growth-inhibitory compounds (e.g. CSPGs) that mimic the inhibitory lesion microenvironment, or if optogenetic stimulation is capable of promoting cell survival when cultured in a cytotoxic environment (e.g. in the presence of H_2O_2 to induce oxidative stress), since some of the events represented in Fig. 20 have been also described to improve cell survival (e.g. ERK1/2 or PI3K/AKT signalling) (Schöneborn *et al.*, 2018). *Ex vivo* experiments transplanting NPCs to spinal cord slices and studying how optogenetic stimulation might affect their integration would be also of great interest before moving to the final *in vivo* assays in SCI animal models.

5. CONCLUSIONS

SCI is a devastating disease with tremendous consequences for affected patients and society. Cell therapy is one of the most promising therapeutic approaches currently under investigation, although still much work is needed to overcome its limitations, which make evident the need for combination with additional strategies. In this work, we propose optogenetics as a candidate to complement cell therapy due to its potential to increase the neuroregenerative capabilities of NPCs used for transplant. The complex neural networks of the nervous system work with a high temporal and spatial precision, and optogenetics acts accordingly with high precision and selectivity, allowing for fewer side effects and potentially great effectivity.

The main objective of this project was to study the effect of *in vitro* optogenetic stimulation on ependymal NPCs. The conclusions drawn from the present work are the following:

- First, delivery of the light-activated cation channel ChR2 to *in vitro* cultured NPCs using an AAV9 vector leads to successful expression of ChR2 in these cells, although transduction efficiency is limited.
- Secondly, expression of mCherry-tagged ChR2 in transduced NPCs does not affect NPC viability.
- Thirdly, optogenetic stimulation does not affect the proliferation and viability of NPCs under proliferation conditions, but it does improve neurite outgrowth via an increased neuritogenesis.
- Fourthly, optogenetic stimulation enhances spontaneous differentiation of NPCs into oligodendrocytes and neurons. Even if optogenetic stimulation does not affect the proportion of NPC-derived astrocytes generated, it influences astrocyte morphological maturation by favouring acquisition of a fibrous morphology over a protoplasmic one.
- Fifthly, optogenetic stimulation promotes neuronal axon growth and branching, yielding longer axons and higher number of axonal branches.

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