Universidad de Navarra

Facultad de Ciencias

"rAAV8-mediated in vivo reprogramming of striatal astrocytes into neurons as a potential therapeutic strategy for neurodegenerative diseases"

José Diego Pignataro López

2017

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Memoria presentada por D. **José Diego Pignataro López** para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Terapia Génica y Neurociencias y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 10 de abril de 2017.

Dr. Gloria González Aseguinolaza

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Funding statement

Supported by FP7-PEOPLE-2011-IAPP - Marie Curie Action: "Industry-Academia Partnerships and Pathways" (ref. 286071 “Brainvectors”), ERC Advanced grant (ref: 340527 “Repropark”), Fundació La Marató TV3 (ref 20141331), Fundación La-Caixa, Terapia génica Sindrome de Dravet and CoEN Pathfinder (Ref: Phase II Call). Diego Pignataro is partially supported by a Jon Zarandona donation.

“What if I say I will never surrender”

Dave Grohl

“Y si digo que nunca me voy a rendir”

Dave Grohl

A mi padre

A mi madre y hermano

A toda mi familia

A todos los que siempre confiaron en mi

A Diegón

A Maider

AKNOWLEDGEMENT

En primer lugar, agradezco a la Universidad de Navarra y al CIMA por abrirme sus puertas y darme la oportunidad de hacer el máster y posteriormente el doctorado en ella. Ha sido una gran experiencia que me ha fortalecido mucho en lo personal y profesional.

Siempre estaré agradecido al Gobierno de Navarra porque gracias a su beca pude cargar mis maletas llenas de sueños, curiosidades y muchas ganas de cumplir esta meta. Gracias por depositar su confianza en las personas que venimos de afuera la verdad. Sin duda alguna hubiese sido imposible empezar esta aventura sin su apoyo inicial. Por otro lado, quiero agradecer al proyecto que co-financió mi tesis: ¨El Brainvectors¨. Su financiación me ha permitido conocer un sin número de personas que tanto a nivel personal como profesional me han dejado muchos valores positivos. Thank you very much Liliane and Marie! Since our first talk I got this ¨feelling¨ and good vibes coming from you two. Despite you did not know me since a long time you always trusted in me, support me in everything you could (even outside the lab) and I will never forget that!

Imposible no mencionar a Cristian, Pepe y todo el laboratorio 4.07 que en el 2011 me abrió sus puertas para empezar esta aventura científica. Cristian y Pepe, ustedes fueron mis grandes mentores cuando llegué a Pamplona. La formación que me dieron durante el año de mi máster ha sido fundamental para mi desempeño en el doctorado, me enseñaron a caminar en el mundo de la investigación biomédica. Gracias por dejarme participar en un proyecto que me formó tanto en lo teórico y práctico. Gracias por las charlas, las reuniones para enseñarme como organizarme con los experimentos, analizar los datos, como organizarme con el cuaderno, como organizarme con las muestras, etc, etc. Tantas cosas que no menciono y que no las he dejado de considerar ni un solo día durante estos últimos cuatro años de la tesis, ni uno solo. Cristian, nunca olvidaré que incluso cambiaste tus vacaciones para corregirme el proyecto y practicar conmigo la presentación. Si algún día tengo un equipo a mi cargo, trataré de darle a mis pupilos lo mismo y más. ¡¡Gracias infinitas!!

Terminé el master y el momento de empezar la tesis llegó. Por temas fuera de nuestro control no pude continuar mi tesis con tu grupo, pero nunca me dejaron de lado y lograron

reubicarme. Nunca olvidaré el momento en el que recibí la llamada de Gloria para decirme si estaba dispuesto en unirme a su grupo: ¨ya lo he hablado con mi grupo y están de acuerdo, sólo falta que tú nos confirmes¨. Por supuesto que dije que sí y tanto ella como Lanci me abrieron las puertas de sus laboratorios para quedarme con ellos estos cuatro años. Ahora que miro atrás, veo lo mucho que he aprendido y el Diego que llegó se ha enriquecido de mucha formación profesional y también a nivel personal. Gracias Gloria y Lanci, a ustedes les debo un agradecimiento especial por aceptarme en su grupo para llevar a cabo este trabajo y cumplir este gran objetivo profesional (prácticamente ya tenía las maletas listas para volverme a casa). Gracias por dirigir este trabajo y siempre plantearme retos que me han enseñado a ser independiente en mi trabajo y desarrollar mi criterio científico. Gracias Gloria por siempre estar disponible para mí sea donde te tocó responder a un email y mensaje. Valoro muchísimo el tiempo que me has dedicado a pesar de no ser directamente de tu grupo. Sin tu apoyo creo que en estos momentos no estaría terminando de redactar la tesis. Gracias Lanci por enseñarme a trabajar con un modelo tan complejo como son los monos y estar casi listo para operar uno si es necesario y por impulsar nuestra participación en congresos y eventos a los que he podido asistir. Gracias a ambos por su comprensión y por preocuparse por mí en los difíciles momentos que me tocó vivir a lo largo de estos años.

Se me vienen tantos momentos a la cabeza que no sé por dónde empezar. Aún recuerdo como si fuera ayer cuando miraba por la ventanilla del avión y veía como mi familia, mi ciudad y todo lo que tengo en Lima iba quedando atrás en medio de la noche para cumplir este sueño. Mirando al cielo decía: “Por favor cuida a mi familia en estos años que estaré fuera, que no les pase nada y podamos estar todos juntos a la vuelta”. La primera meta se cumplió y pude volver a casa a abrazar a los míos y decirles “misión cumplida, terminé el master”. La mirada de satisfacción de mi papá y mi mamá es algo que nunca olvidaré y que me ha llenado de fuerzas durante estos años de tesis, pero sobretodo para mi era la satisfacción de volver cada año a casa y abrazarlos a la salida del aeropuerto y otra vez a disfrutar de mi familia. Las semanas de vacaciones pasaban como si fueran unas pocas horas y otra vez llegaba el momento del abrazo que un hijo o padre no quiere dar. El abrazo del “buen viaje hijo, cuídate mucho” o “chau pá, má, por favor cuídense mucho hasta que vuelva”. Faltaba tan poco para conseguir esta meta y te me adelantaste

mi amado pa. Te fuiste al cielo para cuidarnos desde arriba y el abrazo de meta cumplida quedará pendiente hasta que nos volvamos a unir donde estés. Me dieron todo lo que pudieron y cuando no se pudo, siempre encontraron la forma de compensarlo. Nunca olvidaré cuando me dijiste entre lágrimas que no podría estudiar en una universidad privada, sin embargo juntos sacamos adelante la carrera en mi querida San Marcos y mira dónde estoy terminando el Doctorado.

La vida en estos años me ha traído un sin número de experiencias buenas y no tan buenas, pero de todas ellas se aprende. Mi mamá siempre me ha dicho que tengo una especie de "don" para elegir a mis amigos y la verdad es que no sé cómo lo hago, pero mis grandes orgullos de la vida siempre van a ser mi familia y mis amigos. Considero que he tenido mucha suerte desde el primer día que llegué a Pamplona. Manolo y Anita me recibieron en su casa como si fuera un miembro más de la familia y a pesar que no nos veamos hace un tiempo, los tengo muy presentes y para ellos todo mi cariño y también esta tesis. En mi segundo hogar en Pamplona ya no me recibió una familia, esta vez la familia se formó en ella y ¡qué familia! Maider, Santi, Marcos y el que les habla. Creo que lo de "compis" nos duró poco o nada ¿no?, somos "Diablitas non-stop" para siempre. La paciencia y buen humor de Santi para escuchar nuestras frustraciones durante horas en la cocina ("qué paciencia la de Santi" solíamos decir con Maider. Pero sí que te han servido nuestras conversaciones porque ahora eres el economista que más sabe de modificaciones epigenéticas del mundo mundial! Marquitos, cuando llegaste pusiste la cuota aventurera y cómo dejar de mencionar nuestras largas conversas y proyectos "Nobel prize I y II". Aunque ahora son Nobel prize - failed I y II, no importa, seguiremos intentando po! Gracias por hacer de nuestro día a día una vida de familia. Rom y Victor, ustedes llegaron un poco después, pero igual se integraron a la familia de una forma tan increíble que no sé si es que nuestro agente inmobiliario sabe elegir bien o es que tenemos una suerte única.

Dicen que los zurdos tenemos menos probabilidades de sobrevivir (incluyendo la tesis), pero tuve la gran suerte de contar no sólo con mi mano izquierda, también tuve la mejor mano derecha que alguien pueda tener. Diegón, ¡mi gran partner de mañana, tarde y noche! Desde el momento en que nos presentaron tuvimos esa buena vibra y qué pareja la que se formó. Qué te puedo decir, estoy muy orgulloso de tu avance y de todo lo que hemos hecho juntos. Tus ganas de trabajar, aprovechar al máximo el tiempo y seguir

desarrollando ideas es algo que siempre tomo de modelo y admiro. Gracias por estar siempre allí, fuera o dentro del lab. Somos Los Diegos, seámoslo siempre.

Mi querida "cuadrilla internacional", aunque ahora sólo quedamos 3 quiero agradecerles por ser parte de mi vida y compartir tantos buenos momentos. Luis, Ro, Ali pensar en ustedes sólo me trae risas y carcajadas. Gracias Luis por siempre acudir cuando te he necesitado, ¡mi otro gran partner de la vida y del ukulele!. Gracias por tu apoyo en mis momentos difíciles y escucharme cuando volví, realmente lo necesitaba. No puedo dejar de mencionar a Cris, que gracias a ella tenemos muchas anécdotas que contarles a nuestros hijos. Para ustedes con mucho cariño esta tesis. ¡No se olviden que aún nos queda mucho que remar!

Iria, reconozco que no empecé bien nuestra relación y qué pena no haber disfrutado más tiempo de tu persona porque vales mucho. Muchísimas gracias por todo tu apoyo, concejos, ideas, empuje para que por fin pueda imprimir mi tesis. Valoro mucho lo que has hecho por mi y para lo que necesites estaré y te deseo el mayor éxito profesional, ¡¡te lo mereces!!

Cris y Afri, a ustedes dos todo mi cariño y respeto por el gran trabajo que hacen en el laboratorio. ¡Qué sería de nosotros sin ustedes! A lo largo de estos años se han convertido en una parte muy importante de mi vida. Realmente considero que así como hay el día del padre, la madre, de la mujer científica. Creo que se debería celebrar el día de la técnico. Ya que sin ustedes poco o nada podrías hacer en el laboratorio. Las quiero un montón y gracias por su ayuda y cariño en las buenas y en las malas. Son como unas madres la verdad. ¡Lonchera time por siempre!

Nerea e Itzi, mis Pepa Pigmeas favoritas, gracias por su amistad, por estar pendientes de mi estado mental en los momentos difíciles, por escuchar mis ideas locas de negocio, por siempre remar juntos cada viernes y también disfrutar de la vida conmigo. Con mucho cariño esta tesis y todo lo que pueda darles.

Alberto y Elvira, ¡mil gracias por su apoyo en el laboratorio! Elvira, gracias por enseñarme las inmunos, cómo cortar y montar los cortes de cerebro con tanta precisión y arte. Me has dado desde un pincel hasta un hogar donde vivir. ¡Gracias!. Alberto, muchas gracias por enseñarnos a cuidar de los monos, a medir las escalas por tu ayuda en las cirugías y estar pendiente de reemplazarme en algún turno del PET para que yo pueda seguir escribiendo la tesis. ¡Gracias Alberto!

Tengo tantas personas a las qué agradecer y dedicarles esta tesis. A mis amigos de toda la vida del colegio, de mi universidad, del CIP, a mi GRUpo de Cayetano, mi primer grupo de investigación profesional. A mis tíos, primas a todos los que me quieren, para todo ellos este trabajo. A todos ellos porque a pesar de estar estos 5 años separados siempre se han mantenido cerca como si nunca nos hubiésemos separado.

Este último párrafo va para una de las mejores personas que he podido conocer en esta vida. No sólo eres mi mejor amiga, mi partner, mi colega o ahora mi novia. Eres una hija y hermana ejemplar, tu capacidad para dar con lo poco que puedas tener es increíble y admirable. Gracias por tu apoyo incondicional en estos cuatro años. Tu vocación de científica me inspiró mucho durante estos años, realmente te admiro y cuando sea grande quiero ser como tu. Sé que lograrás cumplir tus objetivos profesionales porque tu talento hay que aprovecharlo y porque ¡eres grande! Gracias por escucharme y por transmitirme esta paz cada vez que hablamos. Gracias por mantener la cabeza fría y ayudarme a poner los pies de vuelta en el suelo cuando lo he necesitado. Gracias infinitas cruzar el mundo y estar a mi lado cuando mi papá se fue a pesar de estar con la escritura de la tesis de por medio, simplemente no lo dudaste y fuiste detrás de mi. Como siempre dices “en las buenas y en las malas estos pelos siempre estarán presentes”. Ahora nos queda cumplir una meta personal muy importante y seguir escribiendo nuestra historia, pero en un libro diferente.

Gracias vida por esta oportunidad. Querido viejo, nos vemos en el cielo para gritar un gol más, y celebrar de una buena vez que no nos volveremos a separar.

¡¡¡Feliz cumpleaños, feliz 10 de abril!!!

LIST OF ABBREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
AAP	Assembly activating protein
AAV	Adeno-associated vector
AD	Alzheimer´s disease
Ad	Adenovirus
AL	Ascl1/Lmx1A
ALN	Ascl1/Lmx1A/Nurr1
AMP	Adenosine monophosphate
BP	Basal plate
bp	Base pair
CAG	Chicken B-actin
CaMKIIa	Calcium-calmodulin kinase-2a
CHRNB2	Neuronal nicotinic receptor B2
CMV	Cytomegalovirus
CNS	Central Nervous System
CNS	Central Nervous System
CSF	Cerebrospinal fluid
D	Diencephalon
DA	Dopaminergic
DBS	Deep brain stimulation
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
DREADD	Designer receptor exclusively Activated by designer drugs
EDS	Excessive daytime sleepiness
eGFP	Enhanced green fluorescent protein
ERAD	Reticulum-associated degradation
ESC	Embrionic stem cells
FBS	Fetal bovine serum
GAPDH	Gliceraldehído-3-fosfato deshidrogenasa
GBA1	Glucocerebrosidase
GC	Genome copies
GD	Gaucher disease
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrilary acidic protein
GP	Globus pallidum
GPe	External globus pallidum
GPi	Internal globus pallidum
GSG	Glycine-Serine-Glycine
H	Hindbrain
hA	human transcription factor Ascl1
hAL	human transcription factors Ascl1/Lmx1A
hGH	Human growth hormone

hN1	human transcription factor Nurr1
hpt	Hours post-transfection
HSV	Herpes Simplex Virus
hSyn	Human synapsin
iPSC	Induced pluripotent stem cell
ir	Immuno reactive
IRES	Internal ribosome entry site
IsO	Isthmic organizer
ITR	Inverted terminal repeat
IV	Intravascular
IZ	Intermediate zone
Kb	Kilobase
KDa	Kilo Dalton
LB	Lewy bodies
M	Midbrain
M&M	Material and methods
mAL	murine Ascl1-Lmx1A
mCh	mCherry
MCI	Mild cognitive impairment
MCS	Multiple cloning site
mFP	Midbrain floor plate
MHB	Midbrain-hindbrain boundary
miDA	Midbrain dopaminergic
MiniPs	Mini promoters
M-MLV	Moloney murine leukemia virus
mRNA	Messenger RNA
MSN	Medium spiny neurons
MZ	Mantle zone
N1	Nurr1
Nabs	Neutralizing antibodies
Nb	Neuroblast
NeuN	Neuronal nuclei
NHP	Non human primate
NO	Nitric oxide
NPC	Neuronal progenitor cell
NPC	Nuclear pore complex
NRTN	Neurturin
NSC	Neural stem cell
NSE	Neuron specific enolase
ORF	Open reading frame
PB	Phosphate buffer
PCR	Polymerase chain reaction
PD	Parkinson´s disease
pr	Promoter

qPCR	Quantitative polymerase chain reaction
rAAV	Recombinant Adeno-associated vector
RBD	REM sleep behavior disorder
RG	Radial glial cells
rh	Recombinant human
RM	Radial migration
RNA	Ribonucleic adic
RrF	Retrorubral field
SC	Stem cell
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
SVZ	Sub-ventricular zone
T	Telencephalon
TBS	Tris buffer solution
TF	Transcription factor
TH	Tyrosine hydroxylase
VM	Ventral midbrain
vp	Viral particles
VP	Viral protein
VTA	Ventral tegmental area
VZ	Ventral zone
WB	Western blot
wpi	Weeks post injection
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
α-syn	alpha synuclein

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INTRODUCTION

1. GENE THERAPY IN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases are a group of disorders in which loss of neurons is observed and are characterized by progressive cognitive and/or motor functions impairment. Disease-modiying treatments aiming at reducing the rate of neurodegeneration or even stopping disease progression have remained elusive in the last years. However, understanding of the pathogenesis is advancing and thereby helping the development of new therapy based approaches.

The field of gene therapy (GT) applied to Central Nervous System (CNS) pathologies has recently witnessed a number of major conceptual changes. Indeed in the last decade many promising approaches have been developed to treat diseases, particularly as future treatments for neurodegenerative diseases such as Alzheirmer´s (AD) or Parkinson´s disease (PD) (Kalia et al., 2015a; Hocquemiller et al., 2016). GT has been used for the treatment of CNS tumors by transferring genes that specifically kill the transduced cells (suicide genes) or inhibit their proliferation capacity (Kroeger et al., 2010). Moreover, it could be also applied to a wide variety of CNS disorders by the delivery of therapeutic genes with the capacity to protect against the development of neurodegenerative diseases or mental injuries (Ruitenberg et al., 2002; Bartus et al., 2013; Hocquemiller et al., 2016).

Gene transfer in the CNS depends on the use of delivery vehicles that selective transduce specific cells, due to the intrinsic characteristic of the vector (cell specific entry) or by the specificity of the promoter that controls the expression of the transgene (Bourdenx et al., 2014; Murlidharan et al., 2014). Although viral and non-viral vectors have been used for CNS GT, in general viral vectors are significantly more efficient than non-viral vector at introducing genes into the cells (Terzi et al., 2008). In particular, Adeno-associated viruses (AAVs) have emerged as promising tools for preclinical and clinical gene transfer in a broad range of neurological disorders with a safety profile and efficiency in transducing a wide range of cell types (Gray et al., 2013; Hocquemiller et al., 2016) and it is the viral vector selected for this thesis.

2. ADENO-ASSOCIATED VIRAL VECTORS MEDIATED GENE THERAPY

2.1 AAV biology, organization and structure

AAV is a member of the *Parvovirus* (*parvo-:* Latin for ¨small¨) family, classified as a *Dependovirus* because it requires the co-infection of a helper virus such as adenovirus (Ad) or Herpes Simplex Virus (HSV) to complete its replicative cycle (Murlidharan et al., 2014; Berns et al., 1987). The name adeno-associated originates in its detection as a contaminant in adenovirus preparations (Atchinson et al., 1965). AAV is naturally replication-defective and nonpathogenic in humans or animals, and it shows a low immunogenicity in comparison with other viruses (Sun et al., 2003).

The 4.7-kilobase (kb) single stranded wild-type AAV genome is composed of genes rep and cap, flanked by two 145 nucleotide inverted terminal repeat sequences (ITRs). The *rep* gene encodes four proteins essential for replication, packaging, transcriptional regulation of viral promoters and site-specific integration. The *cap* gene acts as template for the production of three structural proteins that only differ in their N-terminus: VP1, VP2 and VP3 proteins; these proteins form the capsid at a ratio of 1:1:10, respectively **(Figure 1A)**. An alternative ORF nested in *cap* encodes for an assembly activating protein (AAP), which interacts with the viral capsid proteins VP1, 2 and 3 and is necessary for viral assembly (Murlidharan et al., 2014; Ojala et al., 2015).

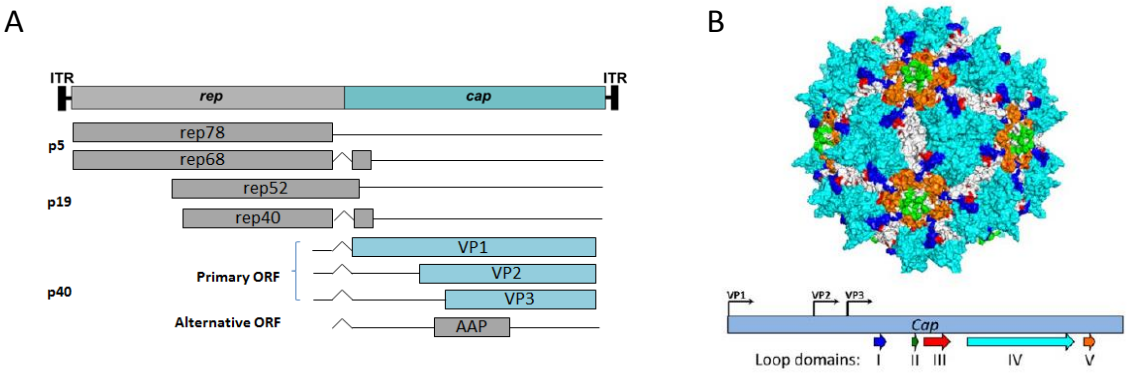


Figure 1. AAV genome organization. (A) Through alternative splicing, rep encodes four proteins — Rep78, Rep68, Rep52, and Rep40 — that are involved in viral genome replication. The protein AAP, which assists in viral capsid assembly, is encoded by an alternative ORF with a nonconventional CUG start codon. Gene expression is driven by the p5, p19, or p40 promoter as indicated; AAP = assembly-activating protein. (B) AAV capsid structure. (adapted from Ojala et al., 2015).

The AAV is a non-enveloped virus with a capsid composed of 60 proteins subunits with an icosahedral architecture of 25 nm in diameter **(Figure 1B)**. The different looped-out domains that are displayed on the surface of the capsid and a slightly varying amino acid composition give rise to the different AAV serotypes (Samulski et al., 2015). At least 12 natural serotypes have been isolated with many additional variants and, surprisingly, over one hundred AAV variants were discovered in human or non-human primate tissues. Interestingly, in humans, more than 80% of the population at the age of 20 years has been exposed to AAV2. This serotype was the first fully characterized and has been used as a gene therapy vehicle since 1984 (Hermonat and Muzyczka, 1984). It has been the serotype most used in the last 30 years (Samulski et al., 2015; Hadaczek et al., 2006; LeWitt et al., 2011).

AAV serotypes have been demonstrated to efficiently transduce a number of somatic tissues like muscle (Hagg et al., 2016), liver (Vanrell et al., 2011), heart (Chu et al., 2004), retina (Harvey et al., 2009) and the CNS (Chtarto et al., 2016; Bockstael et al., 2011). Based on their capsid structures the different AAV serotypes have particular properties pertaining to antigenicity, *in vivo* tropism and receptor interactions **(Figure 2)**.

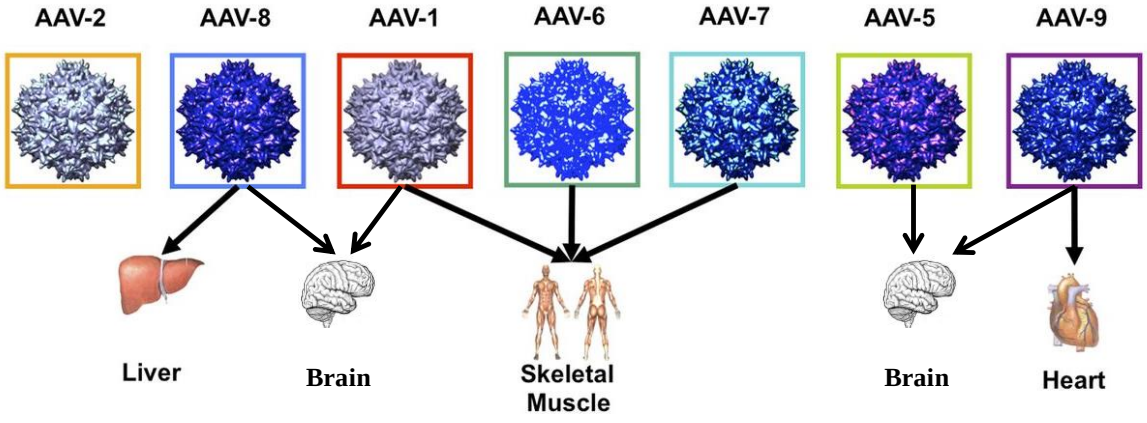


Figure 2. Tropism of wild-type AAV and of recombinant AAV vectors. The capsid sequences are highly conserved, from 60% to >99%, but studies with naturally occurring serotypes and purpose-engineered capsids have shown that even small differences in capsid sequence may affect tissue tropism of a vector and can be exploited to improve therapeutic outcomes. Figure adapted from Arrunda and Xiao, 2013. Brain illustration taken from Dreamstime ®.

2.2 AAVs as gene delivery vectors

The ITRs are the only cis-element of the AAV genome necessary for DNA replication and packaging. The *rep* and *cap* genes can be replaced by any sequence of interest within a size limit of approximately 5 kb. During the vector production process rep and cap are administered in trans (Dong et al., 1996) **(Figure 3)**.

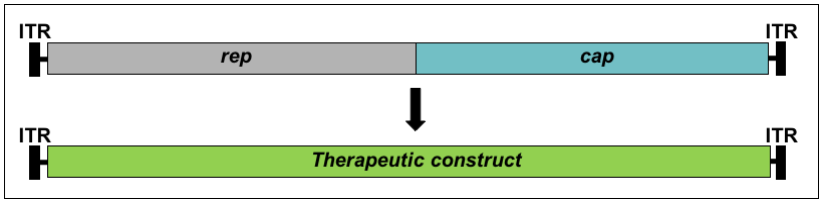


Figure 3. Construction of recombinant AAV (rAAV) for therapeutic approaches.

rAAV vector production begins with transfection of mammalian cells (commonly HEK 293T) with two/three plasmids. The first provides the Cap proteins from the chosen AAV serotype in conjunction with Rep from AAV2. This plasmid lacks ITRs, ensuring that the Rep/Cap sequences are not packaged into AAV capsids and no replication-competent virus is made. The genome plasmid contains the chosen transgene sequence flanked by ITRs. The third plasmid (if used) provides in *trans* the adenovirus genes that are necessary for AAV replication. It is common to see this plasmid and the Rep/Cap plasmid combined in a single large construct for simplified production. After 48 – 72 hours cells are harvested and lysed. Vectors can be purified by either column chromatography or density gradient centrifugation, which can separate AAV from contaminating cellular proteins as well as separating empty capsids from genome-containing particles. Finally the vector quantification is often performed by real-time PCR (Samulski et al., 2015) **(Figure 4)**.

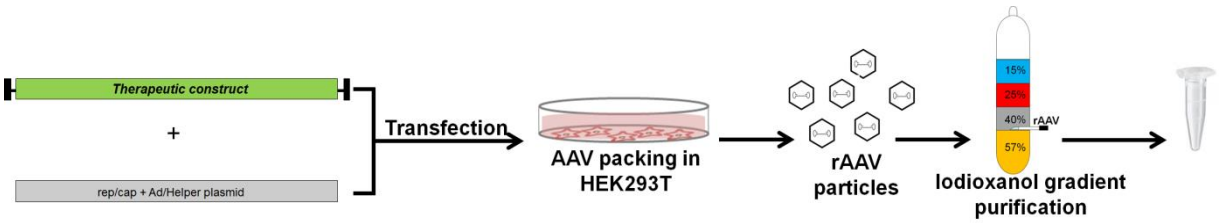


Figure 4. rAAV vector production.

As mentioned above, the vast majority of studies on AAV biology have been performed using AAV2 as a model. The primary attachment receptor for AAV2 is the heparin sulfate proteoglycan (Summerford and Samulski, 1998) and various other cell surface glycans have been identified as the preferred primary receptors for many natural AAVs (Asokan et al., 2012). Accordingly, differences in glycan architecture have been attributed to variations in the efficiency of gene transfer by AAV capsids in different organs including the brain (Huang et al., 2014).

These differences in capsid-receptor interactions play a major role in determining the regional and cellular transduction efficiencies of AAV serotypes across different mammalian organs (reviewed by Murlidharan et al., 2014 and Samulski et al., 2015). In the brain, the transduction efficiency of each strain is different depending on the region, animal model, viral particle purification method and the cell type. Especially for neurons AAV shows a high transduction rate, however, when the target cells are microglia or astrocytes the transduction is limited. Nevertheless the search for and the development of new tools in the past two decades have provided to the scientific community with an ¨arsenal¨ of AAV serotypes with specific features for CNS gene transfer (Lentz et al., 2012). Nowadays it is more feasible to target glioblastoma cells (Maguire et al., 2010); rat, mouse and human neural stem cells (Jang et al., 2011); or even reach specific brain regions after systemic viral vector injection in small and large animal models with AAV9 (reviewed by Murlidharan et al., 2014).

3. TARGETING THE CENTRAL NERVOUS SYSTEM

AAVs have become the most commonly used GT vectors for the CNS because of their safety, nonpathogenic nature and ability to infect dividing and quiescent cells *in vivo*, particularly neurons. Moreover, they have demonstrated long-term expression *in vivo* (Hadaczek et al., 2010). The AAV serotypes most studied in the CNS are 1, 2, 5, 8, 9 and recombinant human (rh)10. AAV2, 5 and 8 are of our particular interest because of their favorable transduction pattern of the motor neurons (Dodiya et al., 2010; Tenembaum et al.,

2004). Neurological disorders like PD are among the most difficult pathologies to treat by gene delivery because of the limited access to the deep brain structures. To bypass the blood brain barrier (BBB) and deliver viral vectors to the CNS, several strategies have been developed: Intraparenchymal administration, cerebrospinal fluid injection (CSF-based delivery) and intravascular administration. Each strategy present advantages or disadvantages in respect to the inhibitory effect of neutralizing antibodies or the viral diffusion (reviewed in more detail by Hocquemiller et al., 2016) (**Figure 5**).

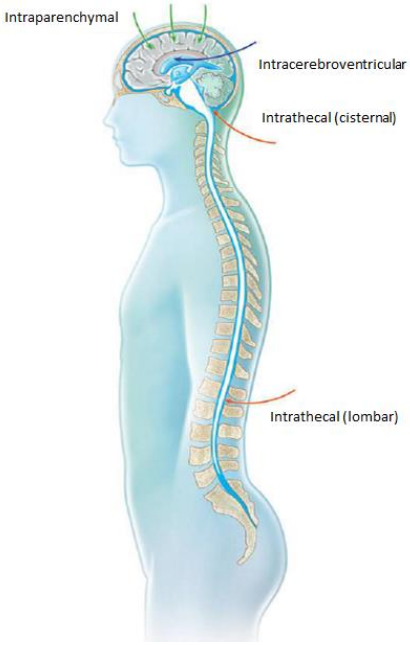


Figure 5. Routes of administration targeting the CNS by direct injection into the parenchyma or by injection into the cerebrospinal fluid via the intracerebroventricular or intrathecal (cisternal or lumbar) route. Taken from Hocquemiller et al., 2016).

4. RECOMBINANT AAV GENOME DESIGNS FOR OPTIMAL TRANSGENE EXPRESSION IN THE CNS

Specific delivery of the genes to the cell type of interest is essential for the success of GT. Cell specificity can be directed by either intrinsic characteristics of the vector (as described above) or the specificity of the promoter that controls the expression of the transgene (Gray et al., 2011). Therefore a correct selection of the promoter plays a very important role. Using

specific promoters we can restrict transgene expression to a particular cell population among those that can be infected by a given AAV serotype.

4.1 Promoters

Promoters are required for an efficient gene expression and are an essential factor in the design of GT vectors (Gray et al., 2011). By definition a promoter is a sequence of DNA where transcription is initiated. It is located upstream of the gene sequence and has a binding sites for the enzymes transcribing messenger RNA (mRNA). Considering that AAVs have a limited cloning capacity due to the small size of their genome, the use of small promoters allows the expression of larger genes or even co-expression of more than one gene from the same vector.

To this end, many GT studies have employed ubiquitous promoters to drive strong expression; however, this strategy can be limited by off-target side effects. The cytomegalovirus promoter (CMV) is one of the constitutive promoters most commonly used in preclinical and clinical studies in the CNS since 1990 (Schmidt et al., 1990; Gray et al., 2011). However the use of viral promoters, such as the CMV promoter, is decreasing because of the ability of eukaryotic cells to detect and silence viral promoters by methylation of cytosines in CpG dinucleotides (Gray et al., 2011). Other studies have conjugated different promoter elements as a simpler means to construct a synthetic promoter. A composite promoter consisting of the CMV immediate early enhancer and the chicken β-actin promoter (CAG) has proven to be a popular and very effective but non-specific alternative to the CMV promoter, with higher expression profiles both *in vitro* and *in vivo* (Papadakis et al., 2004).

4.1.1 Astrocyte and oligodendrocyte-specific promoters

Astrocytes are one of the most abundant cell types in the vertebrate CNS (Sofroniew et al., 2010). The astrocyte-selective targene expression can be accomplished with the GFAP promoter described by Besnard et al., (1991), Brenner et al., (1994) and Lee et al., (2008) in the context of rAAV. Furthermore, Meng et al., (2015) show a specific gene

expression in mouse cortical astrocytes mediated by the 1740bp-GFAP promoter in AAV serotypes 2, 5, 7, 8 and 9. However the size of these promoters is still a disadvantage for cloning large transgenes into a single AAV vector cassette. Considering oligodendrocytes, specific promoters have also been made available elsewhere (Chen et al., 1999; McIver et al., 2005; von Jonquieres et al., 2013; Kagiava et al., 2014).

4.1.2 Neuronal specific promoters

Among the neuronal specific promoters used in GT the rat neuron-specific enolase promoter (NSE) 1.8-kb has been used widely and is well characterized in the rat brain (Fitzsimons et al., 2002; Nagykery et al., 2013). It was first used by Reier (Peel et al., 1997) with rAAV vectors expressing GFP to transduce the rat spinal cord with great results. Another widely used promoter is the human synapsin I (hSyn) promoter (Kügler et al., 2003). Syn is a neuronal protein localized on the surface of synaptic vesicles (Thiel et al., 1991). The regulatory element present in this promoter allow neuron-specific gene expression (Schoch et al., 1996). Furthermore, the mouse calcium-calmodulin kinase-2a promoter (CaMKIIa) also is a feasible alternative to synapsin I for achieving specific neuronal transgene expression (Gerits et al., 2015; Watakabe et al., 2015). Using these promoters only neurons and not glial cells became specifically transduced with the gene of interest. Despite the interesting features, the size of such promoters is a disadvantage for cloning large transgenes into a single AAV cassette. In this context, the development of human DNA MiniPromoters (MiniPs) has been described (de Leeuw et al., 2014).

5. PARKINSON´S DISEASE

PD is a neurodegenerative disorder that affects 1.5% of the global population over 65 years of age (Miller et al., 2015). The origin of PD motor sympthoms is located in a region of the brain called basal ganglia and is characterized by the progressive loss of midbrain dopaminergic (miDA) neurons in the substantia nigra pars compacta (SNpc). Such miDA neurons are characterized by the presence of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine (Björklund and Hökfelt., 2013). The resultant dopamine deficiency leads to the movement disorder called Parkinsonian motor sympthoms.

The basal ganglia are a group of subcortical nuclei controlling voluntary movement. It is composed by: Striatum (St) (putamen and caudate nucleus), external globus pallidus (GPe), internal globus pallidus (GPi), substantia nigra (SN) and subthalamic nucleus (STN). Such structures serve motivation, motor planning and procedural learning functions. The neurotransmitter dopamine is released by midbrain SN neurons into the striatum where it modulates neuronal firing leading to a fine tuning of basal ganglia activity (Jarraya et al., 2009) **(Figure 6)**.

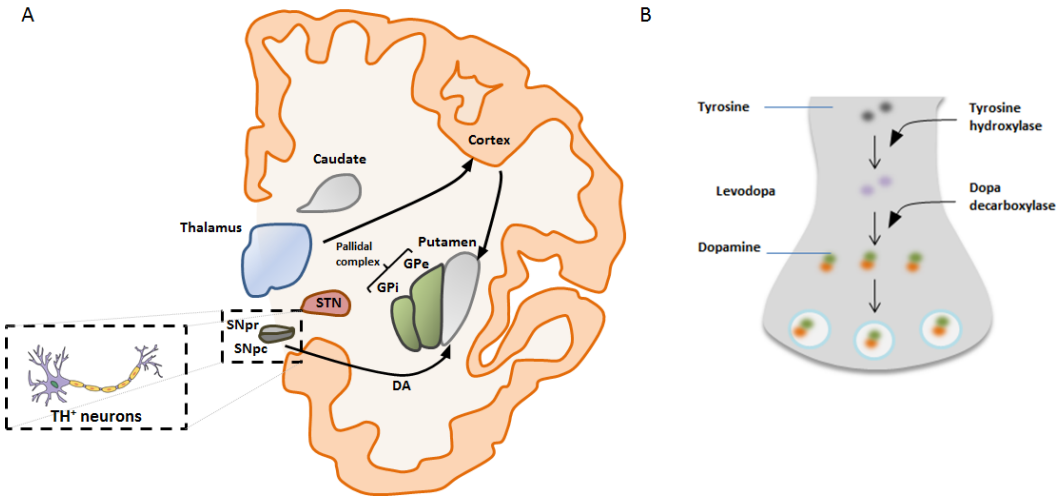


Figure 6. Dopaminergic neurons from the SN are the most affected neurons in PD. (A) SN comprises two neuronal subpopulations, one dopaminergic (SNpc) and one GABAergic (SNpr). DA neurons from the SNpc innervate the striatum (nigrostriatal pathway) taking part in the basal ganglia motor loop. DA neurons from the SN are characterized by the prpesence of tyrosine hydroxylase (TH). **(B)** Enzymatic reaction to produce dopamine. TH converts tyrosine into levodopa which in turn will be converted into dopamine by the action of the enzyme dopa decarboxylase. (Adapted from Calabresi et al., 2014).

5.1 THE MOTOR CIRCUIT DEGENERATES

The deficits in PD have their origin in the basal ganglia circuit. Normal movement depends on the correct levels of dopamine produced by cells in the SNpc and delivered to the dorsal striatum and other compartments within the brain. Information from different cortical areas, thalamus and brain stem reaches the striatum (glutamatergic terminals) and in turn the projection neurons of the striatum (GABAergic) project to the pallidal complex and/or the substantia nigra. The projection neurons of the striatum received the name of striatal medium spiny neurons (MSNs) and project to different output structures. The prevailing model of basal ganglia function describes two circuits, the direct and indirect pathways, which originate from two distinct MSNs populations. The direct and indirect pathways are believed to have opposite effects on movement, specifically, the activity of direct pathway MSNs is postulated to promote movement, whereas the activation of indirect pathway MSNs is hypothesized to inhibit it (reviewed by Haver et al., 2012; Lanciego et al., 2012).

According to this model, cortical activation produce a release of glutamate in the striatum that activates MSNs projecting to the SNpc and the GPi (the striato-nigral output neurons representing the direct pathway (**Figure 7**). MSNs are GABAergic cells, exerting an inhibitory action on neurons of the SNpr that are also GABAergic. This inhibition of the SNpr leads to a disinhibition of the thalamic glutamatergic neurons, which receive SNpr input and project to the cortex. The behavioral result of this chain of events is locomotor activation/inactivation **(Figure 7A)**.

Conversely, activation of striatum-pallidal MSNs, which project indirectly to the SNpr via the GPe and the STN (indirect pathway), inhibits the GABAergic neurons of the GPe, leading to a disinhibition of the glutamatergic neurons of the STN. The increased discharge of these excitatory STN neurons in turn activates the SNpr GABAergic neurons projecting to the thalamus. Ultimately, this effect results in the reduction of locomotor activity movement (**Figure 7B**).

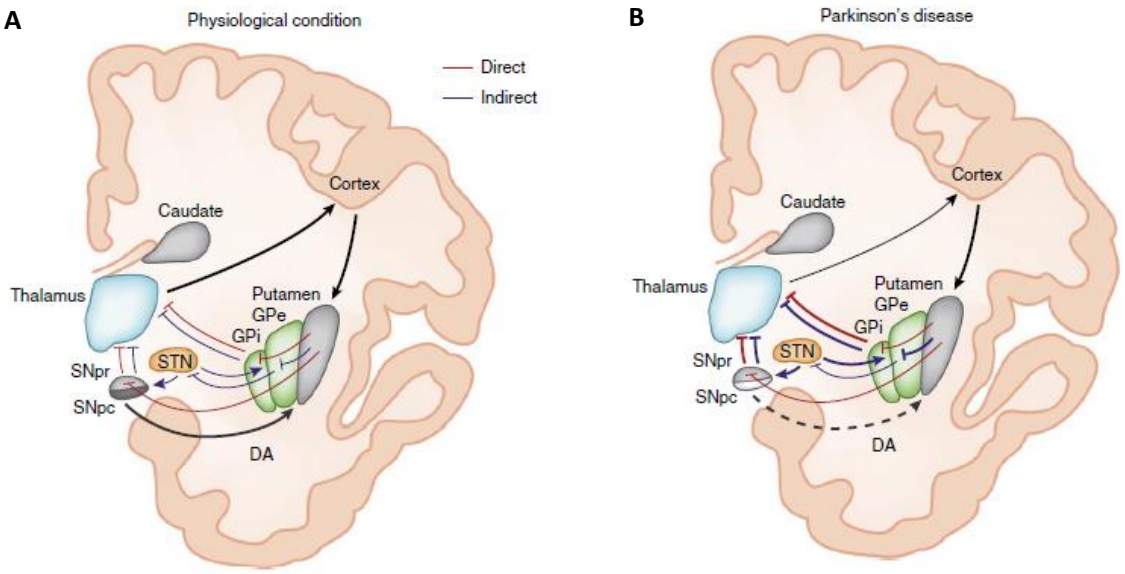


Figure 7. Schematic representation of the direct/indirect pathway classical model in the physiological condition and in PD. (A) In the physiological condition, DA arising from the SNpc is thought to activate D1-expressing striatal MSNs of the direct pathway (red lines) and to inhibit D2-expressing striatal neurons of the indirect pathway (blue lines). The output nuclei Gpi and SNpr project to the thalamus, which in turn sends efferents that complete the cortico-basal ganglia-thalamo-cortical loop. **(B)** In PD, degeneration of nigral neurons reduces DA receptor stimulation in striatal MSNs. The imbalance between direct and indirect pathways results into abnormal activation of output nuclei and over-inhibition of thalamic neurons projecting to the cortex (taken from Calabresi et al., 2014).

In addition to their distinct projections, MSNs of the direct and indirect pathway are characterized by the differential expression of dopamine (DA) receptors. D1 receptors are expressed by direct pathway MSNs, whereas D2 receptors are associated with the indirect pathway. This neurochemical segregation is considered to be further support for a dichotomous effect of the activation of the direct and indirect pathways (reviewed by Calabresi et al., 2014). The DA is produced by cells in the SNpc and delivered to the dorsal striatum though the nigro-striatal pathway; DA, which modulates the activation/inactivation of the direct and indirect pathways, has opposing effects on activity in the direct and indirect pathways. It excites MSNs of the direct pathway through the D1 receptors while inhibits the MSNs of the indirect pathway though D2 receptors.

The adecuate balance between the DA modulation, glutamatergic release and activation of both, direct and indirect pathways is necessary. MSNs are critical for the correct control of wanted movements and also the prevention of unwanted movements.

5.2 MOTOR SYMPTOMS AND GENERAL CAUSES

The parkinsonian motor symptoms include: rigidity, resting tremor, bradykinesia and postural instability. PD is also associated with numerous non-motor symptoms, some of which precede the motor dysfunctions (**Figure 8**). At the onset of the motor symptoms, the striatal dopamine levels are already depleted by 80% and approximately 50-70% of the dopaminergic neurons in the SNpc have been lost (Dauer and Przedborski, 2003).

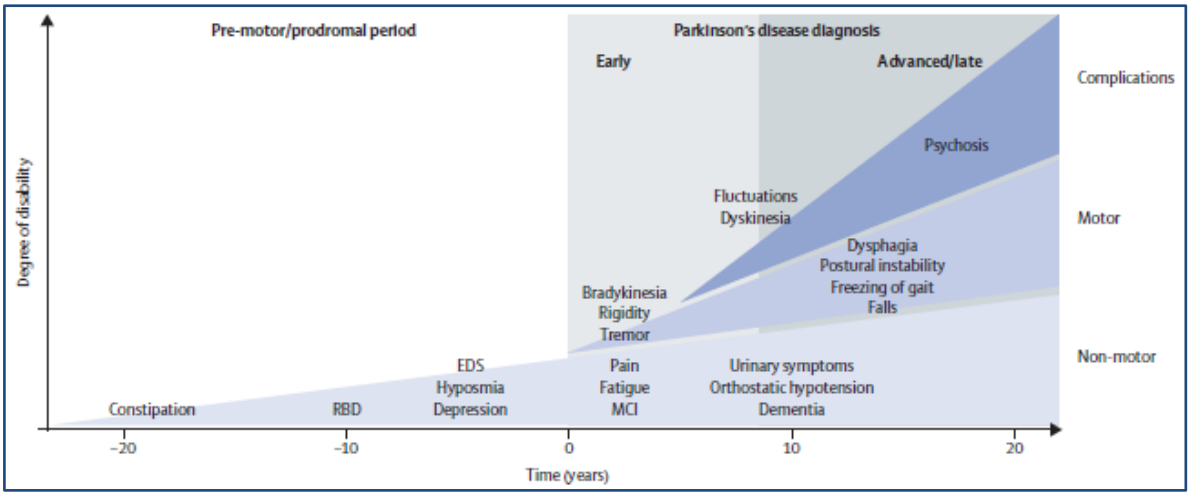


Figure 8. Clinical symptoms and time course of progression of the disease. PD is usually diagnosed with the onset of motor symptoms (time 0 years) but can be preceeded by a pre-motor or prodromal phase of 20 years or more. Non-motor features develop following diagnosis and with disease progression, causing clinically significant disability. Axial motor symptoms, such as instability with frequent falls and freezing of gait, tend to occur in advanced disease. EDS = excessive daytime sleepiness. MCI = mild cognitive impairment. RBD = REM sleep behavior disorder (taken from Kalia and Lang, 2015b).

The majority of PD cases are idiopathic and a minimal fraction of cases can be attributed to a Mendelian inheritance due to several genetic mutations (Kalia and Lang, 2015b). There is an overall consensus in considering the etiology of PD as a mixed scenario comprising genetic susceptibility as well as environmental factors including insecticides, pesticides and herbicides (Van der Mark et al., 2012). Although the majority of PD cases are idiopathic, it has been the familial PD which has provided with important clues to underlying the causes of the illness. Studying different familial PD cases the scientific community has been able to generate different hypothesis related with the factors related with the vulnerability to PD.

Currently the gold standard for diagnosis of PD is the presence of SNpc degeneration with the subsequent presence of cytoplasmic inclusions rich in α-synuclein (α-syn) (Lewy bodies - LB) (Spillantini et al., 1997). The Lewy pathology is a hallmark of PD, both in idiopathic and familiar forms of the disease. The LBs were first described at the beginning of the 20th century but it was at the end of the century when the main component of the LB was identified, the protein α-synuclein (α-syn). The LBs are alpha-synuclein aggregates forming insoluble fibrils (Spillantini et al., 1997). The study of different cases of autosomal dominant familiar forms of early-onset PD (5-10% of cases) demonstrated that the overexpression or the presence of mutant α-syn variants were risk factors to develop PD (Benskey et al., 2016), mutations in the α-syn gene, SNCA, including missense, duplication and triplication mutations are linked to familial PD (Farrer et al., 2006). Since alpha-synuclein is the primary structural component of LBs, the current hypothesis supports that protein aggregation plays a role in idiopathic PD as well.

But α-syn is an abundant protein in non-pathological states; many studies have shown the possible role of α-syn in diverse physiological processes, such as regulation of the synaptic transmission, calcium regulation or mitochondrial homeostasis. However, the reason why this protein forms toxic aggregates, and the precise role and structure of the toxic form of α-syn is still under debate (Abeliovich and Gitler, 2016). Aggregates of α-syn could be the toxic factor that causes the death of the DA of the SNpc that is observed in PD.

Following the same strategy, the study of other proteins related to different familiar genetic mutations causing PD has provided more information about different factors that are also involved in the vulnerability of miDA neurons. Those studies have shown that impairment in mitochondrial function is a common feature of different neurodegenerative diseases, including PD. Proteins encoded by genes such as PARK2, PARK7, PINK1 or LRRK2 are all related with mitochondrial function and all they are associated with familial PD. If mutated they can affect mitochondrial physiology (reviewed in Klein and Westenberger, 2012; Sidransky, 2006), although, the specific mitochondrial target that triggers PD is still missing (Cieri et al., 2016). All these data suggest that the pathology of PD and other neurodegenerative diseases are related to defects in the autophagy pathway (Bahr and

Bendiske, 2002; Menzies et al., 2006), suggesting that mitochondrial dysfunction is an important contributor to miDA vulnerability and neurodegeneration.

Recently different studies related with mutations in the gene that encodes for glucocerebrosidase (GBA1) have also pointed that protein as vulnerability factor to develop PD. Mutations in this gene have been shown to cause Gaucher disease (GD), a lysosomal storage disease. Patients with GD type 1 tend to also develop PD (Neudorfer et al., 1996), which links GBA1 with PD. Although GBA1 was linked to familial PD, recent studies suggest that GBA1 is also reduced in association with early abnormal accumulation of α-syn in sporadic PD causing alterations in the lysosomal chaperone-mediated autophagy pathway. GBA1 alterations alone are not sufficient to cause PD (Gegg et al., 2012; Murphy et al., 2014; Alcalay et al., 2016) but can act as a factor that increase the vulnerability of miDA neurons in the SNpc.

There is also growing consensus that axonal degeneration is predominantly involved at disease onset and, more importantly, it is the progressive axon loss that determines the course of the clinical progression. This data is supported by postmortem studies, functional neuroimaging and toxin-induced animal models (Burke et al., 2013). miDA neurons are highly branched and they have long axonal arbors and it is energetically expensive being a neuron with such long and unmyelinated axons (especially in humans) (Cheng et al., 2010). Other *in vitro* results supporting the hypothesis showed that the mitochondrial oxidative stress is higher in the SNpc miDA axons than in other DA axons such the VTA, which show a less branched structure (Pacelli et al., 2015). The fact that there are neuronal populations with long, highly branched axons with as many release sites as those of SNpc miDA neurons but that are not affected in PD, indicates that a long axon alone is unlikely to be a primary cause of neurodegeneration in PD (reviewed by Surmeier et al., 2016). Another major hypothesis of the vulnerability of the miDA neurons favors involvement of a distinctive pacemaker phenotype that relies upon CaV1 channels and leads to oxidative stress in basal mitochondria (Surmeier et al., 2010). This in turn, when sustained, could be a major factor underlying a decline in mitochondrial function in these SNpc miDa populations. The mitochondrial oxidative stress could lead to an increased rate of mitophagy that may

compromise other functions such as the degradation of misfolded proteins in the lysosomes (reviewed by Surmeier et al., 2016).

Neuroinflammation is another feature of PD and it could be another factor for degeneration if maintained for long periods of time. It is caused by the presence of active inflammatory responses in the brain mediated primarily by resident astrocytes and microglia (reviewed by Kalia and Lang, 2015a). Neuroinflammation may affect the mitochondria. Activated astrocytes and microglia produce nitric oxide (NO). NO and peroxynitrite (a product derived from the reaction of NO with the free radical superoxide) can cause damage in the electron transport chain of the mitochondria (Bolaños et al., 1994; Stewart et al., 2000).

There are many unanswered questions and challenges related to the mechanisms involved in the selective degeneration of miDA neurons and pathogenesis in PD. We need a deeper understanding of the molecular and physiological mechanisms involved in the functionality and development of our brain. To obtain such knowledge the cooperation between the fields of stem cell biology, embryology, virology and neuroscience is necessary (or would be desirable) to push towards an efficient treatment.

5.3 MIDBRAIN DOPAMINERGIC NEURONS (miDA NEURONS)

DA neurons are found in specific subpopulations throughout the mammalian CNS including the ventral midbrain (VM). miDA neurons are arranged in three distinct nuclei: the SNpc (A9 group), the ventral tegmental area (VTA, or the A10 group) and the retrorubral field (RrF, or A8 group) (Bjorklund and Hokfelt, 2013) **(Figure 9A)**. The different populations of miDA neurons project to distinct areas and control or modulate specific functions depending on their targets. For instance, groups 8 and 10 project to the ventromedial striatum which regulates emotional behavior, natural motivation, reward and cognitive functions, whereas the A9 group projects to the dorsal striatum which regulates motor function (**Figure 9B**).

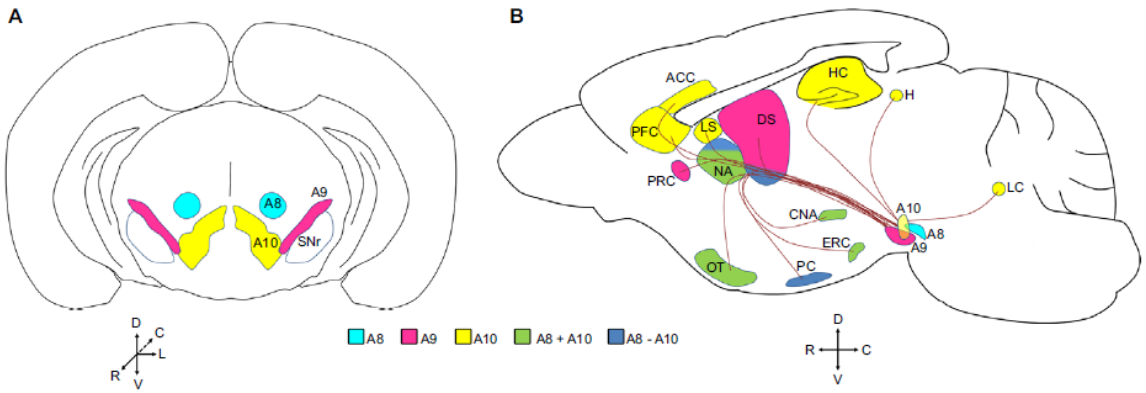


Figure 9. Distribution of miDA neurons and their projections in the adult mouse brain. (A) Coronal section of the adult brain at the midbrain level, showing the position of the three miDA nuclei: R.R/A8. SNpc/A9 and VTA/A10. (B) Sagittal view of the adult brain with a schematic representation of miDA neurons and their projection areas. A8, retrorubral; A9, substantia nigra; A10, ventral tegmental area; A8+A10, structures innervated by A8 and A10; A8-A10, structures innervated by A8, A9 and A10; ACC, anterior cingulate cortex; CNA, central nucleus of the amygdala; DA, dorsal striatum; ERC, entorhinal cortex; H, habenula; HC, hippocampus; LC, locus coeruleus; LS, lateral septum; NA, nucleus accumbens; OT, olfactory tubercle; PFC, prefrontal cortex; PRC, perirhinal cortex; PC, pyriform cortex (adapted from Arenas et al., 2015).

It is known that PD involves the degeneration of multiple neuronal subtypes in many other brain regions: locus ceruleus, nucleus basalis of Meynert, pedunculopontine nucleus, raphe nucleus, dorsal motor nucleus of the vagus, amygdala and hypothalamus (Kalia and Lang 2015b). However, the cells most affected and responsible for many of the motor degenerative features in PD are miDA neurons of the SNpc. In order to recover SNpc DA neurons, we must first understand the molecular mechanism that controls their development and how these neurons are specified, differentiated and maintained in the adult brain. This knowledge is essential for the development of therapies based on inducing *in vivo* reprogramming and the development of future regenerative medicine for PD.

5.3.1 Midbrain dopaminergic neurons development

The vertebrate brain consists of an enormous number of neurons and glial cells that establish a highly complex pattern of fibers and connections. The CNS develops from a small number of highly plastic cells that proliferate, differentiate and produce different cells types of neurons and glia. During development through the neurulation, when the top layers of

the embryonic germ disc elevate as folds and fuse in the midline, the neural tube is formed. This tube is the embryonic structure that ultimately forms the brain and spinal cord. The phenomenon is complex and involves numerous cell processes, requires a perfectly orchestrated pattern of expression of transcription factors (TFs) and morphogen signals. These cell fate decisions are dictated and sustained by the TFs that act as master regulators, chromatin regulators and associated cellular networks. TFs bind and activate *cis*-regulatory elements that modulate transcription and thereby direct specific gene expression programs (Lee and Young et al., 2013; Suvà et al., 2014).

At early developmental stages the central portion of the ectoderm of the embryo forms the neural plate. During the primary neurulation, the cells surrounding the neural plate direct the neural plate cells to proliferate, invaginate, and pinch off from the surface to form a hollow tube. Afterwards, in secondary neurulation, the neural tube arises from a solid cord of cells that sinks into the embryo and subsequently hollows out (cavitates) to form a hollow tube, that eventually form the entire CNS.

The neural tube patterns along the antero-posterior and dorsal-ventral axis establish defined compartments of neural progenitor cells that lead to distinct classes of neurons. This patterning occurs early in development and results from the activity of several secreted signaling molecules. Induction of specific neuronal fates is restricted in time and space in the developing CNS through integration of extrinsic morphogen signals and intrinsic determinants. Morphogens impose regional characteristics on neural progenitors and establish distinct progenitor domains. Such domains are defined by unique expression patterns of fate determining TFs. Once the cells acquire their identity, they migrate to their final positions and establish the connections through axonal growth, axon guidance and dendrite extensions that eventually form synapses with neighboring cells.

The patter along the rostral-ventral axis of the rostral region of vertebrate neural tubes produces three distinct primary brain vesicles: forebrain, midbrain and hindbrain. These three primary vesicles go on to subdivide into a series of five secondary brain vesicles. The forebrain (prosencephalon) and hindbrain (rhombencephalon) are subdivided into the

telencephalon/diencephalon and metencephalon/myelencephalon, respectively, whereas the midbrain (mesencephalon) remains undivided (**Figure 10**).

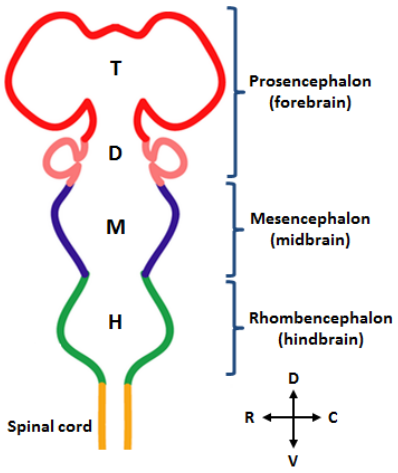


Figure 10. Main subdivisions of the embryonic vertebrate brain. These regions will later differentiate into forebrain, midbrain and hindbrain structures.

The neural tube is also organized in a dorsal-ventral pattern, the notochord establishes a secondary signaling center—the floor plate cells— on the ventral side of the neural tube, the epidermis establishes a secondary signaling center by inducing BMP4 expression in the roof plate cells of the neural tube. In the region of interest for the present study two main signaling centers are present, participating during the development of the midbrain, the isthmic organizer (IsO) controlling the antero-posterior axis, and the floor plate (mFP) (Placzek and Briscoe, 2005) controlling the dorsal-ventral axis. The isthmic organizer forms a boundary between the midbrain and hindbrain (MHB), and controls patterning of the midbrain and the anterior hindbrain. It is essential for the specification and normal development of dopamine neurons and serotonin neurons (Brodski et al., 2003).

The neurogenesis of miDA neuron begins in the ventral zone (VZ), where the new neurons are generated; once they reach the postmitotic state they begin their migration in the intermediate zone (IZ) towards the mantle zone their final position in the SNpc, VTA or in the RrF, where they continue to differentiate and become tyrosine hydroxylase expressing cells.

As was stated above the onset of the miDA development requires several extrinsic factors, mainly Shh, Fgf8 and Wint1, which are produced by the organizing centers of the developing CNS, the IsO and the mFP (Prakash and Wurst, 2006; Smits et al., 2006; reviewed

by Alavian et al., 2008, 2014). The combination of Shh and Fgf8 is necessary for the induction of dopamine neurons in the rostral forebrain and the lateral midbrain (Ye et al., 1998). Both Shh and Fgf8 appears to maintain normal development of the midbrain and hindbrain by regulating TFs such as engrailed-1 (En1), En2, and Pax5 and Foxa2 (Ferri et al., 2007). Those TFs positively regulate determinants of dopamine neurons while repressing ventrolateral genes in midbrain dopamine progenitors. To date the regulatory transcription cascade that determines the miDA fate has only partially been identified (reviewed by Doucet-Beaupré et al., 2015) and are summarize in the **Figure 11.**

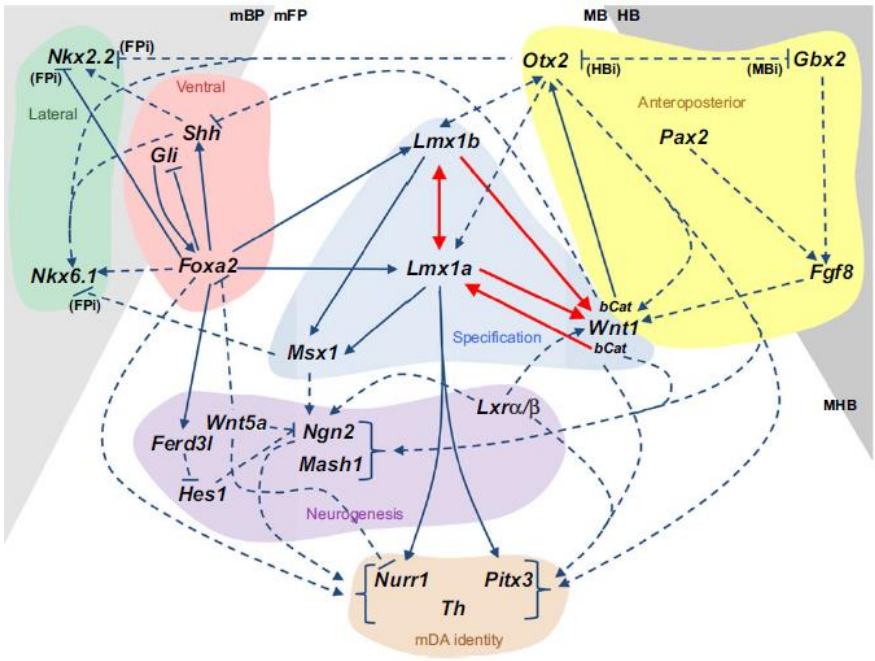


Figure 11. Genetic networks controlling the development of the midbrain hindbrain and mDA neurons in the mouse brain. Antero-posterior patterning (yellow area): The mutually repressive activities of Otx2 in the midbrain (MB) and Gbx2 in the hindbrain (HB) establish the midbrain-hindbrain boundary (MHB), where Otx2 is inhibited in the hindbrain (HBi) and Gbx2 in the midbrain (MBi). Midbrain floor plate (mFP) specification (blue area): LMX1B expression in the midbrain mFP directly regulates the expression of Wnt1 and Lmx1a, which also regulate each other via CTNNB (bCat) or directly (LMX1A), forming an auto-regulatory loop (shown by red arrows). Wnt1 regulates Otx2 and Lmx1a via β-catenin, and Lmx1a/b regulate Msx1 (Wnt1-Lmx1a/b-Msx1 network). Ventral patterning (pink area): FOXA2 regulates Shh, which feeds back onto Foxa2 via GLI (Shh-Foxa2 network). FOXA2 also directly regulates Lmx1a/b, to coordinate the specification of mDA neurons. Lateral phenotypes (green area): Midbrain basal plate (mBP) markers (Nkx2-2 and Nkx6-1) are inhibited in the mFP (FPi) by Foxa2 and Otx2 (Nkx2-2), and by Msx1 (Nkx6-1). Neurogenesis (purple area): The expression of Ngn2 is indirectly regulated by Wnt5a, Foxa2 (via Ferd3l and Hes1), Lmx1a/b (via Msx1) and Lxr alpha/beta (Nr1h3/Nr1h2). Wnt1/bCat and Otx2 regulate both Ngn2 and Mash1 (Ascl1). mDA neuroblasts and neurons (beige area): LMX1A directly regulates the expression ofmDApostmitotic genes, such as Nurr1 and Pitx3, which in turn regulate Th. These postmitotic genes are also regulated by Foxa2, Ngn2, Wnt5a, Lxr alpha/beta, Wnt1/β-catenin and Otx2. Solid lines indicate direct interactions as demonstrated by chromatin immunoprecipitation. All other interactions, whether direct or indirect, are shown by dashed lines. Arrowheads indicate activation and perpendicular lines denote inhibition. (Taken from Arenas et al., 2015)

From the *in vitro* data we know that apart from other TFs, early miDA progenitors along the mFP express, Lmx1a, an early TF that contributes to the development of DA populations (Smidt et al., 2000; Yan et al., 2011). Lmx1a is essential for the proliferation, specification and differentiation of miDA progenitors into DA neurons (Puelles et al., 2004; Andersson et al., 2006; Yan et al., 2011).

Lmx1a activates Msx1 and once activated both TFs collaborate in the activation of Ngn2, a key cell autonomous regulator of neurogenesis (Andersson et al., 2006). Apart from Lmx1a and Ngn2, miDA progenitors also express Mash1 (Ascl1); Mash1, like Ngn2, is a proneural gene. Data from *in vitro* studies suggest that Mash1 can regulate proliferation during early neurogenesis while Ngn2 is involved in the final steps of differentiation and maturation of postmitotic miDA neurons by activating Sox2 in miDA progenitors that later will become Nurr1 positive ($Nurr1^{+}$) postmitotic cells (Yi et al., 2008). As was described earlier the potsmitotic cells that express Nurr1 migrate towards the mantle zone reaching their final destination, the SNpc, VTA or the RrF, differentiating and become tyrosine hydroxylase expressing cells (TH^{+}) (reviewed by Alavian et al., 2008, 2014; Doucet-Breaupé et al., 2015) (**Figure 11**). It is important to highlight that each of the steps described above is strictly controlled by multiple pathways that involves the up-/down-regulation of many TFs, morphogens, specific timings and neuronal migration processes regulated by cell adhesion molecules (for detailed information see Arenas et al., 2015). In summary, the combined actions of the TFs and the morphogens from the IsO and the mFP play a key role in determining the regional identity of ventral midbrain, the specification and proliferation of the miDA and finally the differentiation and survival of those miDA neurons (reviewed by Arenas et al., 2015) **(Figure 12).**

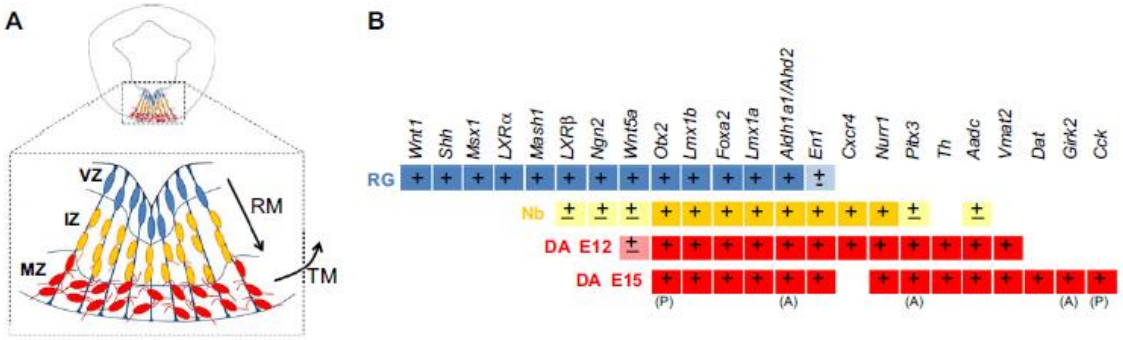


Figure 12. Gene expression in the miDA lineage. (A) Schematic representation of a section through the mFP at E11.5. The ventricular zone (VZ) contains radial glia cells (RG, blue) that undergo neurogenesis to generate postmitotic neuroblasts (yellow) that migrate radially through the intermediate zone (IZ), over the processes of the RG and differentiate into mDA neurons (red) in the marginal zone (MZ). As cells become mDA neurons, they migrate tangentially towards the substantia nigra pars compacta (SNc). Arrows indicate radial migration (RM) of neuroblasts and tangential migration (TM) of mDA neurons. (B) RG (blue) cells express morphogens, proneural and early transcription factors, some of which are also expressed in neuroblasts (Nb, yellow) and mDA neurons, defining the entire mDA lineage. ±, low levels of expression; +, expressed; A, mainly anterior midbrain; P, mainly posterior midbrain.

However, some of those TFs remain present in the adult miDA, and are required to maintain the identity of the miDA neurons. Among those TFs that have been described in the adult miDA population are Nurr1 and Lmx1A (Zetterstrom et al., 1997; Backman et al., 1999; Zou et al., 2009; reviewed by Alavian et al., 2008; Alavian et al., 2014;). On **Table 1** we present a brief description of the main functions described for each of the TFs Ascl1, Lmx1A and Nurr1 (ALN).

Table 1. Summary of the main functions described for Ascl1, Lmx1A and Nurr1

TFs	Description	Reference
Ascl1	In adult mouse brain is present in cells with long-term neurogenic potential (SVZ and SGZ) Essential for neuronal differentiation during development Also present in the adult brain (role is not fully understood) It seems that is not present in any differentiated neuronal population like miDA neurons	Kim EJ et al. 2011
Lmx1A	Not specific description of subcellular location	Laguna et al. 2015; Doucet-B. et al. 2016
	Expression in post-mitotic neurons diminishes with age	Laguna et al. 2015
	Involved in autophagic and lysosomal functions	Doucet-Beaupré et al. 2016
	Maintenance of mitochondrial functions of miDA neurons	Laguna et al. 2015; Doucet-B. et al. 2016
Nurr1	Maintenance and maturing of the miDA	Kadkhodaei et al. 2009
	Expression starts after the induction of neurons in the midbrain	Zetterstrom et al. 1997
	Induce the differentiation of embryonic stem cells into DA cells	Chung et al. 2002; Martinat et al. 2006
	Regulate nuclear-coded mitochondrial genes	Kadkhodaei et al. 2013
	In adult life is involved in the synthesis, packing, axonal transport and reuptake of dopamine	Kim KS et al. 2003; Smits et al. 2003
	Expression is mostly nuclear but also present in the cytoplasm	Garcia-Yagüe et al. 2013
	Cytoplasm-nuclear trafficking	Garcia-Yagüe et al. 2013
	In response to oxidative-stress is exported to the cytoplams	Garcia-Yagüe et al. 2013

We know that TFs regulate which genes are turned on and off determining the levels of proteins expression, and determining the cell fate. In vitro data have suggested that the differentiated cells are plastic and can be reprogrammed to a different cell fate. A subset of transcription factors possesses the remarkable ability to reprogram one type of cell into another (reviewed by Srivastava and DeWitt, 2016). Direct cell-reprogramming technology is based on the dominant action of cell-lineage TFs in converting adult somatic cells into different cell types (Graf and Enver, 2009). Previous studies in vitro have shown that the combination of Ascl1, Lmx1A and Nurr1 are able to transform fibroblast into miDA (Caiazzo et al., 2011; Pfisterer et al., 2011) and also one group have converted the striatal NG2 glia into neurons that remain stable over a long period of time in vivo using a set of TFs (Torper et al., 2015). This technique represents a promising avenue in the field of regenerative medicine, with the potential to generate cellular sources suitable for cell replacement therapies (Chambers and Studer., 2011).

5.4 CURRENT THERAPIES FOR PD

The main treatment for PD is pharmacological. The most efficient drug is the dopamine precursor levodopa (Bricha et al., 2014) but other agents including dopamine agonists, catechol-o-methyl-transferase (COMT) and monoamine oxidase type B (MAOB) inhibitors, as well as non-dopaminergic agents, such as antidepressants or cholinesterase inhibitors for dementia, are also prescribed. The chronic use of levodopa is associated with motor complications, including fluctuations and dyskinesias whereas dopamine agonist can cause behavioral alterations (reviewed by Arenas et al., 2015). Deep brain stimulation (DBS), targeting the thalamus, subthalamic nucleus and globus pallidus is currently used in PD patients whose motor symptoms cannot be adequately controlled by medication.

Although all these treatments relieve some symptoms of PD, they do not slow down disease progression or reverse the damage of mDA neurons and the treatment loses efficacy. Cell and gene therapy have thus gained interest in the last years as a therapeutic complementary option, as they have the potential to change the course of neurodegenerative diseases such as in PD.

5.1.2 ADVANCES THERAPIES FOR PD

As described above, PD is, by large, an idiopathic neurodegenerative disorder with origin in basal ganglia and sustained by the progressive loss of miDA neurons located in the SNpc. This neuronal loss after injury or disease in the human brain is irreversible and often leads to functional impairments. In the literature, neural protection and axon regeneration are often conflated with neuroregeneration in the CSN, which it has been proven to be difficult despite decades of research. The old dogma that CNS neurons cannot be regenerated in the adult mammalian brain has been overturned; however, endogenous adult neurogenesis appears to be insufficient for brain repair. In this regard, several approaches have been described in the last years and will be described below:

5.4.1.1 Stem cell therapy

It has been believed that neurogenesis occurs only during the embryonic development. Nowadays we know that neural stem cells (NSCs) are located in two specific brain regions in the adult human and mouse brain, referred to as “niches” [the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus] (Shen et al., 2008; Lim and Alvarez-Buylla, 2016). However, it is still unknown which factors trigger the neurogenesis in an adult brain. Brain injury and a disease condition induce neurogenesis that begins throughout the promotion of NSC proliferation and their migration towards the injury/diseased zone. It is here where the final cell maturation and integration in the local circuit take place (reviewed by Ming and Song, 2011). Unfortunately, neurogenesis is limited and not always can response to injury or illness (Li and Chen, 2016) and this is when external stem cell (SC) transplantation appears as an alternative approach to generate new neurons in the CNS. In this regard, two long-term clinical trials demonstrated the cell replacement capacities using human fetal midbrain cells (human Embrionic Stem Cells – hESCs) or human fetal ventral mesencephalic tissue in the striatum and in the SN as restorative treatment for PD treatment (Mendez et al., 2005; Kefalopoulou et al., 2014). Despite the promising results ethical concerns regarding the use of human fetal dopaminergic cells from elective abortions are an issue. Furthermore, many studies required immune-deficient mice or the use of

immune suppressors, creating challenges for translating this technique into effective therapies for patients already with serious illness and compromised immune systems.

5.4.1.2 Neuronal cells obtained from Induced Pluripotent Stem Cells (iPSCs)

IPSCs technology has the advantage of overcoming the ethical and resource limitations of human ESCs. In 2006, Yamanaka and colleagues demonstrated the capacity to dedifferentiate an adult cell (mouse or human fibroblasts) to their pluripotent state, thereby obtaining the iPSCs, by using four transcription factors: Oct4, Sox2, Klf4 and C-myc (Takahashi and Yamanaka, 2006; Takayashi et al., 2007). This revolutionary discovery allowed, initially, overcoming the ethical issues and the limited resources of hESCs and the later likelihood of immune rejection against the graft. Nevertheless, a much deeper understanding of the process is needed because the final fate is not always reached and results in genetic instabilities in the iPSCs. For example, Araki et al., (2013) described a limited immune response of newly differentiated skin and bone marrow tissues derived from mouse iPSCs, although their findings were not conclusive. Moreover, in another study, the low immunogenicity of neural progenitor cells differentiated from iPSCs that had been derived from human umbilical cord mesenchymal cells, provided a new concept to generate functional lineages with lower immunogenicity. But again the results suggest further research is necessary to properly assess this possibility (Liu et al., 2013). Two years earlier, the group of Kikuchi et al., (2011) described a feeder-free neural differentiation method from human iPSCs and analyzed the NPCs at different stages of predifferentiation into primate brain. Their results showed that after 6 months of follow-up the NPCs survived as DA neurons generating big grafts compared with NPC pre-treated with Sonic hedgehog and fibroblast growth factor-8 followed by glial cell–derived neurotrophic factor, brain-derived neurotrophic factor, ascorbic acid, and dibutyryl cyclic AMP (Kikuchi et al., 2011). Despite the increasing evidence of iPSCs as an alternative to NCS, there are scientific and clinical challenges that must be overcome. Even though, iPSCs can be induced to differentiate into DA neurons and serve as an "*in vivo*" platform for drug screening and cell-replacement therapies. However, if the purpose is to reach patients, the existing scientific data must be validated by robust and precise evidence (which to date does not exist). iPSCs technology has suffered drawback by causing tumorigenesis and immunogenicity after transplantation

(Lee et al., 2013; Gao et al., 2016; Li and Chen, 2016; Xiao et al., 2016). In this context, the now improved *in vivo* reprogramming technique has become one of the best available alternatives and is presented below. In **Table 2**, a summary of the main TFs used for lineage reprogramming in mouse (*in vivo* and *in vitro*) is given and in **Table 3** the respective TFs used for humans in the CNS (adapted from Xu et al., 2015; Li and Chen, 2016).

Table 2. Summary of Lineage Reprogramming in Mice since the discovery of iPSCs

In vivo / vitro	Initial Cell Population	Target Cell Type	Reprogramming Factors	Reference
In vitro	Fibroblast	Astrocytes	Nfia, Nfib, Sox9	Caiazzo et al.,, 2014
In vitro	Fibroblast	Neural precursor cells	Brn2, Sox2, FoxG1	Lujan et al., 2012
In vitro	Fibroblast	Neural progenitor cells	VPA, CHIR99021, RepSox (616452) under hypoxia	Cheng et al., 2014
In vitro	Fibroblast	Neural stem cells	Brn4, Sox2, Klf4, c-Myc, E47	Han et al., 2012
In vitro	Fibroblast	Neural stem cells	Sox2, Klf4, c-Myc, Oct4 (limiting activity at initial stage)	Thier et al., 2012
In vitro	Fibroblast	Neural stem cells	Sox2	Ring et al., 2012
In vitro	Sertoli cells	Neural stem cells	Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-Myc, Klf4	Sheng et al., 2012
In vivo	Astrocytes	Neuroblasts	Sox2	Niu et al., 2013
In vitro	Hepatocytes	Neurons	Ascl1, Brn2, Myt1l	Marro et al., 2011
In vitro	Fibroblast	Neurons	PTB repression	Xue et al., 2013
In vitro	Fibroblast	Neurons	Ascl1	Chanda et al., 2014
In vivo	Astrocytes	Neurons	Ascl1, Brn2, Myt1l	Torper et al., 2013
In vivo	Astrocytes	Neurons (GLUT)	NeuroD1	Guo et al., 2014
In vitro	Fibroblast	Neurons (DA)	Ascl1, Pitx3, Lmx1a, Nurr1, Foxa2, EN1	Kim et al., 2011
In vitro	Fibroblast	Neurons (DA)	Ascl1, Lmx1a, Nurr1	Caiazzo et al., 2011
In vitro	Fibroblast	Neurons (DA)	Lmx1a, Foxa2, Ascl1, Brn2 or Lmx1b, Otx2, Nurr1, Ascl1, Brn2	Sheng et al., 2012
In vitro	Astrocytes	Neurons (GABA)	Ascl1, Dlx2	Heinrich et al., 2010
In vivo	NG2 cells	Neurons (GLUT + GABA)	NeuroD1	Guo et al., 2014
In vitro	Fibroblast	Neurons (GLUT)	Ascl1, Brn2, Myt1l	Vierbuchen et al., 2010
In vitro	Astrocytes	Neurons (GLUT)	Ngn2	Heinrich et al., 2010
In vitro	Fibroblast	Neurons (motor)	Brn2, Ascl1, Myt1l, Lhx3, Hb9, Isl1, Ngn2	Son et al., 2011
In vitro	Fibroblast	Oligodendrocyte progenitor cells	Olig1, Olig2, Nkx2.2, Nkx6.2, Sox10, ST18, Gm98, Myt1	Najm et al., 2013
In vitro	Fibroblast	Oligodendrocyte progenitor cells	Sox2, Olig2, Zfp536	Yang et al., 2013

Table 3. Summary of Lineage Reprogramming in Human since the discovery of iPSCs

Initial Cell Population	Target Cell Type	Reprogramming Factors	Reference
Fibroblast	Neural crest cells	SOX10	Kim et al., 2014
Fibroblast	Neural crest cells	SOX2	Ring et al.,2012
Fibroblast	Neurons	ASCL1, NGN2, CHIR99021, SB431542	Ladewig et al., 2012
Fibroblast	Neurons (GLUT)	NGN2, Forskolin, Dorsomorphin	Liu et al., 2013
Fibroblast	Neurons	ASCL1	Chanda et al., 2014
Pericyte-derived cells	Neurons	SOX2, ASCL1	Karow et al., 2012
Fibroblast	Neurons (DA)	ASCL1, BRN2, MYT1L, LMX1A, FOXA2	Pfisterer et al., 2011
Fibroblast	Neurons (DA)	ASCL1, LMX1A, NURR1	Caiazzo et al., 2011
Fibroblast	Neurons (DA)	MASH1, NGN2, SOX2, NURR1, PITX3	Liu et al., 2012
Fibroblast	Neurons (GLUT)	ASCL1, BRN2, MYT1L, NEUROD1	Pang et al., 2011
Fibroblast	Neurons (GLUT)	BRN2, MYT1L, miR-124	Ambasudhan et al., 2011
Fibroblast	Neurons (GLUT-GABA)	ASCL1, MYT1L. NEUROD2, miR-9/9, miR-124	Yoo et al., 2011
Fibroblast	Neurons (motors)	BRN2, ASCL1, MYT1L, LHX3, HB9, ISL1, NGN2	Son et al., 2011

5.4.1.3 Direct lineage reprogramming

The idea to use TFs to reprogram somatic cells into stem cells has inspired to find shortcuts for obtaining terminally differentiated cells directly from fibroblast or other easily accessible cells without going through a stem cell stage (Li and Chen, 2016). The conversion of skin fibroblast into neurons has been described (Xu et al., 2015). Besides fibroblast cells, cultures glial cells such as astrocytes or NG2 glia have also been reprogrammed into functional neurons in vitro using neurogenic TFs. (Heinrich et al., 2010). The disadvantage of direct lineage compared to iPSCs is the difficulty storage and longterm use after reprogramming. For CNS repair, whether the trans-differentiated neurons can integrate successfully into the neural circuit after transplantation still needs to be thoroughly evaluated.

6. GENE THERAPY

In addition to cell therapies, gene therapy has emerged as a promising alternative. Gene therapy has strong potential for treating a variety of genetic disorders, as demonstrated in recent clinical trials. To date, approximately two thirds of the 1800 gene therapy clinical trials completed worldwide have used viral vectors (Ojala et al., 2015). Vectors based on AAV are particularly promising gene delivery vehicle in large part because they exhibit low immunogenicity, can mediate long-term gene expression in both dividing and non-dividing

cells and have a low risk of insertional mutagenesis (Murlidharan et al., 2014; Ojala et al., 2015).

It has been shown the controlled release of neurotrophic factors through viral vectors, mostly focused on glial cell-derived neurotrophic factor (GDNF) and Neurturin (NRTN; a close relative of the GDNF family). Another strategy often used consists in overexpressing the enzyme converting L-dopa to dopamine, the aromatic L-amino acid decarboxylase (AADC). This strategy showed a 50% improvement in L-dopa responsiveness and lasting results for at least 8 years (reviewed by Hocquemiller et al., 2016). In a clinical trial conducted by Ceregene, delivery of CERE-120 (an AAV2 vector encoding the neurotrophic factor neurturin under a constitutive CAG promoter) to the putamen and SN resulted in an excellent safety profile for up to 5 years (Marks et al., 2016). To date, several preclinical **(Table 4)** and clinical trials **(Table 5)** are ongoing to evaluate the safety and effectiveness of several rAAVs overexpressing GDNF, AADC, NRTN as well as others. Because of the lack of efficacy of several drugs in phase III, such as creatine and ubiquinone, rAAV gene therapy for PD seems promising and could offer an interesting alternative (reviewed by Hocquemiller et al., 2016). In particular, the use of in vivo reprogramming as an emerging technology to regenerate functional neurons has been demonstrated successfully in different organs and has the potential to revolutionize regenerative medicine by using a patient´s own internal cells for tissue repair (Li and Cheng, 2016).

Table 4. Preclinical studies of gene therapy for PD

Model			Injection site	Serotype	Transgene	Promoter	Dose, min vg	Dose, max vg	Volume µL	Volume µL/min
Intraparenchymal	Rodent	Mice	Str/Hip/SN	2 or 5	GBA1/a-synuclein	Synapsin	2×10^{10}		10	1
			SN	2	Nurr1 &/or Foxa2	CMV	1×10^{9}		1	0.66
			SN	1/2	shRNA anti ROCK2 or LIMK1	Synapsin	2.5×10^{7}	1×10^{8}	1	0.5
			Str	2	NTN	CAG	4×10^{9}		4	0.2
	Large animal	NHP	Cau/Put	2	AADC	NA	3.6×10^{11}		180	NA
			Put	2	GDNF	CMV	9.9×10^{11}		150	NA
			Put/SN	2	GDNF	CMV	8.3×10^{10}	8.3×10^{11}	50-75	NA
			Cau/Put	2	NTN	CAG	3×10^{11}		150	2
			STN	NA	GAD	NA	6×10^{10}	1.2×10^{11}	20	NA
			Put	2	hAADC	CMV	6×10^{9}	5×10^{11}	200	0.1 - 1
			Put	2	hAADC-2	CMV	3×10^{11}		200	1
			Str/SN	NA	GDNF	CAG	8.4×10^{10}		21	0.25

Cau, Caudate; Hip, Hippocampus; Str, Striatum; SN, Substantia Nigra; STN, Sub Thalamic Nucleus. The CAG promoter designation includes the CBA and CB promoters (adapted from Hocquemiller et al., 2016).

Table 5. Summary of AAV Clinical trials for PD

	Injection site	Clinical trial	Inclusion	Serotype	Transgene	Promoter	Dose, min vg	Dose max vg	Volume µl	Speed µl/min	IS	Status	Identifier
Intraparenchymal	StN (n=2)	Phase II	16	2	GAD	CAG	2.0×10^{12}		70	0.23	NA	C	NTC00643890
	Str (n=4)	Phase I	10	2	AADC	CMV	9.0×10^{13}	3.0×10^{11}	200	1	NA	C	NTC00229736
	Put (n=8)	Phase I/II	70	2	NTN(CERE-120)	CAG	1.3×10^{11}	5.4×10^{11}	80	2	NA	C	NTC00252850
	Put (n=6) / SN (n=4)	Phase I/II	57	2	NTN(CERE-120)	CAG	9.4×10^{11}	2.4×10^{12}	360	2/3	NA	0	NTC00985517
	StR (n=2)	Phase I	24	2	GDNF	CMV	9.0×10^{10}	3.0×10^{12}	NA	NA	NA	0	NTC01621581
	StR (n=2)	Phase I	10	2	AADC	NA	7.5×10^{11}	1.5×10^{12}	NA	NA	NA	0	NTC01973543
	Put (n=4)	Phase I/II	6	NA	AADC	NA	3.0×10^{11}	9.0×10^{11}	200/600	3	NA	0	NTC02418598
	Put (n=2)	Phase I	10	2	AADC	NA	NA	NA	NA	NA	NA	0	NTC01395641

C, completed; IS, immunosuppressor; max vg, maximum vector genome; min vg, minimum vector genome; N, no; NA, not available; 0, ongoing; Put, Putamen; SN, substantia nigra; StN, Sub thalamic Nucleus; Str, striatum. The CAG promoter designation includes the CBA and CB promoter.

6.1 *In vivo* reprogramming using gene delivery strategies

The concept of *in vivo* reprogramming is based on the idea of using endogenous cells (either neurons or glial cells) as an unlimited autologous source for the generation of new neurons with the desired phenotype and without the development of rejection phenomena. This type of "phenotypic switch" is termed *in vivo* reprogramming. While earlier studies modulated adult neurogenesis in the CNS by manipulating endogenous neuroprogenitors cells, it was not until recent years that a clear concept of direct *in vivo* reprogramming emerged as the conversion of reactive glial cells into functional neurons for brain repair. The concept in vivo reprogramming is in part inspired by iPSCs and direct lineage reprogramming that were initially developed in *in vitro* cultures. In vivo reprogramming in the CNS is largely based on the fact that glial cells react to injury and become proliferative and hypertrophic in response to neuronal injury. Such reactive glial cells are one of the most prevalent pathological hallmarks associated with a wide variety of neurological disorders (Li and Cheng, 2016).

The first successful demonstration of this approach in adult animals was carried out in the pancreas, by reprogramming pancreatic exocrine cells into insulin-secreting beta cells using a combination of three transcription factors (Zhou et al., 2008). Similar approaches have been carried out in other organs, such as the heart and liver (Qian et al., 2012; Song et al., 2012, 2016; Rezvani et al., 2016). Within the CNS, a priori it sounds reasonable to focus on astrocytes instead of neurons for *in vivo* reprogramming purposes, thus directly converting astrocytes into different types of neuronal-like phenotypes. Among others, the

genes coding for a number of transcription factors such as neurogenin 2 (Ngn2), NeuroD1, Sox2, Ascl1, Lmx1a/b, Nurr1, Bcl2, FGF2 (or combinations herein) have been used in different viral vectors to promote the *in vivo* reprogramming of astrocytes in the CNS with low and high reprogramming efficiency (Guo et al., 2014; Grande et al., 2013; Gascon et al., 2016; Liu et al., 2015; Torper et al., 2013, 2015; Niu et al., 2013). In particular, the work of Toper et al., (2015) describes the in vivo conversion of NG2 into functional adult neurons using the TFs Ascl1, Lmx1A and Nurr1 (ALN). Nevertheless, they could detect any TH-immunoreactive cell, the use of ALN resulted in functionally mature neurons in larger proportions than previously reported for conversion of resident glial using Sox2 or in combination with Ascl1 (Heinrich et al., 2014). Moreover, the results obtained were without the need for treatment with neurotrophic factors (see also **Table 6**).

Table 6. *In vivo* reprogramming of different glial cells into different subtypes of neurons with different TFs

Glia Source	Transcription Factor	Neuronal Subtype	Reference	Efficiency	Vector	Promoter
Astrocyte	NeuroD1	Glut	Guo et al. 2014	High	Retrovirus	GFAP
	Ngn2 + FGF2 + EGF	Not determined	Grande et al. 2013	Low	Retrovirus	?
	Ngn2 + Bcl2	Glut	Gascon et al. 2016	High	?	?
	Ascl1	GABA	Liu et al. 2015		AAV	GFAP
	Ascl1 + Brn2 + Myt1	?	Torper et al. 2013	Low	LV	GFAP
	Sox2	NB	Niu et al. 2013	Low	LV / Retrovirus	GFAP / CAG
NG2	NeuroD1	Glut/GABA	Guo et al. 2014	Medium	Retrovirus	GFAP
	Ascl1 + Lmx1a + Nurr1	GABA	Torper et al. 2015	Low	AAV	CBA / SYN / NG2
	Sox2	GABA	Heinrich et al. 2014	Low	LV	GFAP

NB, Neuroblast; LV, lentivirus; ? not specified.

6.1.1 Advantages of *in vivo* reprogramming for CNS repair

- **Capacity of avoiding an immune response:** The unique feature of using endogenous cells for regeneration and repair makes this technique the most valuable option to date. Now we have the capacity of targeting a specific cell type within the patient ´s brain by using viral vectors and, moreover, we can restrict the transgene expression (of TFs) by choosing the correct cell-specific promoter, thus avoiding the undesirable rejection response caused by external cell transplantation. Although more research is needed for CNS repair, the reprogramming efficiency still low. The highest efficiency reached (90%) was reported with TF Neuro-D1 (Guo et al., 2014) but most of the published work

shows an efficiency of no more than 50% or even less than 20% (Table 7). Nevertheless, according to people with vast experience in this field, any in vivo reprogramming with >50% efficiency should have significant impact on repairing tissue (Li and Chen, 2016).

- **Capacity to regenerate using neighboring cells:** Minimal interference from outside the injury site is the most economic recovery mechanism (Li and Chen 2016). In the CNS, astrocytes in the mouse cortex are different from their counterparts in the striatum in terms of reprogrammability and neuronal identity despite expressing the same TFs (Niu et al., 2013).

- **Capacity of targeting proliferative cells:** For the CNS, reactive glial cells are the best candidates for in vivo reprogramming because of their maintained ability to divide and regenerate. Although it is important to point out that only a fraction of the reactive glial cells will be reprogrammed into functional neurons, the remaining cells will keep their own properties (like proliferative capacity).

Thus, given efficacy of AAV vectors to delivery transgenes into the CNS with a safety profile it is our selected gene therapy vehicle to deliver the TFs Ascl1, Lmx1A and Nurr1 in order to convert astrocytes into dopaminergic neurons *in vivo*.

HYPOTHESIS AND AIMS

HYPOTHESIS AND AIMS

1. Hypothesis

CNS neurodegenerative disorders represent a major health problem worldwide. In PD, the progressive loss of miDA neurons leads to an irreversible dopamine deficiency in the striatum, ultimately triggering the appearance of the cardinal motor symptoms that typically characterize this movement disorder of basal ganglia origin. Although stem cell therapies once held great promise for generating large quantities of DA neurons, a number of limitations tuned down the initial enthusiasm. Among others, rejection of transplanted cells by the host, failure to achieve long-term integration and potential oncogeneicity have all impaired pushing forward these initiatives towards the implementation of clinical therapies. Accordingly, the so-called *in vivo* direct reprogramming has recently emerged as an appealing technical choice to further generate functional DA neurons from endogenous glial cells in situ. In this regard, here we are taking advantage of newly-designed adeno-associated viral vectors in an attempt to overexpress a number of transcription factors within striatal astrocytes to compensate the lack of dopamine in this brain region. Therefore the present work has the following aims:

2. General aim

The main goal of this project is the development of new gene therapy tools to drive gene expression in specific cell populations of the CNS for future applications in neurodegenerative diseases including Parkinson.

Specific objectives

1. To construct and characterized *in vitro* and *in vivo* a set of AAV vectors carrying small and specific astrocytes or neuronal promoters driving transgene expression.

2. To study *in vivo* the reprogramming capacity of astrocyte-selective expression of transcription factors with neuron reprogramming capacity in mouse striatum using AAV8 vectors.

CHAPTERS

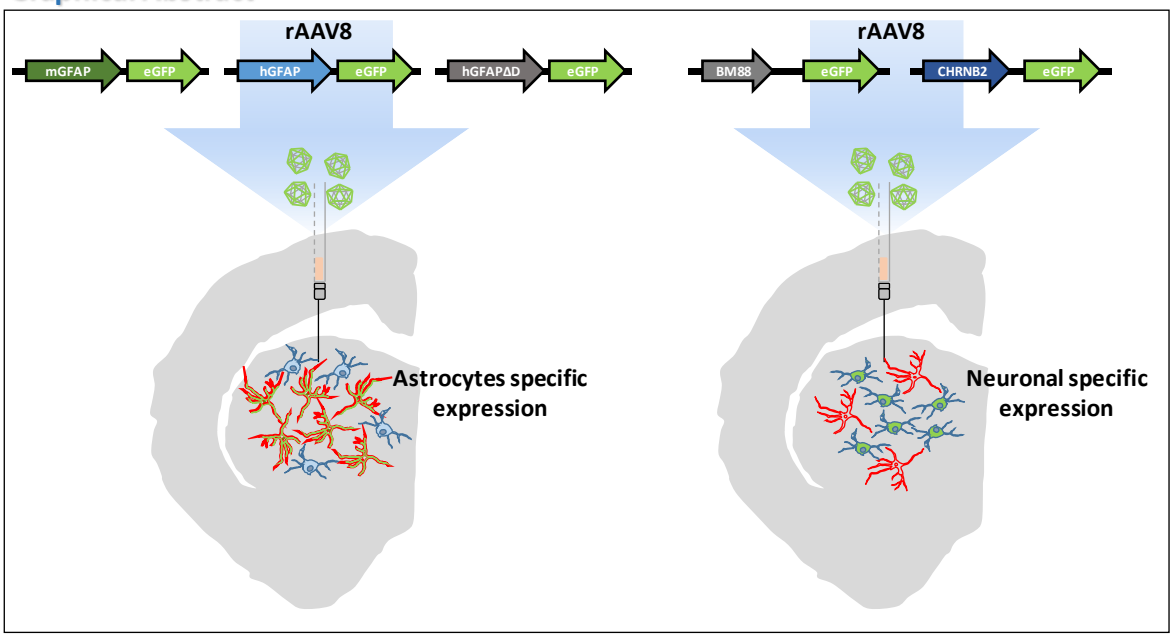
Chapter one

Published in Frontiers in Neuroanatomy

doi: 10.3389/fnana.2017.00002.

"Adeno-associated Viral Vectors Serotype 8 for Cell-Specific Delivery of Therapeutic Genes in the Central Nervous System"

Graphical Abstract



Adeno-Associated Viral Vectors Serotype 8 for Cell-Specific Delivery of Therapeutic Genes in the Central Nervous System

Short title: AAV vectors for CNS delivery

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Key words: AAV, CNS, promoters, basal ganglia, Parkinson's disease, gene therapy (5-8)

Supported by FP7-PEOPLE-2011-IAPP - Marie Curie Action: "Industry-Academia Partnerships and Pathways" (ref. 286071 "Brainvectors") and ERC Advanced grant (ref: 340527 "Repropark"). CoEN Pathfinder Ref: Phase II Call. Diego Pignataro is partially supported by a Jon Zarandona donation.

Acknowledgments

We thank Elena Ciordia, Alberto Espinal, and CIFA staff for animal care **and** vivarium management. Furthermore, we would like to thank Elvira Roda for help with the histological procedures.

Abstract

Adeno-associated viruses (AAVs) have become highly promising tools for research and clinical applications in the central nervous system (CNS). However, specific delivery of genes to the cell type of interest is essential for the success of gene therapy and therefore a correct selection of the promoter plays a very important role. Here AAV8 vectors carrying enhanced-GFP (eGFP) as reporter gene under the transcriptional control of different CNS-specific promoters were used and compared with a strong ubiquitous promoter. Since one of the main limitations of AAV-mediated gene delivery lies in its restricted cloning capacity, we focused our work on small-sized promoters. We tested the transduction efficacy and specificity of each vector after stereotactic injection into the mouse striatum. Three glia-specific AAV vectors were generated using two truncated forms of the human promoter for glial fibrillar acidic protein (GFAP) as well as a truncated form of the murine GFAP promoter. All three vectors resulted in predominantly glial expression; however we also observed eGFP expression in other cell-types such as oligodendrocytes, but never in neurons. In addition, robust and neuron-specific eGFP expression was observed using the minimal promoters for the neural protein BM88 and the neuronal nicotinic receptor β2 (CHRNB2). In summary, we developed a set of AAV vectors designed for specific expression in cells of the CNS using minimal promoters to drive gene expression when the size of the therapeutic gene matters.

Introduction

Longevity coincides with an increased prevalence in neurodegenerative disease and a concomitant increase in the burden on health systems around the world (Checkoway H et al., 2011). The need for treatment options has fuelled research, with the field of gene therapy applied to CNS pathologies being on the forefront. Despite having recently witnessed a number of major conceptual changes – such as gene delivery of specific transcription factors or micro-RNAs for *in vivo* reprogramming of different cells to neurons (Ghasemi-Kasman et al., 2015; Caiazzo et al., 2011; Colosante et al., 2015; Niu et al., 2013, 2015) – the more traditional approach of using viral vectors for the delivery of therapeutic genes still offers one of the most promising options (Terzi and Zachariou, 2008; Bartus et al., 2013; Kalia et al., 2015).

Although viral and non-viral vectors have been broadly used for CNS gene therapy, viral vectors, including AAVs and lentiviruses (Blessing and Déglon, 2016), are generally significantly more efficient than non-viral vectors at delivering genes into the cells of interest (Nayerossadat et al., 2012). Cell-specificity can be directed by either intrinsic characteristics of the vector (Nayerossadat et al., 2012; Kantor et al., 2014; Maguire et al., 2014) or the specificity of the promoter that controls the expression of the transgene (Gray et al., 2011). AAVs have emerged as the most promising tool for gene transfer in the CNS (Bourdenx et al., 2014; Aschauer et al., 2013; Klein et al., 2007) as they are able to transduce dividing and non-dividing cells and induce stable, long-term gene expression in the absence of inflammation and/or toxicity. Since neurons are post-mitotic cells, the capability of AAV vectors to transduce non-dividing cells is of vital importance in the context of neurodegenerative disease gene therapy (Bartlett et al., 1998).

AAV serotype 8 (AAV8) in particular has been demonstrated to be one of the most effective vector in some structures of the CNS, producing the highest rate of transgene transduction in the striatum compared with other serotypes, in the absence of neurotoxicity (Aschauer et al. 2013). Moreover, in a number of studies in different animal models it was observed that this serotype was actively transported along axons (Aschauer et al., 2013; Löw et al., 2013; Masamizu et al., 2011; Masamizu et al., 2010). Due to its small size (4.7 kb) one

of its limitation is its cloning capacity, however, the use of minimal specific promoters facilitates the expression of larger genes or co-expression of more than one gene from the same vector. In pre-clinical and clinical studies the use of AAV as delivery vehicles was confirmed to result in robust and long-term gene expression (reviewed by Hocquemiller et al., 2016).

In the present work we describe the characterization of a series of astrocyte- and neuron-specific small promoters in the context of an AAV8 vector with the aim of using these vectors for future therapeutic applications in neurodegenerative disease including Parkinson`s disease (Coune et al., 2012). Astrocytes were chosen as they are one of the most abundant cell type in the vertebrate CNS (Colombo and Farina, 2016) and contribute to the pathogenesis of neurodegenerative disorders - hence they may be an ideal cellular target for the delivery of therapeutic genes (Pekny and Nilsson, 2005). Because the anatomy of the striatum is affected in many neurodegenerative diseases, such as Parkinson's disease, we characterized the expression pattern and specificity of the different vectors by stereotaxic injection into the mouse striatum. Robust and specific neuronal transgene expression was *achieved using neuron-specific promoters, while astrocyte-specific promoters drove* expression in astrocytes and oligodendrocytes but not in neurons.

Materials and methods

Animals and stereotaxic AAV injection

Eighteen C57BL/6 male mice (six to eight weeks old) were purchased from Harlan Laboratories (Barcelona, Spain). Animal handling was conducted in accordance with the European Council Directive 2010/63/UE, as well as in agreement with the 'Policy on the Use of Animals in Neuroscience Research' issued by the Society for Neuroscience. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra (protocol Ref: 102-16). Anesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The coordinates for targeting the striatum were 0.5 mm rostral, 2 mm lateral and 3.5 mm ventral from the bregma (Paxinos et al. 2001). All animals received two pressure injections: one of 2 µl of PBS/5% sucrose

containing AAV vector on the left side (4 x 10^9 vp), and a second of vehicle alone on the right side of the striatum. Injections were performed using a Hamilton syringe driven by a syringe pump at a flow rate of 0.2 µl/min. Following the injection, the needle was left in place for 2 minutes prior to being slowly retracted to avoid vector leakage from the injection tract. After surgery, animals were kept under constant monitoring with *ad libitum* access to food and water.

Cells

Human embryonic kidney fibroblast (HEK-293) cells, were purchased from the ATCC and were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 µg/ml) and streptomycin (100 U/ml) (all supplements were from Invitrogen, Pisley, Scotland, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids

cDNA encoding enhanced green fluorescent protein (eGFP) was isolated from the vector pBSKII-CMV-EGFP and inserted into the multiple cloning site (MCS) of an rAAV2 plasmid, which contained AAV2 inverted terminal repeats (ITR), to obtain rAAV2-eGFP. Upstream of the eGFP coding sequence different promoters were inserted: a constitutive hybrid promoter composed of the CMV immediate-early enhancer fused to chicken ß-actin promoter (CAG pr) (Niwa et al., 1991), two reduced versions of the human GFAP promoter (hGFAP pr, 587bp, containing the A, B, C_1 and D elements) (Lee et al., 2008) and hGFAPΔD (512bp), in which the D sequence of was removed. This sequence was previously shown to play an important role in the functionality of the promoter (Bresnard et al., 1991). Furthermore, using the structure of the human gfaABC_1D promoter and the sequence of the murine GFAP promoter, a reduced version of the murine gfaABC_1D promoter was constructed (581 bp, mGFAP pr). The proximal promoter of murine BM88 (88bp) (Papadodima et al., 2005) and the minimal promoter driving neuron-specific expression of the ß2 subunit of the nicotine acetylcholine receptor (CHNRB2 pr, 177 bp) (Bessis et al., 1995), were used for neuron-specific targeting. Moreover, the human growth hormone (hGH) poly A signal and the ß-globin intron were cloned into the plasmid (Figure 1). Minipreps and maxipreps were prepared using commercial kits according to the

manufacturer´s instructions (Macherey-Nagel, Düren, Germany). In order to study the functionality of the constructs, HEK-293T cells were transfected with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). Transfections were performed according to the manufacturer's protocols. 1-2 µg of plasmid was transfected, depending on the size of the culture plate used (6- or 12 wells). Expression was analyzed 24-48 hours post-transfection (hpt) under a microscope equipped with epifluorescent illumination (Nikon Eclipse 800).

Viral vector production

Recombinant single-stranded AAV8 vectors were purified from HEK-293T cells that had been co-transfected using linear polyethylenimine 25 kDa (Polysciences, Warrington, PA, USA) with two different plasmids: a plasmid containing ITR-flanked transgene constructs and a plasmid containing the adenoviral helper genes plus AAV2 rep and AAV8 cap (named pDP8.ape, Plasmid Factory, Bielefeld, Germany) as described (Durocher et al., 2002). Seventy-two hpt the supernatant was collected and treated with polyethylene glycol solution (PEG8000, 8% v/v final concentration) for 48-72 hours at 4°C. Supernatant was then centrifuged at 3000 rpm for 15 minutes. Pellet containing particles from the supernatant was resuspended in lysis buffer and kept at -80°C. Cells containing AAV particles were collected and treated with lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100) and kept at -80°C. Three cycles of freezing and thawing were applied to both supernatant and cell lysate. Viral particles obtained from cell supernatant and lysate were purified by ultracentrifugation in an iodioxanol gradient according to the method of Zolotukhin et al. (1999). The viral batches were then concentrated further by passage through centricon tubes (YM-100; Millipore, Bedford, MA). All vector stocks were kept at -80°C until used.

AAV vector titers (viral particles (vp)/ml) were determined by quantitative PCR for viral genome copies extracted from DNAase-treated viral particles (High Pure Viral Nucleic Acid Kit, Roche). The primers used in the q-PCR were Forward-eGFP: 5´-GTCCGCCCTGAGCAAACA-3´ and Reverse-eGFP: 5´-TCCAGCAGGACCATGTGATC-3´. Vector titers obtained ranged from 2 x 10^{12} to 9 x 10^{12} vp/ml.

Histological procedures

Mice were sacrificed three weeks post-surgery by transcardiac perfusion with saline Ringer solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were dissected and stored for 48 hours in a cryopreservation solution containing 10% glycerin and 2% dimethylsulphoxide (DMSO) in 0.125 M PB, pH 7.4 at 4 ºC. Frozen serial coronal sections (40 µm thickness) were obtained using a sliding microtome and collected in cryopreservation solution in series of 10 adjacent sections.

Free-floating sections were rinsed with Tris buffer pH 7.4 (TBS) and then incubated in a blocking solution containing 1% cold fish gelatin (Sigma), 1% bovine serum albumin (BSA) and 0.05% Triton X-100 in TBS for one hour; sections were then incubated overnight at room temperature (RT), with the appropriate primary antibodies diluted in blocking solution.
The following primary antibodies were used in double immunofluorescent stains: 1) rabbit anti-GFAP (1:400, Dako, Glostrup, Denmark; catalog number Z0334). 2) mouse anti-GFAP 1:400, AbD Serotec, Killington, UK; catalog number 4650-0309). 3) mouse anti-neuronal nuclear antigen (NeuN) (1:500, Millipore, Darmstadt, Germany; catalog number MAB 377). 4) goat anti-olig2 (1:200, R&D systems, Minneapolis, MN; catalog number AF2418). 5) rabbit anti-Iba1 (1:500, Wako, Neuss, Germany; catalog number 019-19741). After rinsing with TBS, sections were incubated with the appropriate fluorescent secondary antibodies diluted as before for one hour. The following secondary antibodies were used (all purchased from Molecular Probes and used 1:200): Alexa Fluor® 633 donkey anti-rabbit IgG (#A21070), Alexa Fluor® 633 donkey anti-mouse IgG (#A21050); Alexa Fluor® 546 donkey anti-mouse IgG (#A10036); Alexa Fluor® 633 donkey anti-goat IgG (#A21080); Alexa Fluor® 555 donkey anti-rabbit IgG (#A31572), Alexa Fluor® 546 goat anti-rabbit (#A11010) Alexa Fluor® 546 goat anti-mouse (#A11003). Finally, sections were rinsed in PBS and mounted on SuperFrost Ultra Plus® slides, dried at RT and coverslipped with Depex (VWR International). As negative control and to verify the specificity of the secondary antibodies, the same immunohistochemistry procedure was performed omitting the primary antibodies. No staining was observed. Furthermore, all antibodies used here were used in other publications (see Eng et al. 2000; Talbott et al., 2008; Gil-Perotin et al., 2009; Lalancette-Hebert et al. 2012; Seto et al. 2014; Haberl et al. 2015). Sections were inspected under a confocal laser-scanning microscope (LSM 800; Zeiss, Jena, Germany). To ensure appropriate

visualization of the labeled elements and to avoid false positive results, the emission from the argon laser at 488 nm was filtered through a band pass filter of 505–530 nm and color-coded in green. The emission following excitation from the helium laser at 543 nm was filtered through a band pass filter of 560–615 nm and color coded in red. A long-pass filter of 650 nm was used to visualize the emission from the helium laser at 633 nm and color coded in pale blue.

As the main goal of this study was to determine the specificity of the chosen promoters in the context of AAV8-mediated gene delivery to the striatum, we focused our analysis on the transduced area only. The numbers of eGFP-positive cells infected with each vector were determined on images of 8 random areas within the transduced striatum regions (i.e. containing at least one $eGFP^+$ cell) per mouse using a 40x objective and ImageJ software. Percentages were calculated based on the total number of transduced cells (number of $eGFP^+NeuN^+$/total $NeunN^+$, $eGFP^+GFAP^+$/total $GFAP^+$ or $eGFP^+Olig2^+$/total $Olig2^+$, respectively).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analyses were performed using the software GraphPadPrism. To test for difference in transduction efficacy, a non-parametric one-way ANOVA with Tukey post-test was applied, except for Figure 5 where we used Chi square analysis. All tests were considered significant if $p < 0.05$.

Results

In vitro analysis

A total of six recombinant AAV genomes carrying an eGFP reporter gene were constructed (Figure 1; for a more detailed description of the vectors see 'Materials and Methods'). In brief, five constructs carried CNS cell-specific promoters and one a ubiquitous promoter, CAG pr, was used as control. Three promoters targeting astrocytes were tested: hGFAP pr, hGFAPΔD pr and mGFAP pr, as well as two neuronal ones, BM88 pr and CHNRB2 pr. The expression of eGFP, driven by the different constructs, was first analyzed *in vitro* by plasmid transfection of HEK-293T cells (Figure 2). All promoters were able to drive the expression of the fluorescent protein, however, important differences in their transcriptional activity were found. Of the neuronal promoters, BM88 was stronger than CHNRB2, while the astrocyte promoter mGFAP was better than hGFAP, and the level of eGFP expression was not diminished upon deletion of the D region. Non-transfected controls were eGFP negative whereas the majority of cells transfected with plasmid containing eGFP under the control of CAG pr were strongly positive.

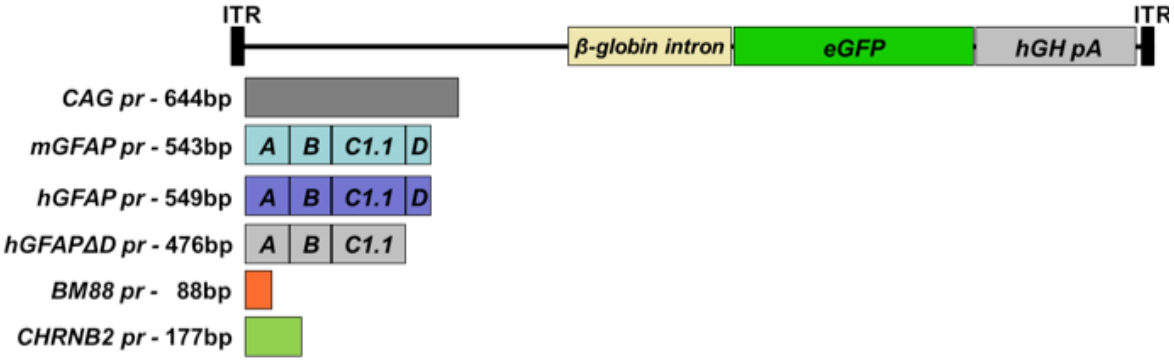

Figure 1. Schematic representation of the genomic structures of the AAV vectors. AAV vectors carry reduced versions of the human or murine astrocyte-specific GFAP promoters, the minimal neuronal promoters BM88 or CHNRB2, which control the expression of the reporter gene enhanced GFP (eGFP). The expression cassettes also contain the ß-globin intron downstream of the promoter sequence and the human growth hormone polyadenylation signal sequence (hGH-polyA).

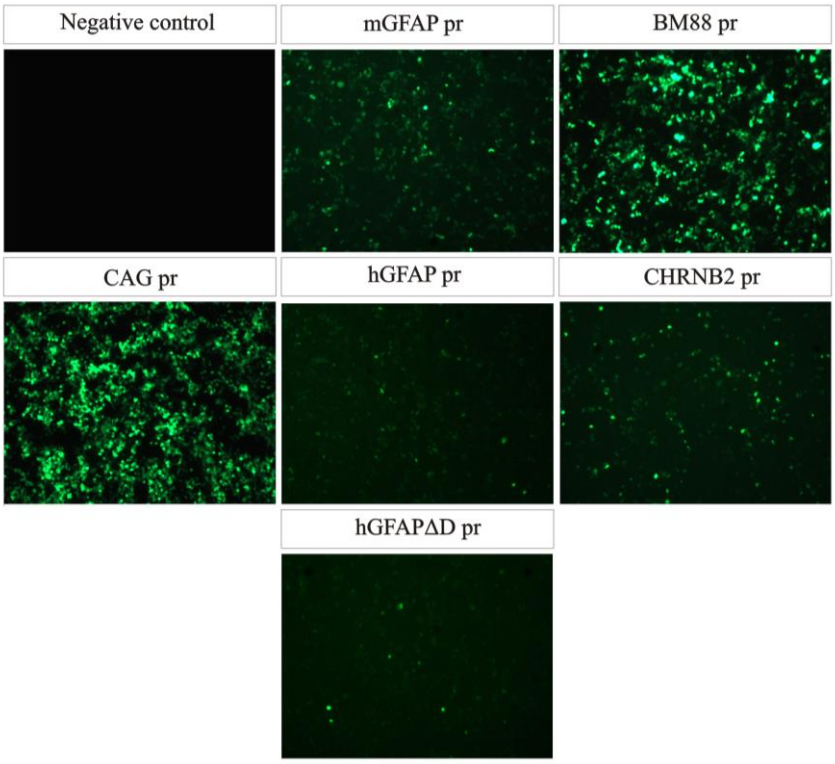

Figure 2. **All plasmid constructs are functional *in vitro***. HEK-293T cells were transfected with the same amount of each plasmid and 48 hours later eGFP expression was analyzed. All promoters were able to drive transgene expression, the strongest one being BM88 and the weakest one hGFAP. As positive control, cells were transfected with a plasmid expressing GFP under the control of the strong and ubiquitous promoter CAG.

Analysis of the transduction efficacy of the different vectors in mouse striatum

After testing the functionality of the different plasmids *in vitro,* we produced the recombinant AAV8 vectors for *in vivo* studies. A dose of 4 x 10^9 vp of each AAV8-eGFP vector was injected into the left striatum by stereotactic injection (n = 3 per group). The mice did not display any adverse reaction or behavioral changes after the intracranial surgery or during the subsequent period until sacrifice. However, no long term studies were performed to test the potential toxicity of sustained transgene expression. Three weeks after vector injection mice were euthanized and eGFP expression was analyzed in both the right and left striatum. In all groups eGFP expression was detected in the left striatum as well as along the injection tract but never in the right brain hemisphere (See Suppl. Fig. 1). The number of eGFP-positive cells/μm^2 varied depending on the promoter. The highest number of transduced cells was observed with the vector carrying the neuron-specific promoter BM88 pr (Figure 3). Transduction was significantly less efficient using the three variants of GFAP,

CHNRB2 or CAG as promoter. The lowest levels of expression were consistently found with the construct containing CHRNB2 pr.

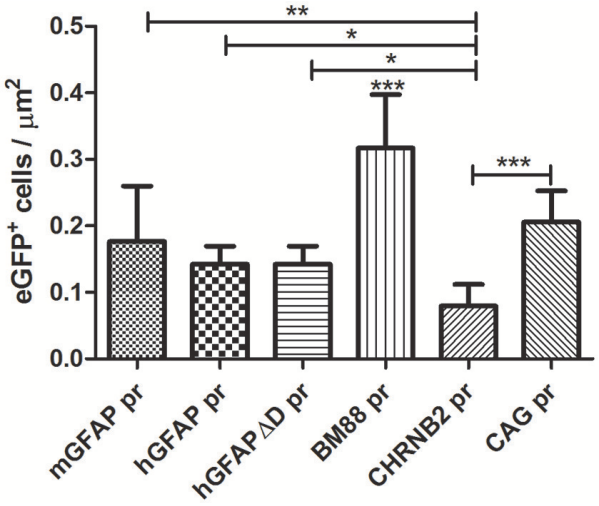

Figure 3. Analysis of the transduction efficacy of the AAV8 vectors carrying different promoters. Mice were treated with the different vectors ($4x10^9$ vp/mouse) by stereotaxic surgery in the left striatum. Twenty-one days later mice were sacrificed and the number of eGFP-positive cells in the transduced area was quantified (mGFAP = 648 cells; hGFAP = 360 cells; hGFAPΔD = 365 cells; BM88 = 808 cells; CHRNB2 = 204 cells; CAG =525 cells). Mean ± SD are shown. Differences in the number or GFP^+ cells were statistically evaluated by One-way ANOVA. Results were considered $p < 0.05$ and levels of significance are indicated as follows: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

Transduction of neurons and astrocytes by CAG-driven AAV8

CNS tissue is highly heterogeneous and consists of different cell types including neurons and glia cells. Following the delivery of eGFP expressing vectors under the control of the constitutive promoter CAG, eGFP-expressing cells with different morphologies were observed. To further identify the type(s) of cells transduced by this vector, a triple immunofluorescence stain using both anti-eGFP as well as cell-specific markers was performed. Astrocytes were identified by their expression of GFAP, whereas for neurons the pan-neuronal marker of neuronal nuclei (NeuN) was used (Figure 4). AAV8-CAG-eGFP mainly transduced neuronal cells and to a lesser extent also astrocytes and oligodendrocytes. Quantification of eGFP-expressing cells revealed that the number of eGFP-positive neuronal cells was eight-fold higher than the number of astrocytes (Figure 5). These results indicate that both neurons and astrocytes are transduced by AAV8 after stereotactic injection into the striatum, albeit with a greatly varying efficacy.

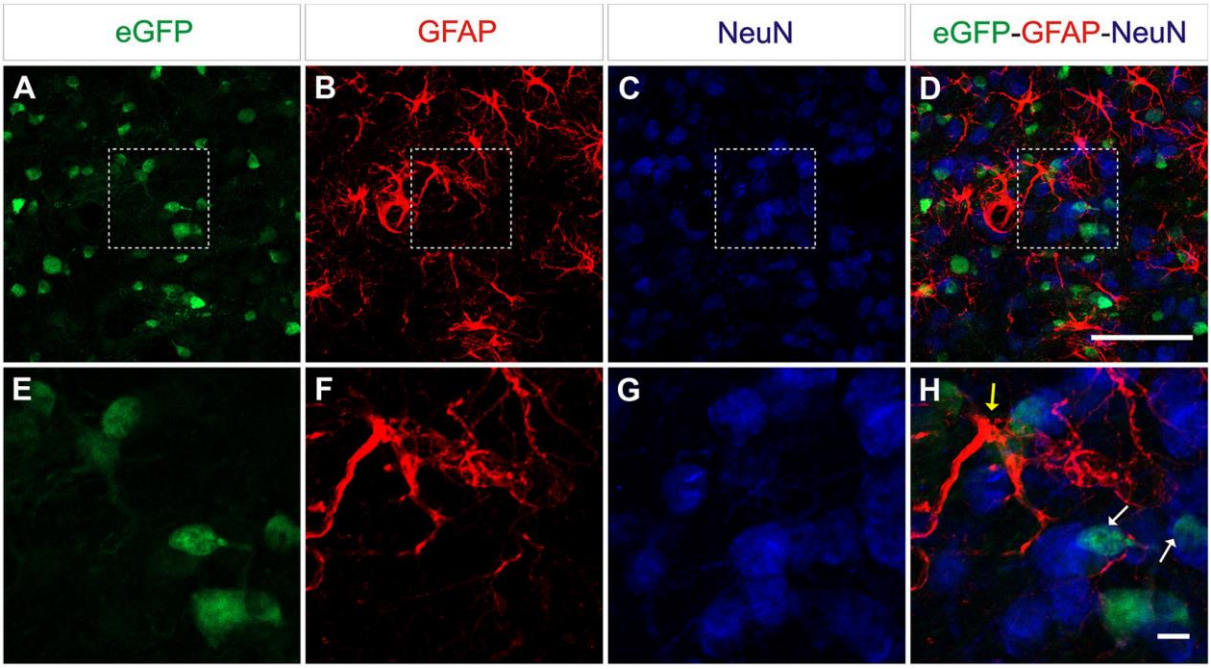

Figure 4. AAV8 expressing eGFP under the control of an ubiquitous promoter transduce both neurons and astrocytes efficiently. Adult mice were injected with $4x10^9$ vp/mouse and killed 3 weeks post-injection to determine $eGFP^+$cells (green) in the striatum. Neurons were labeled with an anti-NeuN antibody (blue) and astrocytes were labeled with an anti-GFAP antibody (red). Arrows indicate eGFP double-positive cells (yellow = $eGFP^+/GFAP^+$, white $GFP^+/NeuN^+$). Scale bars 5 µm.

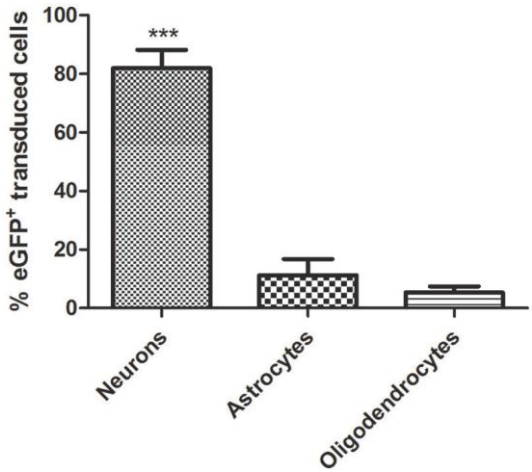

Figure 5. Quantitative analysis of AAV-CAG-eGFP transduction. Although the AAV8 vector transduces both neurons and astrocytes and CAG is a ubiquitous promoter, neuronal cells are transduced significantly better. (Neurons = 1311/1577 $eGFP^+$; astrocytes = 180/1577 $eGFP^+$cells; oligodendrocytes = 86/1577 $eGFP^+$). Mean ± SD are shown. Analysis was restricted to the transduced area of the striatum as described in Methods. Differences in the number of $eGFP^+$ cells were statistically evaluated by Chi square analysis. Results were considered significant when $p < 0.05$ (*).

Astrocytic transgene expression is driven by GFAP promoters

Next we wanted to analyze the specificity of the vectors containing either neuron- or astrocyte-specific promoters. In mice injected with either of the AAV-GFAPpr variants most of the eGFP-positive cells were astrocytes (Figure 6), while neurons were never found to express eGFP (Figure 7).

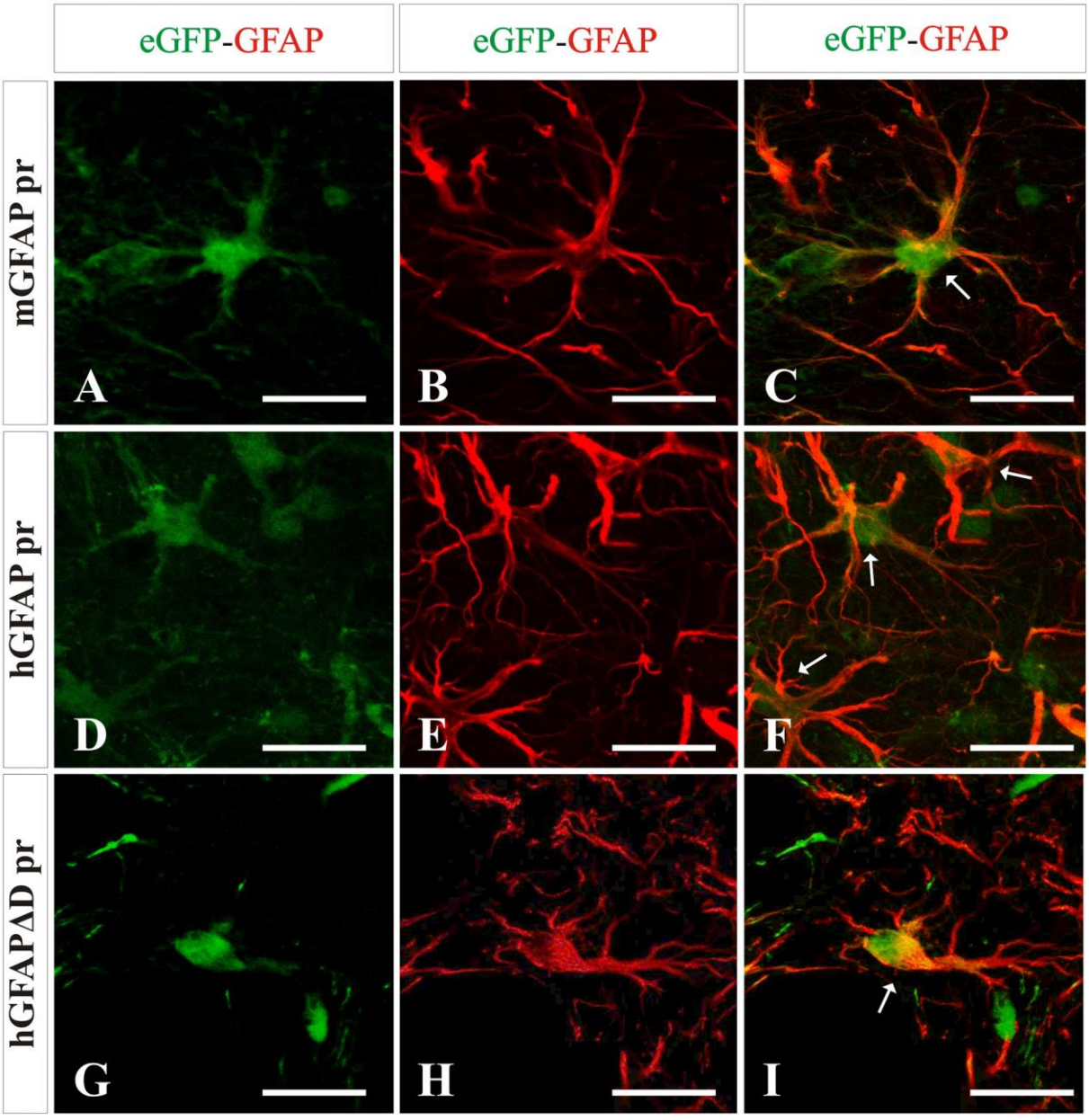

Figure 6. Analysis of brain transduction by AAV8 carrying eGFP under the transcriptional control of astrocyte-specific promoters. Mice were treated with the different vectors carrying astrocyte-specific promoters mGFAP pr (A-C), hGFAP pr (D-F) and hGFAPΔD pr (G-I) at the same dose by stereotaxic surgery in the left striatum. Twenty-one days later mice were sacrificed and the number and type of $eGFP^{+}$ cells (green) were analyzed. Astrocytes were labeled with an anti-GFAP antibody (red). A clear co-localization of GFAP and transgene expression is indicated by arrows. Scale bars 20 µm.

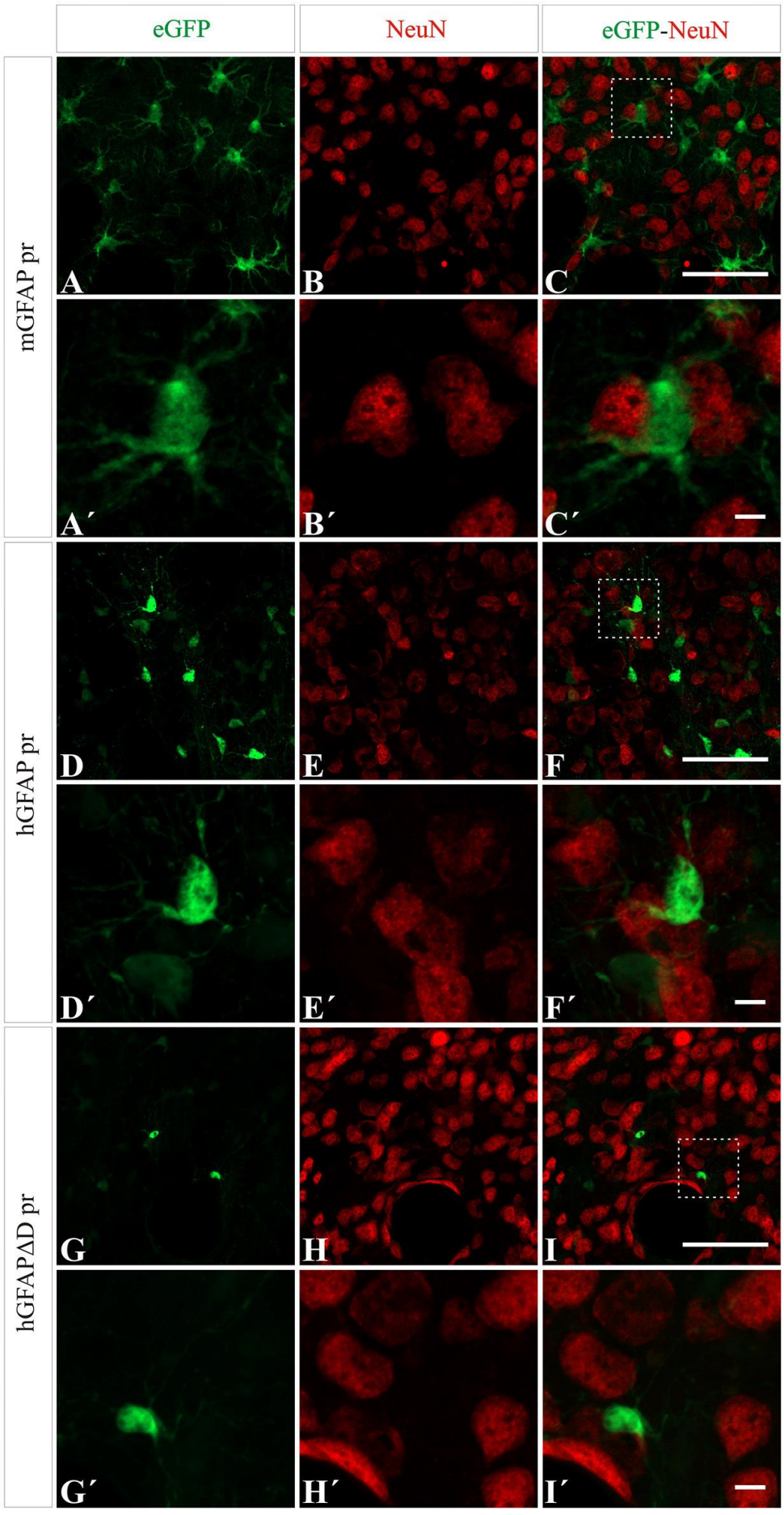

Figure 7. No transgene expression in neurons is detected when using AAV8 carrying GFAP derived promoters. NeuN expression (red) did not co-localize with eGFP expression (green) after injection with AAV8 carrying the mGFAP (A-C), hGFAP (D-F) and hGFAPΔD (G-I) promoters. Scale bars: low magnification - 20 µm (A-I) and high magnification - 5 µm (A'-I').

population of eGFP-positive cells lacking GFAP expression was detected. These cells were lacking the morphological features that typically characterize astrocytes and their small size and morphology were consistent with an oligodendroglial phenotype. In an attempt to properly identify the exact nature of these cells, we labeled brain sections with anti-eGFP, anti-Olig 2, an oligodendroglial marker, and anti-Ibal, a microglial marker. As shown in Figure 8, eGFP co-localized with Olig2-expressing cells but not with Ibal.

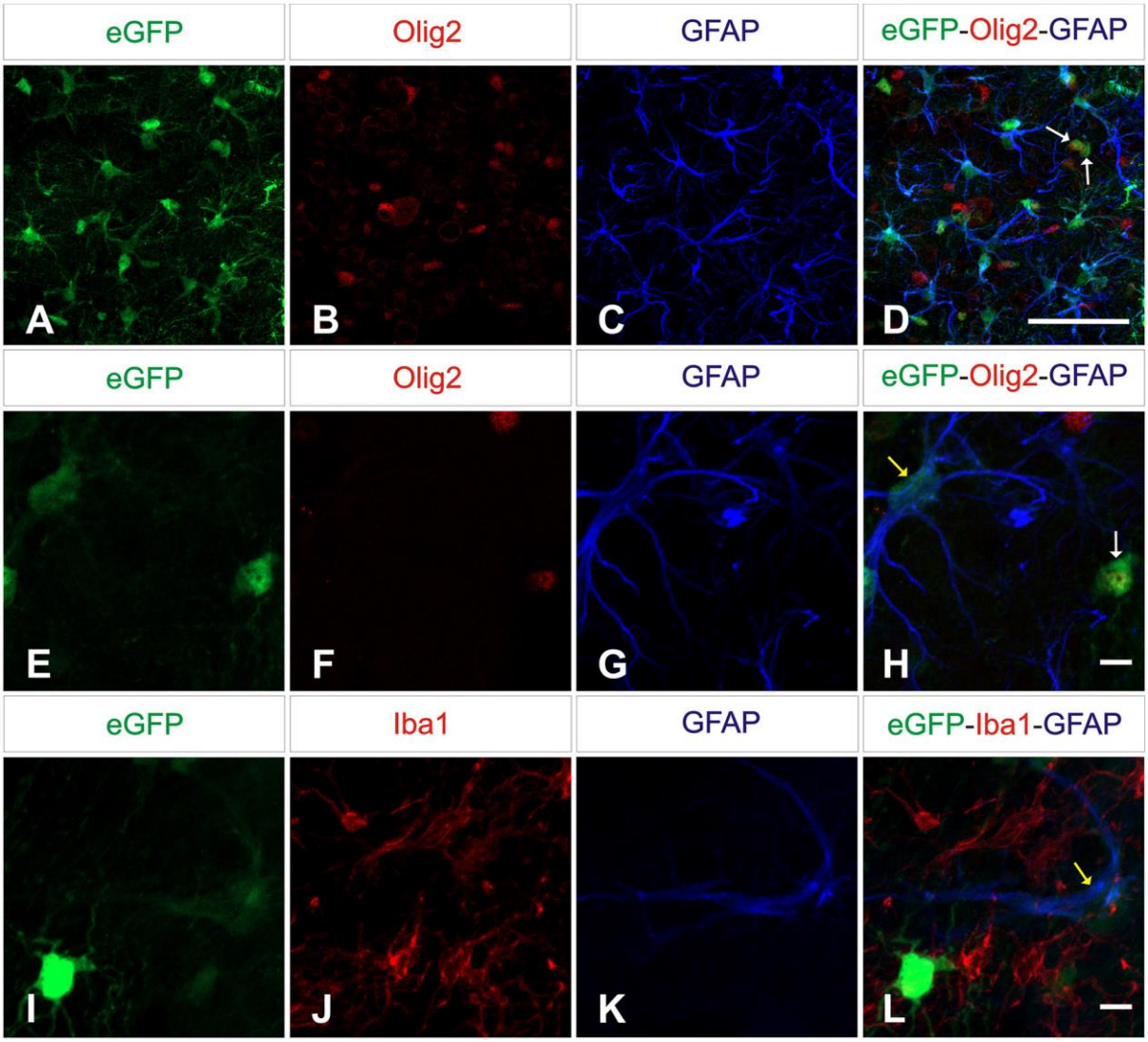

Figure 8. AAV8 carrying GFAP-derived promoters drive transgene expression in oligodendrocytes. Brain sections were labeled with anti-Olig2 (red; upper and middle panels) or anti-Iba1 (red; lower panels) and anti-GFAP (blue) to differentiate microglial, oligodendrocytes and macroglial. Olig2 immunoreactive cells (D,H) and astrocytes (D, H, L) showed co-expression of eGFP (green; arrows: yellow = $eGFP^{+}/GFAP^{+}$, white = $eGFP^{+}/Olig2^{+}$) while Iba1-positive cells were negative. Scale bars 20 µm (A-D) and 5 µm (E-L).

Mice injected with the vector carrying the mGFAP promoter had the highest levels of transduction. A strong fluorescence was observed in cell bodies throughout the dorsal area of the striatum. Furthermore, the number of positive cells was similar in the groups injected with either hGFAP or hGFAPΔD, indicating that the D element is dispensable for the transcriptional activity of the promoter (no significant difference was observed between these two promoters) (Figure 9A). This is in line with what was suggested by our *in vitro* results. Three weeks after viral injection, 81.5% of astrocytes in AAV8-mGFAP recipients were eGFP positive. With 55.5% of positive cells in AAV8-hGFAP- and AAV8-hGFAPΔD-injected mice, respectively, these promoters were less efficient (Figure 9A). Moreover, co-localization of reporter gene expression and the oligodendroglia marker Olig2 in the AAV8-mGFAP group was the lowest with 17.4% (Figure 9B). With the other variants 20% (AAV8-hGFAPΔD) and 26.6% (AAV8-hGFAP) stained positive for both eGFP and Olig2. These results indicate that the mGFAP promoter works best for astrocyte-specific transgene expression.

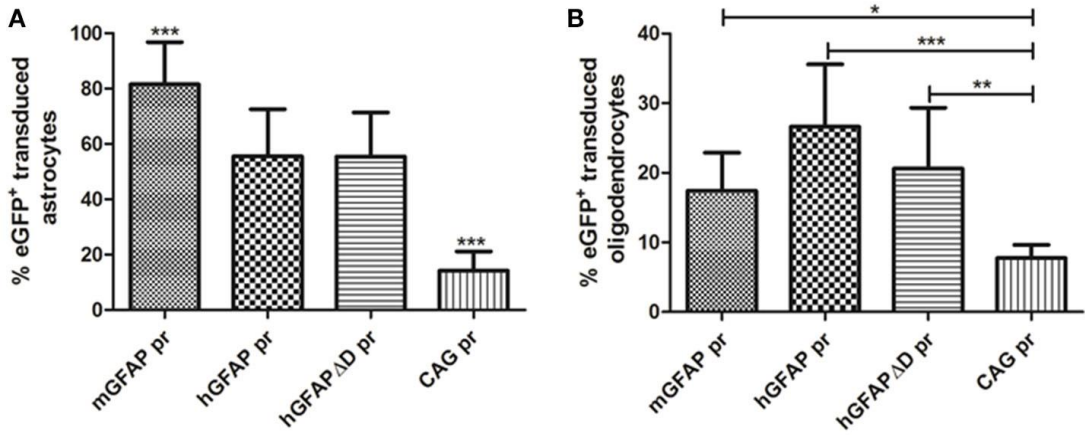

Figure 9. Quantification of the percentage of eGFP+ astrocytes and oligodendrocytes transduced by AAV8 carrying GFAP-derived promoters. A) The vast majority of transgene expressing cells after AAV-GFAP-eGFP injection were astrocytes. The percentage of $eGFP^+$ astrocytes/total $GFAP^+$ cells is plotted (mGFAP = 550/648 cells; hGFAP = 206/360 cells; hGFAPΔD = 98/186 cells; CAG = 28/224 cells). B) A smaller fraction of oligodendrocytes was $eGFP^+$ (mGFAP = 22/131 cells; hGFAP = 41/157 cells; hGFAPΔD = 40/186 cells; CAG = 16/224 cells). Mean ± SD are shown. Counting was restricted to the transduced area of the striatum as described in Methods. Differences in the number or $eGFP^+$ cells were statistically evaluated by One-way ANOVA. Results were considered $p < 0.05$ and levels of significance are indicated as follows: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***).

CHB2RN and BM88 promoters induce neuronal transgene expression

Selective neuronal transduction was observed in mice having received the vector in which eGFP had been placed under control of the CHNRB2 or BM88 promoters (Figure 10). Widespread expression of the transgene was observed throughout the dorsal region of the striatum. At higher magnification specific eGFP expression was identified within NeuN-positive cells (Figure 10), suggesting that eGFP-expressing cells accounted for a neuronal phenotype. This is supported by the fact that co-localization of eGFP and GFAP was never found. The percentage of NeuN-positive cells co-expressing eGFP was significantly higher in animals injected with the BM88 vector (63.4%) than in those injected with the CHNRB2 promoter (15.9%), thus identifying the former as the more useful one for expressing transgenes in neuronal cells (Figure 11).

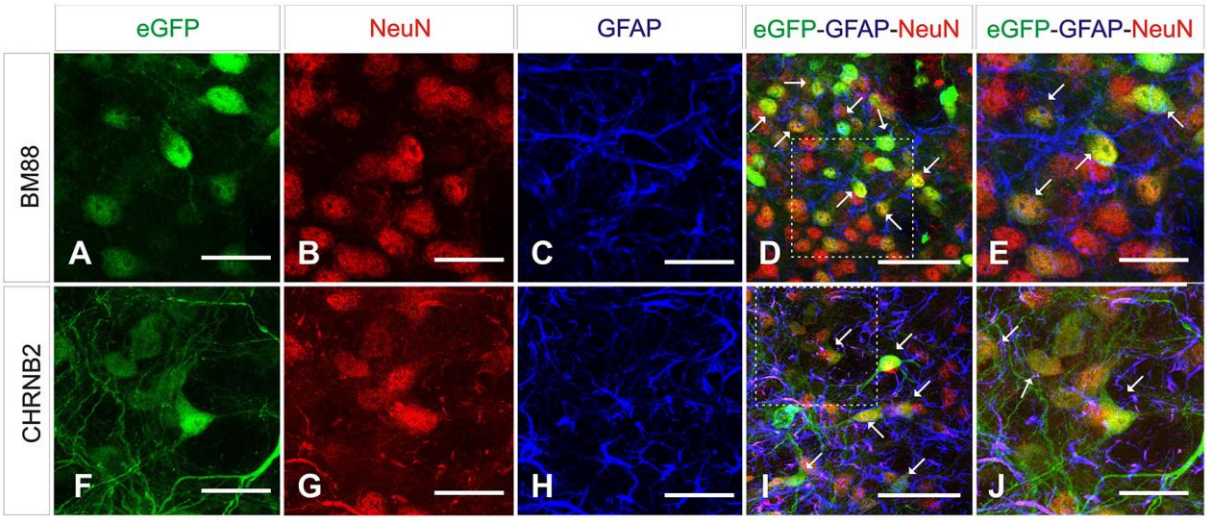

Figure 10. Analysis of brain transduction by AAV8 carrying eGFP under the transcriptional control of neuronal promoters. Mice were treated with AAV8 carrying the neuronal promoters BM88, CHB2RN and the eGFP reporter gene as before. Twenty-one days later mice were sacrificed and the number and type of $eGFP^+$ cells was analyzed. $NeuN^+$ cells (red) showed a clear co-expression of eGFP (green; arrows), while astrocytes expressing GFAP (blue) did not express eGFP (green), We also observed $NeuN^+$ neurons that were not expressing eGFP. Scale bars: D, I 20 µm. All others 50 µm.

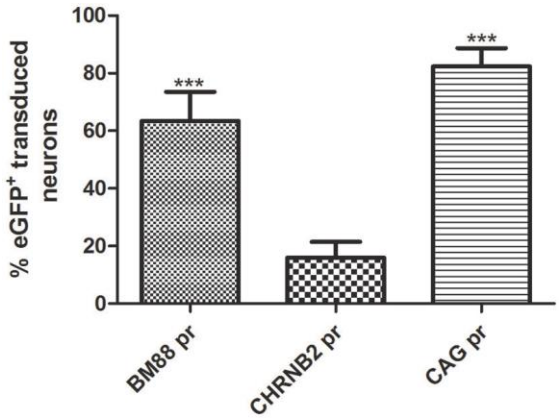

Figure 11. **Quantification of the percentage of neuronal cells expressing eGFP after AAV8 injection carrying BM88 and CHB2RN promoters.** A high percentage of neuronal cells are transduced using the minimal promoter BM88, higher neuronal transduction efficiency was obtained in comparison to CHBRN and CAG promoters. The percentage of $eGFP^+$neurons/total $NeuN^+$ cells is shown (BM88 = 808/1302 cells; CHRNB2 = 204/1250 cells; CAG = 473/525 cells). Mean ± SD are plotted. Counting was restricted to the transduced area of the striatum as described in Methods and differences in the number or $eGFP^+$ cells were statistically evaluated by One-way ANOVA. The significance level was set to $p < 0.001$ (***).

Discussion

Here we described the development and transduction efficacy of AAV-based gene delivery vectors for cell-specific transgene expression in the CNS.

In order to achieve a successful delivery and expression of the therapeutic gene, selection of both delivery vehicle and an optimal expression cassette is essential. As vehicle we chose AAV serotype 8 as it was previously shown to more efficiently transduce cells of the CNS than other serotypes (Aschauer et al., 2013) and to also infect a larger area than the best described and most commonly used serotype 2 (Watakabe et al., 2015). The packaging capacity of serotypes 2 and 8 (or any other AAV serotype) does not differ and expression cassettes are generally based on AAV2. However, having by far the smallest packaging capacity amongst the viruses used for gene therapy (4.4-4.7 kb), a need for a reduction in promoter elements is obvious in order to allow the packaging of larger genes or multiple genes. Accordingly we here focused on the identification of small or minimal cell-specific promoters for CNS applications.

The functionality of the newly generated plasmids was first characterized *in vitro* by transfection into HEK-293T cell lines. Although derived from human embryonic kidney cells,

previous reports demonstrated that this cell line expresses significant amounts of proteins found in the CNS, such as neurofibroblast subunits and α-internexin, (Shaw et al., 2002). HEK-293T cells also express different neuronal receptors and electrophysiological studies have shown the presence of endogenous voltage-activated ion currents (Shaw et al., 2002), which supports the use of this cell line for testing the performance of the CNS-specific promoters. We indeed observed that all the "cell-specific" promoters were able to drive the expression of the reporter gene. That the transduction of HEK-293T cells with AAV carrying the neuron-specific promoter BM88 was significantly more efficient than with all other promoters – including the ubiquitous promoter CAG – is easily explained by the fact the observed expression pattern of CNS-specific proteins in HEK-293T cells is similar to that of a typical early differentiating neurons or neuronal stem cells.

AAV8 has been described to be highly efficient in driving eGFP expression in astrocytes and neurons in the striatum (Taymans et al., 2007) and we first tested the transduction efficiency and efficacy of an eGFP-expressing AAV8 vector under the control of the ubiquitous and highly potent promoter CAG. After stereotactic delivery into the mouse striatum both astrocytes and neurons were found to be transduced with our vector, the latter even more efficiently.

Interestingly, previous experiments using an AAV8 with a similar construct showed a better performance for astrocyte- rather than neuronal transduction in the striatum (Aschauer et al., 2013). Using a ubiquitous promotor in macaques resulted in transduction of certain neuronal cell subtypes but not glia (Masamizu et al., 2010). The main difference between our study and that of Aschauer et al. is that they purified the AAV vector by CsCl density gradient centrifugation, whereas we here performed an iodixanol gradient. Differences in transduction-efficacy and -specificity were shown previously to not only depend on the AAV capsid serotype but to also be related to the production and purification methods (Ayuso et al., 2010).

Astrocytes are the most abundant cell type in the vertebrate CNS and hence involved in many degenerative diseases. We therefore characterized reduced forms of the human and murine GFAP promoters, which mainly drive transgene expression in astrocytes. Importantly,

because GFAP is not expressed in neurons, these promoters cannot drive neuronal expression transgene of the transgene. The reduced form of the hGFAP promoter used in this study was previously characterized *in vitro* by Lee et al., (2008). We further reduced its size by removing the D element located at the 3' end of the promoter sequence. Deletion of the D element was reported to severely reduce transcription (Besnard et al., 1991; Lee et al., 2008), however, our *in vivo* data indicates that this element is not essential for the transcriptional activity of the promoter in astrocytes. In addition we tested the transcriptional activity of the reduced version of the mGFAP promoter, which was designed using the reduced version of the hGFAP (Lee et al., 2008) as a model. The murine version of the promoter was more active and specific than the human one.

Co-localization of eGFP/GFAP was observed in the striatum of all mice injected with either of the three GFAP promoters, demonstrating that they all efficiently transduce astrocytes. We also found a proportion of eGFP-expressing GFAP-negative cells that were subsequently identified as oligodendrocytes. Previous work reported weak neuronal expression using GFAP promoter, but oligodendrocyte expression has not been reported (Lee et al., 2008). Currently we do not have an explanation for this finding. Even though expression of hGFAP (but not mGFAP) was shown in an oligodendrocyte precursor cell (Casper and McCarthy, 2006), the infection and subsequent maturation of these precursors can be excluded as the same precursor can give rise to neuronal cells and eGFP expression was not seen in either neurons or microglial cells. Interestingly, while the transduction efficacy of astrocytes was significantly better with the murine GFAP promoter, transduction of oligodendrocytes did not significantly differ between the three GFAP variants.

While we observed a degree of axonal transport of both AAVs, more experiments need to be performed to determine whether this was retrograde or anterograde. In mice, vector transport along astrocytes has been described in previous studies and was found to be serotype dependent (anterograde: AAV2, Salegio et al., 2012; retrograde: AAV5, Aschauer et al., 2013). Other studies done in marmoset and macaque using AAV8 revealed preferential retrograde transport of this serotype (Masamizu et al., 2011). Future experiments will allow us to confirm the direction of the axonal transport. Another of our future aims is to use GFAP promoters for the astrocyte-selective expression of genes coding for several

transcription factors with the ultimate goal of conducting *in vivo*–reprogramming of these astrocytes into neurons.

For neuron-specific expression we used two very small promoters with different transcriptional potencies, BM88 and CHNB2. The minimal promoter derived from the neural protein BM88 had a stronger transcriptional activity and in the transduced area up to 63% of striatal neurons were $eGFP^+$. This can be explained by the expression pattern of BM88: it is not only widely expressed in proliferating neuronal precursors, but also at an even higher level in their post-mitotic neuronal progeny in the developing as well as in the adult brain (Koutmani et al. 2004). In contrast, the minimal CHRNB2 promoter was also neuron specific but transgene expression was a lot lower and found in only less than 20% of $NeuN^+$ cells. This result was somehow surprising since the CHNB2 pr controls the expression of the nicotinic receptor β-subunit, which is expressed in the majority of neurons in the brain. However, it can possibly be explained by the fact that we are using a reduced version of the promoter, whose activity is significantly lower than that of the original promoter (Bessis et al., 1995). Thus we are most likely unable to detect eGFP expression in all the cells that have been transduced. Moreover, the distribution of the nicotinic acetylcholine receptors subtypes expressed in the CNS will have a direct effect on the promoter activity (Gotti et al., 2006). Additional studies will be needed to better characterize of the type of neurons transduced by either promoter. The small size of these promoters allows the expression of larger genes or more than one gene in neurons. Diseases caused by the deficiency of large genes are not uncommon among the spectrum of neurological disorders, such as for instance in autism spectrum disorders, intellectual disability or Dravet syndrome, in which mutations in several genes are involved. Thus, development of vectors allowing the insertion of multiple genes would be of paramount importance for the adequate development of gene therapy approaches when dealing with these diseases.

In conclusion, we have developed and characterized AAV-vectors with a relatively large cloning capacity for the cell-specific delivery of therapeutic genes to the CNS. Albeit yet needing further characterization these cell-specific AAVs represent promising tools with a great potential use for the development of gene therapy approaches for neurodegenerative disorders.

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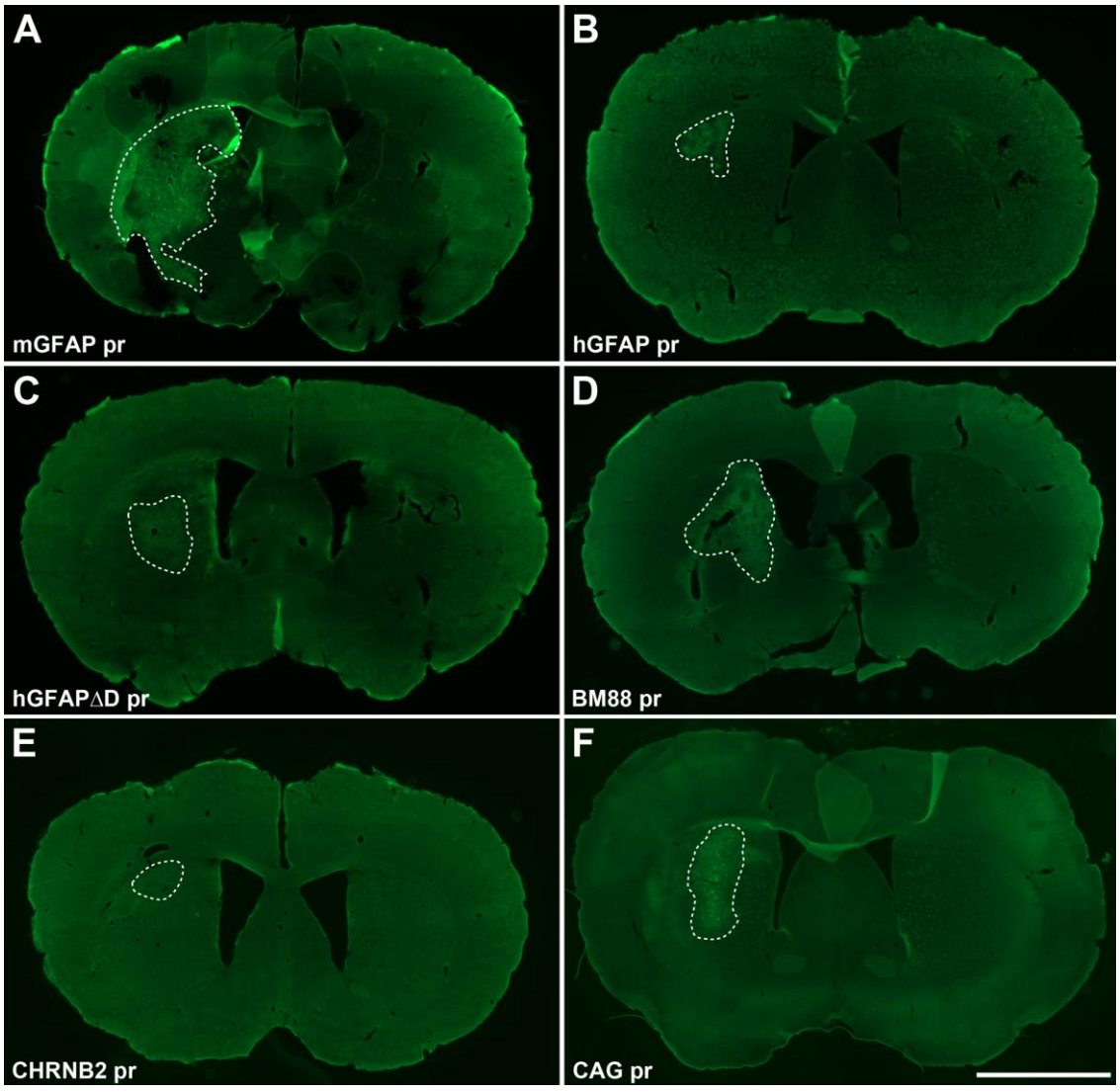
Supplementary Figure 1.

Striatal transduction area for each viral vectors

Representative images showing the different patterns of viral spread in mice striatum. Scale bar: 2000 μm.

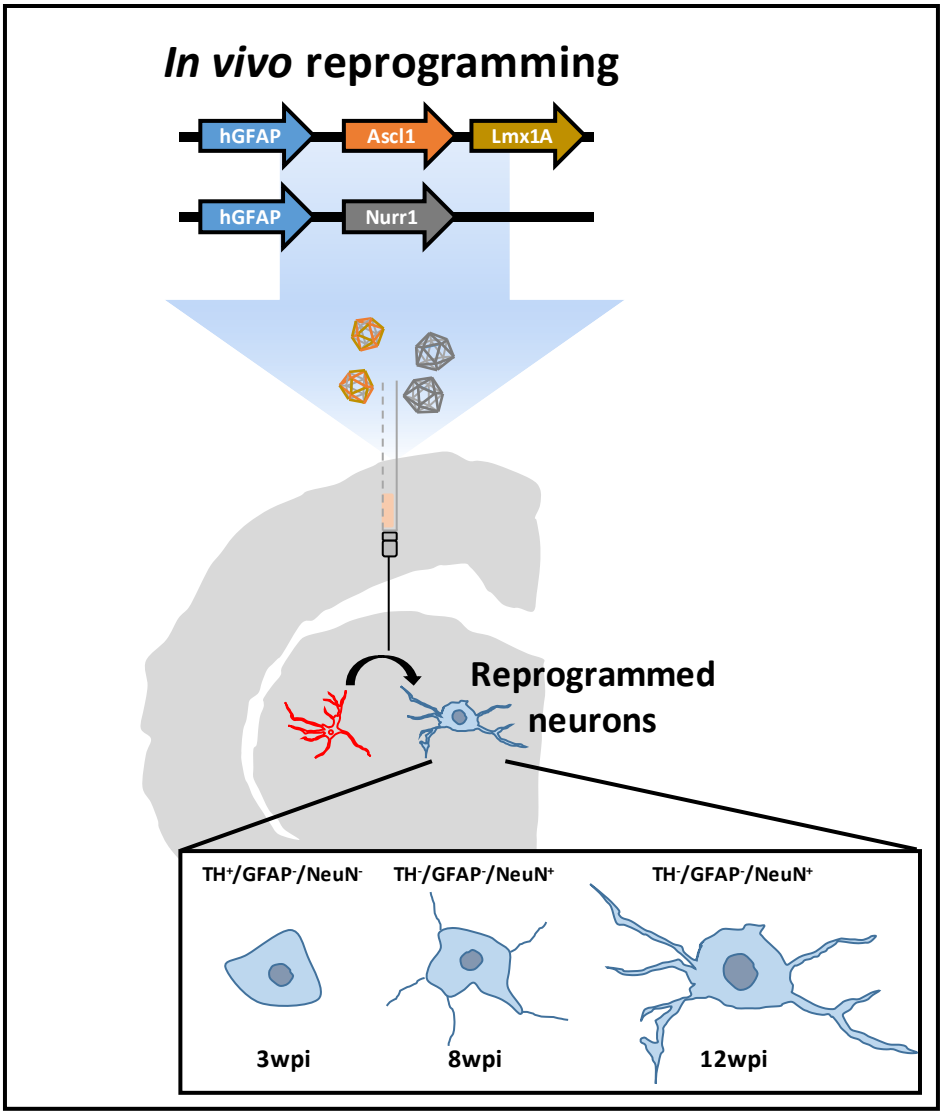


Chapter two

In preparation

"*In vivo* reprogramming of astrocytes into neurons in the mice striatum"

Graphical Abstract



In vivo reprogramming of astrocytes into neurons in the mice striatum

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Key words: AAV, CNS, _in vivo_ reprogramming, basal ganglia, Parkinson's disease, transcription factors, gene therapy

Funding

ERC Advanced grant (ref: 340527 "Repropark"). CoEN Pathfinder Referencia: (ref: Phase II Call). Diego Pignataro is partially supported by a Jon Zarandona donation.

INTRODUCTION

The dramatic cells lost observed in patients with Parkinson's disease (PD) had evidenced the selective vulnerability of the midbrain dopaminergic (miDA) neurons of the substantia nigra pars compacta (SNpc). miDA are characterized by the expression of tyrosine hydroxylase (TH) that is the rate-limiting enzyme in the synthesis of dopamine. miDA death is associated with different environmental and genetic factors but the mechanism is still not fully understood (reviewed by Smidt 2009). The cell death observed in the SNpc leads to the subsequent reduction of striatal dopamine (DA) levels (Dauer and Przedborski et al., 2003), which is the main cause of the motor symptoms such as rigidity, resting tremors and bradykinesia observed in PD patients (reviewed by Lanciego et al., 2012). Therefore, since neuronal lost in PD is initially confined to a small cohort of functionally related neurons, a valuable therapeutic option for this disease could be based on the repopulation of this area of the brain with a source of homologous cells (Mendez et al., 2005; Kefalopoulou et al., 2014). Over the past few years different strategies based on this idea have been pursued. Neuronal replacement injecting new cells have been tried. Despite the initial promising results using embryonic cells a number of important aspects need to be addressed for its application. Ethical concerns have been raised, due to the origin of the cells (human fetal DA cells obtained from elective abortions) and the necessity for immune suppression. Hence, a safe, renewable and standardized method to obtain DA neurons that can be applied for PD treatment needs to be developed. Toward this objective, embryonic stem (ESCs) and induced pluripotent stem (iPSC) cells-derived DA neurons have shown to be efficient in restoring motor symptoms when transplanted in PD animal models (Kim et al., 2002; Kriks et al., 2011). Importantly, the iPSCs offer the possibility to obtain the cells from the same patient and this strategy will avoid immune-mediated rejection. Thus the patients will not require immunosuppressive treatment. However, there are still limitations that have to be solved for making this strategy a clinical reality in the treatment of PD, such as likelihood of tumor formation, reprogramming efficiencies, cell identity, maturation and mutagenesis (Ang and Wernig, 2014). In addition, some studies have recently reported the accumulation of chromosomal aberrations, gene mutations and genomic alterations accumulate during iPSCs reprogramming and following *in vitro* expansion.

The *in vivo* reprogramming or direct cell fate reprogramming have proved that is possible to overcome the epigenetic barriers and change the identity of differentiated cells in different organs including the brain (Heinrich et al., 2015; Zhou et al., 2008; Niu et al., 2013, 2015; Guo et al., 2014). In this regard, since resident glial cells are the most abundant cell type in the Central Nervous System (CNS), they represent an attractive candidate to be reprogrammed into neuronal cells. Different studies have shown that glial cells can be reprogramed, directly or indirectly, and converted into functional neurons in the adult brain and spinal cord (Guo et al., 2014; Heinrich et al., 2014; Niu et al., 2013; Su et al., 2014; Torper et al., 2013).

A way to tackle this reprograming strategy is using the information we have from the neuronal developmental studies. During neuronal development different transcription factors (TFs) collaborate in an orchestrated way to establish the different neuronal subpopulations; Ascl1, Lmx1A and Nurr1 have been described as key factors to generate functional miDA neurons during development (reviewed by Doucet-Beaupré et al., 2016). Those TFs have been also used in several studies to generate miDA neurons directly from mouse and human fibroblast without the need to bring the cells to a progenitor stage (Caiazzo et al., 2011) *in vitro* or *in vivo* (Liu et al., 2015; Torper et al., 2015).

As previously indicated in PD the progressive loss of miDA neurons in the SNpc leads to the subsequent loss of striatal DA levels. In order to increase the DA in the striatum the following strategy was pursued in the present work: to transform astrocytes of the striatum into dopamine producing neuronal cells throughout the specific expression of the TFs, Ascl1, Lmx1A and Nurr1. For that purpose AAV vectors expressing Ascl1 (A), Lmx1A (L) and Nurr1 (N) under the control of the astrocyte specific promoter hGFAP were produced and their reprogramming capacity was tested after *in vivo* injection into the mouse striatum.

MATERIALS AND METHODS

Animals and cell lines

Experiments were performed in 6-8 weeks-old male C57BL/6J mice purchased from Harlan Laboratories (Barcelona, Spain). Animal handling was conducted in accordance with the European Council Directive 86/609/EEC, as well as in agreement with the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research. Mice were bred and maintained under pathogen-free conditions in the animal facility of the University of Navarra. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra. (Ethical protocol 102-16). A total of 21 animals were used and divided in seven treatment groups (n=3 per group).

HEK-293T were purchased from the ATCC and were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 µg/ml) and streptomycin (100 U/ml) 100 µg of penicillin/ml and 100 U of streptomycin/ml (all supplements were from Invitrogen, Pisley, Scotland, UK). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Viral genome constructions

All cloning steps were first simulated using Snapgene 3.1 software and primers were designed with the same program. All basic DNA cloning protocols were essentially performed according to the Molecular Cloning A Laboratory Manual, third edition (Sambrook and Rusell, 2001). The constructs developed in this study contained the AAV2 inverted terminals repeats (ITR) flanking the expression cassettes. The plasmids carrying the full-length cDNA of each of the human (h) and murine (m) TFs Ascl1, Lmx1A, and Nurr1 (ALN1), were gently provided by Dr. Vania Brocolli (phAscl1, phLmx1A, phNurr1 and the pmALiresN1). First, from the plasmid pmALiresN1, the murine TFs (mAL) were replaced by the human homologous, obtaining the intermediate plasmid phALires.N1. To obtain the human Glial Fibrillary Acidic Protein (hGFAP) hGFAP.AAV constructions (pAAV.hGFAP.hAL), the hAL sequence from the intermediate plasmid phALires.N1 was inserted into an AAV-cassette containing the hGFAP promoter (hGFAP-587bp). To generate the pAAV.hGFAP.hN1,

the hN1 from the plasmid phNurr1 was inserted in the same manner as hAL in another AAV-cassette.

Vector production and purification

Recombinant AAV vectors, serotype 8 (rAAV8), were produced and purified from HEK-293T cells that were co-transfected using linear polyethylenimine 25 kDa (Polysciences, Warrington, PA, USA) with two different plasmids: the plasmid containing ITR-flanked transgene constructs and the plasmid contained the adenovial helper genes plus AAV2 rep and AAV8 cap named pDP8.ape (Plasmid factory, Bielefeld, Germany) as described (Durocher et al., 2002). Seventy-two hours post-transfection (hpt), the supernatant was collected and treated with polyethylene glycol solution (PEG8000, 8% v/v final concentration, St. Louis, Missouri, USA) for 48-72 hours at 4°C. Cells containing AAV particles were collected and treated with lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 2 mM MgCl2, 0.1% Triton X-100) and kept at -80°C. After 48-72 hours, supernatant was centrifuged at 3000 rpm for 15 minutes. Pellet containing particles from the supernatant was resuspended in lysis buffer and kept at -80°C. Three cycles of freezing and thawing were applied for both supernatant and cell lysate. Viral particles were purified by ultracentrifugation in an iodioxanol gradient according to the method of Zolotukhin et al., (Zolotukhin et al., 1999). The viral batches were then concentrated further by passage through Centricon tubes (YM-100; Millipore, Bedford, MA). All vector stocks were kept at -80°C until used. Viral titers (viral particles/ml) were determined by quantitative PCR for viral genomic copies extracted from DNAse-treated viral particles (High Pure Viral Nucleic Acid Kit, Roche) in triplicate at three different dilutions. The primers used for the qPCR are described in **Table 1**.

Table 1. Primer sequences used to obtain viral titer by qPCR

Name	Fw (5´ → 3´)	Rv (5´ ← 3´)
hAscl1	CTTGAACTCCATGGCCGGCTC	AAAGAAACAGGCTGCGGGC
hLmx1A	TGTCGATCGCGCTTTGGAAGTTC	GACTGTTTCCTAGCAACCTCAGAAGC
hNurr1	CCCGCTTCTCAGAGCTACAG	TTCAGTGTTGGTGAGGTCCA
hGFAP	GTAACATATCCTGGTGTGGAG	CATTGTGTCTGTGCCAAGG

Stereotaxic AAV injections

Surgical anesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Selected coordinates for targeting the striatum were 0.5 mm rostral, 2 mm lateral and 3.5mm ventral from bregma. Each group of animals received one pressure injections, one on the left side with 2 µl ($2x10^9$ GC/µl) of AAV vector at a flow rate of 0.2 µl/min through a Hamilton syringe drove by a syringe pump. Following the viral injection, the needle was left in the place for 2 minutes prior to being slowly retracted from the brain. After surgery, animals were kept under constant monitoring and with ad libitum access to food and water.

***In vitro* assays**

Cell transfection

Cultured cells were transfected via DNA plasmid using Lipofectamine 2000 Reagent (Invitrogen). Transfections were performed essentially according to manufacturer's protocols. 2µg of plasmid were transfected in 6 wells cultured plates and cells were collected 24-48 hrs post-transfection (hpt).

Western blot analysis

For transgene expression, 48 hpt, cells were scraped with a sterile disposable cell scraper (Costar), transferred to an Eppendorf tube and centrifuged at 14,000 RPM at 4°C for 5 min. A total protein concentration of the lysate was determined by microBCA kit (Pierce). Equal amounts of protein (10-20 µg) were analyzed in a 12% SDS/polyacrylamide gel (Bio-Rad Labs) and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham-Pharmacia, UK). The membrane was then probed with the corresponding antibodies and then incubated with appropriate secondary antibodies conjugated with horseradish peroxidase and detected with a chemiluminescent substrate (Pierce). The following antibodies were used: mouse anti-ASCL1 (556604; 1:1000; BD Pharmingen) and rabbit anti-LMX1A (AB105331; 1000; Millipore).

RNA Extraction and Real-Time qPCR

Total RNA was isolated from HEK-293T cell line with Trizol reagent (Invitrogen) and genomic DNA was removed. RNA was reverse transcribed using random priming and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Each cDNA was diluted 1:10 and 1 µl was used for each quantitative real-time PCR and were amplified by iQ SYBR® Green Supermix (BIO-RAD) according to the manufacturer's instructions in C1000 Thermal Cycler (10 µl final volume). qPCR was performed with the primer for hNurr1 (Table 1).

Histological procedures

Mice were sacrificed three, eight and twelve weeks post-injection (wpi) by transcardial perfusion with saline Ringer solution followed by 4% paraformaldehyde in phosphate buffer (PB) 0.1M. Brains were dissected and stored for 48 hr in a cryoprotectant solution containing 10% glycerin and 2% dimethylsulphoxide (DMSO) in 0.125 M PB, pH 7.4 at 4°C. Finally, frozen serial coronal sections (40 µm-thick) were obtained on a sliding microtome and collected in 0.125 M PB cryoprotectant solution containing 20% of glycerin and 2% DMSO in 0.125 M PB, pH 7.4, as 10 series of adjacent sections.

The following primary and secondary antibodies were used: mouse anti-ASCL1 (556604; 1:1000; BD Pharmingen), rabbit anti-LMX1A (AB10533; 1:1000; Millipore), mouse anti-NURR1 (ab41917; 1:100; abcam), rabbit anti-GFAP (Z0334; 1:400; Dako), goat anti-GFAP (ab53554; 1:1000; abcam), mouse anti-NeuN (MAB377; 1:100; Millipore), rabbit anti-NeuN (ab1777487; 1:1000; abcam), chicken anti-GFP (ab13970; 1:1000; abcam), mouse anti-TH (T2928; 1:1000; Sigma-Aldrich), goat anti-TH(C-20)(SC-7847; 1:50; Santa Cruz). As secondary antibodies we used different Alexa-Fluor 488, Alexa-Fluor 546 and Alexa-Fluor 647-conjugated from Thermo Scientific.

Free floating sections were rinsed with Tris buffer pH 7.4 (TBS) and then incubated in a blocking solution containing 1% of cold fish gelatin (Sigma), 1% bovine serum albumin (BSA) and 0.05% Triton X-100 in TBS for an hour; after that, sections were incubated overnight at room temperature with the appropriate primary antibody/antibodies diluted in a solution of 1% cold fish gelatin, 1% BSA and 0.05% Triton X-100 in TBS.

After being washed in TBS, sections were incubated with the corresponding secondary antibody/antibodies prepared in a solution also containing 1% cold fish gelatin, 1% BSA and 0.05% Triton X-100 in TBS. The sections were incubated for two hours at room temperature. Finally, sections were rinsed in TBS and mounted on SuperFrost Ultra Plus ® slides and dried at RT in the dark, dehydrated rapidly in toluene and coverslipped with DPX.

Images were taken using a LSM 800 (Zeiss, Jena, Germany) laser-scanning confocal microscope. The appropriated negative controls were performed, primary antibodies were omitted to test the presence of nonspecific background. Staining was not observed in any of the series used as a negative control. All the primary antibodies used in the present study has been published and validated for use in immunohistochemestry in mice (see Ivaniutsin et al., 2009; Hayes et al., 2011; Navarro et al., 2013; Eng et al., 2000; Jukkola et al., 2013; Zhang et al., 2015; Korner et al., 2015; Korotkova et al., 2005). For quantification of the numbers of immunofluorescence positive cells infected with the vectors, confocal images were taken using a 40X objective. In each animal three sections with immunopositive cells were selected, and 8 independent fields of view (40X) were randomly acquire and manually counted, to determine the extent of the transduction *in vivo*, with the assistance of Zen Lite software. The numbers of positive cells infected with each vector were determined on images of 8 random areas within the transduced striatum regions (i.e. containing at least one positive cell) per mouse using a 40x objective and ImageJ software

Statistical analysis was performed using the software GraphPadPrism. To test for differences between each group, a non-parametric one-way ANOVA with Tukey post-test was applied. All test were considered significant if $p<0.05$.

Results

A Homology between transcription factors

Since our final goal is to translate this reprogramming strategy to non-human primates (NHPs) and subsequently to humans, we decided to use the human version of the TFs for our constructs. The analysis of the gene and protein sequences homology between the murine/human, macaque/human and macaque/murine sequences of the three TFs: Ascl1, Lmx1A and Nurr1 were performed with the BLASTN and BLASTP tool, respectively. As described in **Table 2** (sequences in Supplementary data 1), due to the high degree of conservation of these TFs, we believe that the three hTFs will be active in mice and macaques.

Table 2. Percentage of homology of Nucleotide and amino acid sequences between mouse-macaque and macaque-human TFs.

	Homology (%)					
	Mouse/Macaque		Macaque/Human		Mouse/Human	
	DNA	Protein	DNA	Protein	DNA	Protein
Ascl1	90	89	95	97	90	90
Lmx1A	91	97	97	100	91	97
Nurr1	94	99	98	99	94	99

B Generation and characterization of AAV vectors carrying the transcription factors Ascl1, Lmx1A and Nurr1 under the control of the transcriptional control of hGFAP promoter

Due to the restricted cloning capacity of the AAV vectors it is not possible to express the three TFs from a single vector, thus we have to divide the TFs in two vectors. The first vector express Ascl1 and Lmx1 and the second one Nurr1 alone, in both cases expression was controlled by a reduced version of the hGFAP which is transcriptionally active mainly in

astrocytes and to a lesser extend in oligodendrocytes but not in neurons (Pignataro et al., 2017). A brief description of each vector is provided bellow (see also **Figure 1**):

1. **pAAV.hGFAP.hAL:** This vector contains the sequence of the hAscl1 and hLmx1A (hAL) using the self cleaving 2A peptide (2A) in between the thwo TFs under the control of the hGFAP promoter (**Figure 1A**).
2. **pAAV.hGFAP.hN1:** This vector contains the sequences of the hNurr1 under the control of the hGFAP promoter (**Figure 1B**).

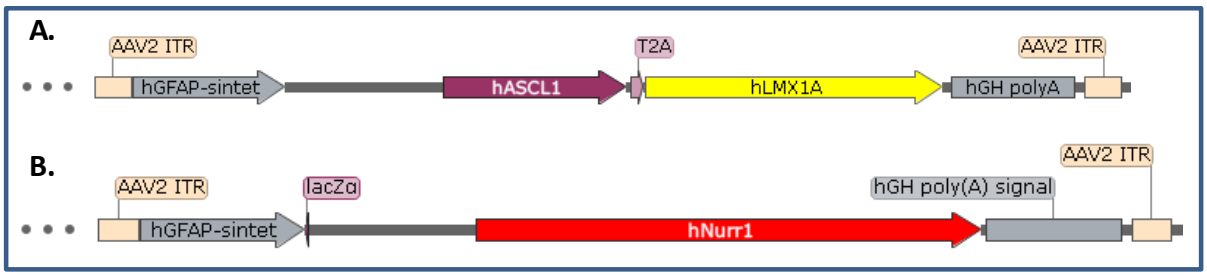

Figure 1. Schematic representation of the genomic structures of the AAV expression cassettes (A) Bicistronic construct carrying the transcription factors hAscl1 and hLmx1A under the control of the hGFAP promoter. (B) Construct carrying only the transcription factor hNurr1 under the control of the hGFAP promoter.

B.1 *In vitro* characterization

The constructs generated were analyzed by sequencing of the full expression cassette and the integrity of the ITRs by restriction assay using *XmaI.The* restriction pattern was in agreement with the one expected (**Figure 2A**).

To check transgene expression HEK-293T cells were transfected with pAAV.hGFAP.hAL and Ascl1 and Lmx1A expression was analyzed by Western Blot (WB). Specific bands with correct molecular weight were detected (**Figure 2B**). To determine the expression of hNurr1 in transfected cells, mRNA expression was analyzed and quantified by Real-Time qPCR using specific primers. HEK-293T cells were transfected with pAAV.hGFAP.hN1 and with a plasmid expressing hNurr1 fused to the mCherry reporter (mCh) under the control of the strong CAG promoter. Higher levels of hNurr expression were detected in cells transfected with pCAG-hNurr-Cherry than in cells tranfected with or pAAV.hGFAP.hN1 and no expression was detected in control cells (**Figure 2C**).

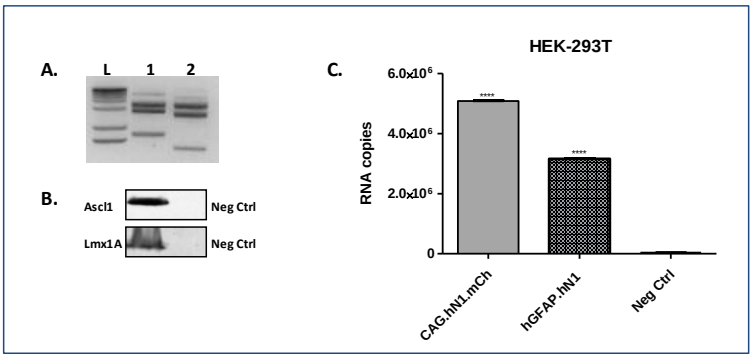

Figure 2. *In vitro* characterization of AAV cassettes. (A) Digestion of maxipreps (200ng) with *XmaI* for one clone of the pAAV.hGFAP.hAL (1) and one clone for the pAAV.hGFAP.hN1 (2) in an agarose gel (1.5%). **(B)** WB analysis for Ascl1 (34KDa) and Lmx1A (43KDa). **(C)** Number of RNA copies for hNurr1 in HEK-293T cells transfected with the plasmid phGFAP.N1.

B.2 *In vivo* analysis

pAAV.hGFAP.hAL and pAAV.hGFAP.hN1 were packaged into AAV capsids by co-transfecting HEK-293T cells with each of these plasmids and the plasmid containing the adenoviral helper genes plus AAV2 rep and AAV8 cap. Cells were collected after 72hpt and recombinant AAVs were purified by ultracentrifugation as described. AAV8.hGFAP.hAL and AAV8.hGFAP.hN1 were produced with a titer of 2.67 x 10^{12} GC/µl and 9.78 x 10^{12} GC/µl respectively. To test its reprogramming capacity *in vivo*, we administered the vectors by stereotaxic injection in the left striatum of 6-8 weeks-old C57BL/6J mice. Mice were divided in seven groups as described in **Table 3**. Control animals, groups 1 and 2, received 4 x 10^9 vp of AAV8.hGFAP.eGFP and 2µl of phosphate-buffered saline (PBS), respectively. These two groups were used to discard any effect derived from vector injection. A third group received 4 x 10^9 vp of the AAV8.hGFAP.hAL, the fourth group received 4 x 10^9 vp of the AAV8.hGFAP.hN1. and groups 5, 6, and 7 received 4 x 10^9 vp of AAV8.hGFAP. hAL + AAV8.hGFAP.hN1 and were sacrificed 3, 8 and 12 weeks post injection (wpi) respectively. No adverse reactions or behavioral changes were observed after the intracranial surgery or during the subsequent period until sacrifice.

Table 3. Summary of groups for evaluation of AAV vectors effect in mice striatum

Group	AAV8 vector(s)	Dose (vp)	Animals (n)	Weeks
1	hGFAP.eGFP	4×10^9	3	3
2	Saline	-	3	3
3	hGFAP.hAL	4×10^9	3	3
4	hGFAP.hN1	4×10^9	3	3
5	hGFAP.hAL + hGFAP.hN1	4×10^9	3	3
6	hGFAP.hAL + hGFAP.hN1	4×10^9	3	8
7	hGFAP.hAL + hGFAP.hN1	4×10^9	3	12

B.2.1 AAV-mediated eGFP overexpression or the injection procedure does not induce the generation of TH immunoreactive (TH-ir) cells in the mice striatum

Immunofluorescence (IF) analysis was performed to detect the eGFP co-localization with astrocytes (GFAP) and neurons (NeuN) in the 3 mice of the group 1 (**Figure 3**). Brain slides were analyzed by confocal microscopy, most of the eGFP-ir cells were GFAP immunoreactive (GFAP-ir) as it is shown in **Figure 3D** (yellow arrows), while none of them co-localized with (NeuN-ir) (**Figure 3H**).

No TH-ir cells were detected in the striatum as it is shown in **Figure 3 (C, G)**. No TH-ir or eGFP-ir cells were detected in the contralateral striatum (**Figure 4**). It is worth noting that red points shown on **Figure 4 (C-G)** and the rest of TH immune labeling are TH-ir fibers that come from the SN.

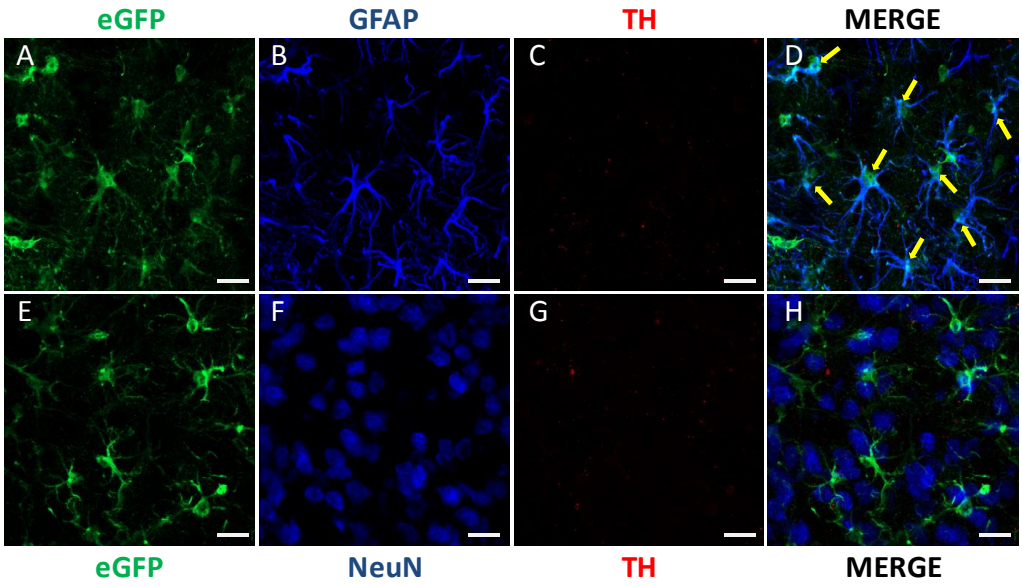

Figure 3. No TH expression was detected when in the striatum of mice receiving AAV8.hGFAP.eGFP. GFAP expression (A-D) or NeuN expression (E-H) did not co-localize with TH (red) after injection with AAV8.hGFAP.eGFP. Co-localization of eGFP (green) and GFAP (blue) is only detected with astrocytes (arrows: yellow = GFP-ir/GFAP-ir). Scale bar 20µm (A-H).

Next the expression of those markers was studied in the striatum of the mice receiving PBS (group 2). Furthermore in these mice we analysed the expression of Ascl1 or Nurr1, by IF. Triple IF, combining antibodies against GFAP, TH and Ascl1 or Nurr1 (**Figure 5**) or combining NeuN, TH and Ascl1 or Nurr1 were performed (**Figure 6**). Ascl1, Nurr1 or TH expression was not detected in the striatum of PBS injected mice (**Figures 5 and 6**).

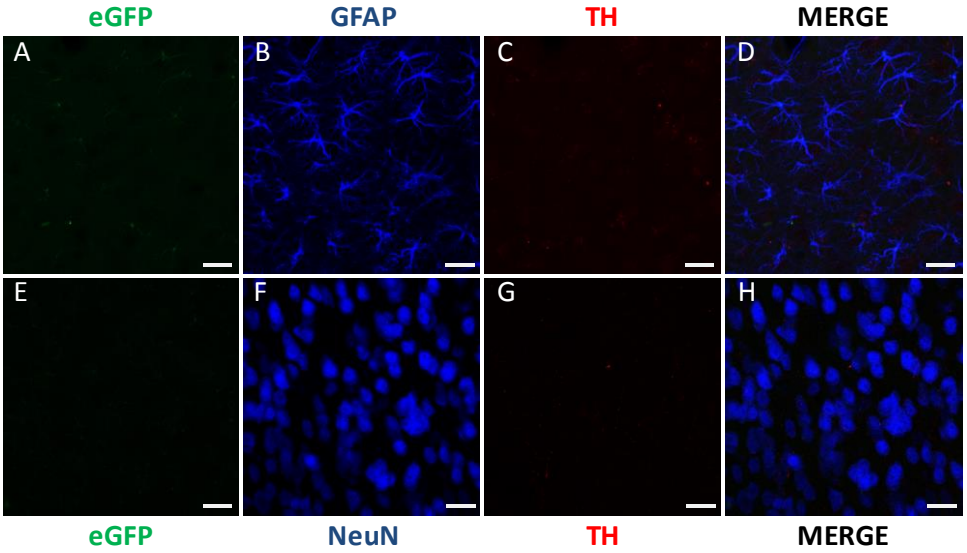

Figure 4. Negative controls for eGFP and TH in the contralateral striatum of mice injected with AAV8.hGFAP.eGFP. Triple IF against eGFP (green), GFAP/NeuN (blue) and TH (red). Images were taken on the contralateral of the injection site. Scale bars 20µm (A-H).

Similar results were obtained in the contralateral striatum. No Ascl1, Nurr1 or TH ir-cells were detected (Supplementary data 2A, B). Thus, with these results we conclude that no TH-ir cells or endogenous Ascl1 or Nurr1 are detectable in the mice striatum of control mice. The presence of Lmx1A-ir cells was also analyzed and it was not detectable in the mice striatum (Supplementary data 3).

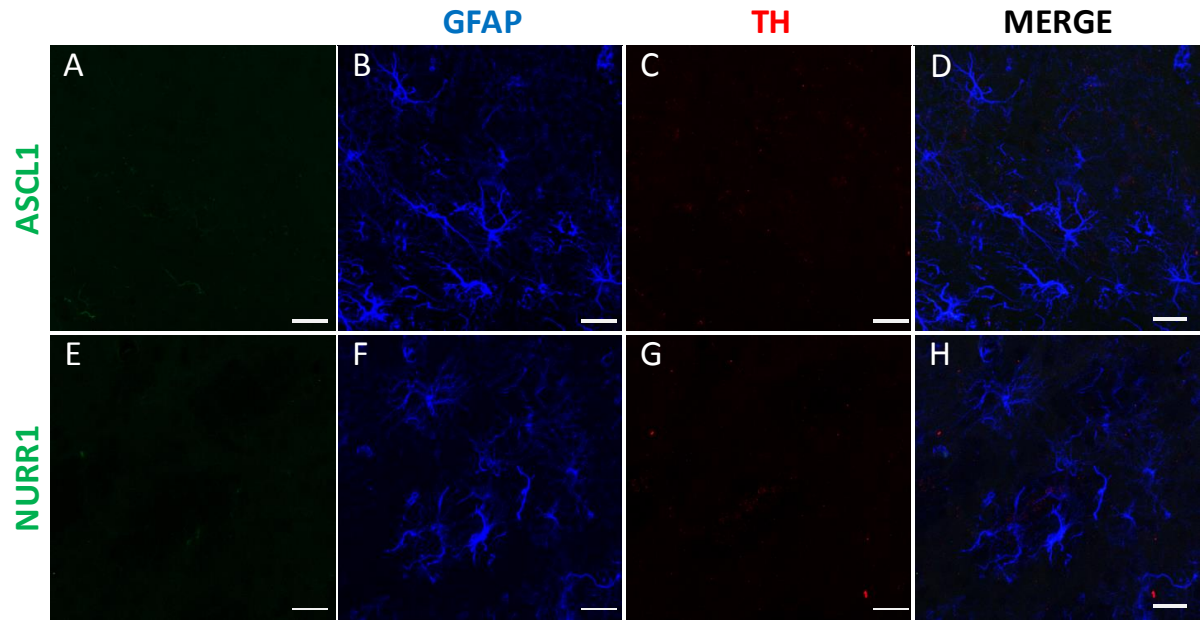

Figure 5. Analysis of Ascl1 and Nurr1 expression in mice injected with saline. Triple IF against Ascl1 (green), Nurr1 (green), GFAP (blue) and TH (red). Images were taken along the injection site. Scale bars 20µm (A-H).

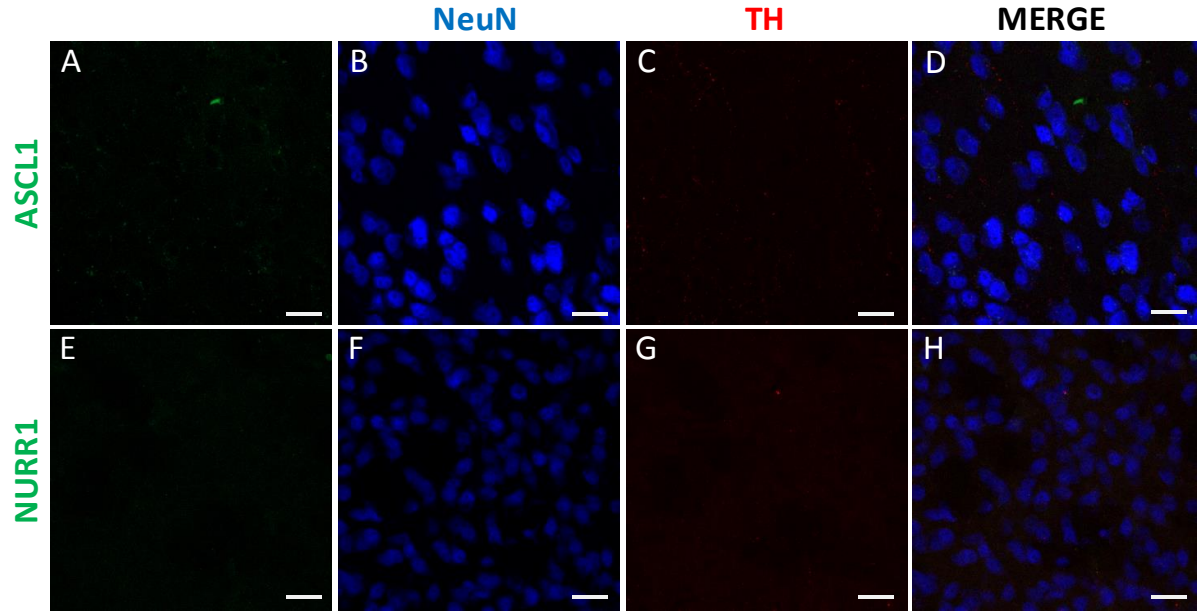

Figure 6. Negative controls for Ascl1 and Nurr1 in mice injected only with saline. Triple IF against Ascl1 (green), Nurr1 (green), NeuN (blue) and TH (red). Images were taken along the injection site. Scale bars 20µm (A-H).

B.2.2 Detection of endogenous populations of Ascl1, Nurr1 and TH

In order to exclude that the negative results obtained before were due to the lack of reactivity of the antibodies against mouse antigens, Ascl1, Nurr1 and TH were analyzed in other brain areas where those molecules are normally expressed. Endogenous populations of Ascl1 are present on the Subventricular Zone (SVZ) (**Figure 7**). As it is shown in the **Figure 7D and 7H** Ascl1-ir cells in the SVZ do not co-localize with NeuN or TH. Since, Ascl1 is a neurogenesis marker, the lack of co-localization of NeuN or TH was expected. Endogenous populations of Nurr1 were detected in the SN (**Figure 8**). As it is shown in the **Figure 8D and 8H**, Nurr1 co-localized with TH.

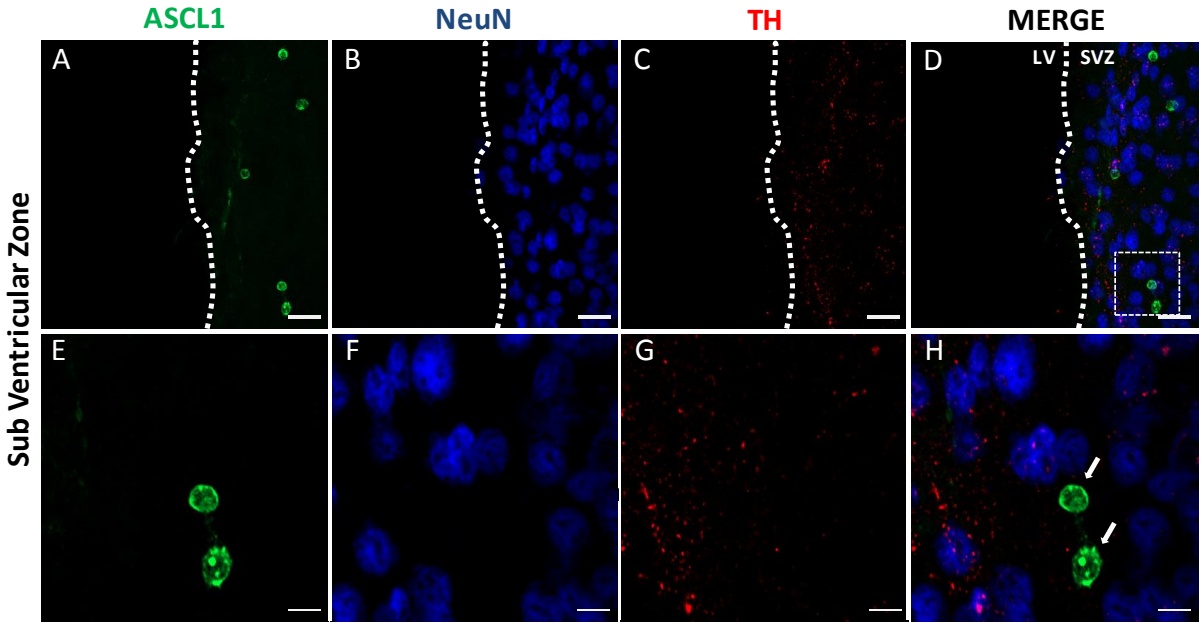

Figure 7. Analysis of Ascl1 expression in the SVZ. Endogenous populations of Ascl1-ir cells were detected (green) in the SVZ. Scale bar low magnification 20µm (A-D) and high magnification 5µm (E-H). White arrows: Ascl1 not co-localizing with NeuN or Th. LV: Lateral ventricle.

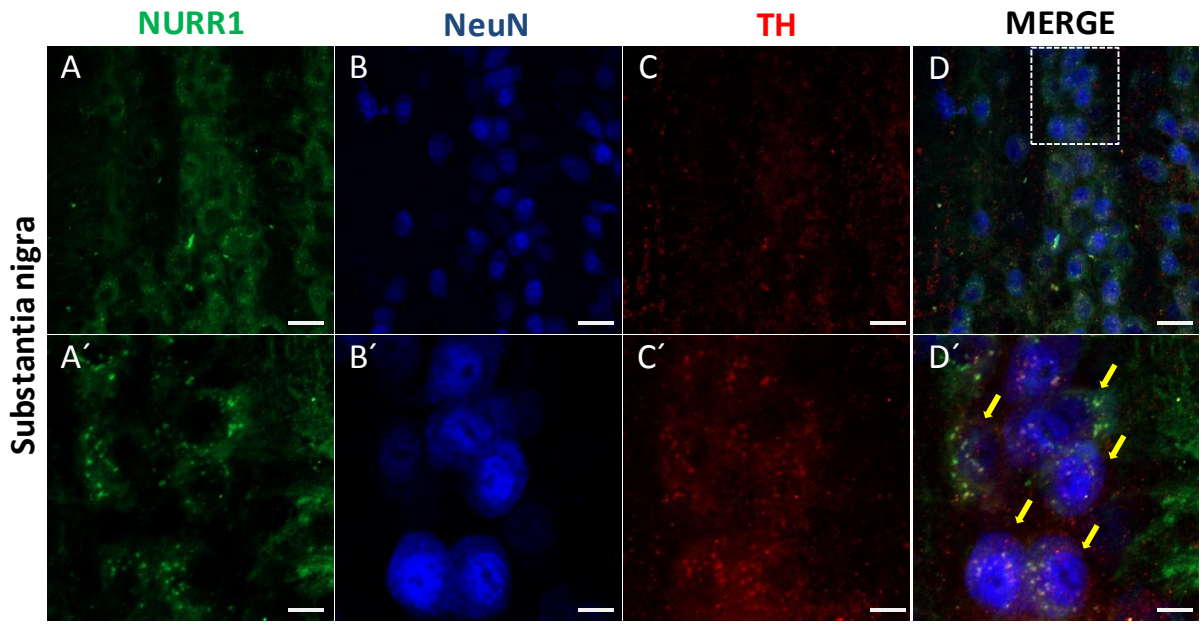

Figure 8. Analysis of Nurr1 expression in the SN. Endogenous populations of Nurr1-ir cells were detected (green) in the SN. Yellow arrows: co-localization of Mash1/NeuN/TH. Scale bar low magnification 20µm (A-D) and high magnification 5µm (E-H).

Numerous TH-ir cells co-localizing with the neuronal marker NeuN were observed in the SN and the VTA (**Figure 9C; 10H**) where DA neurons are present.

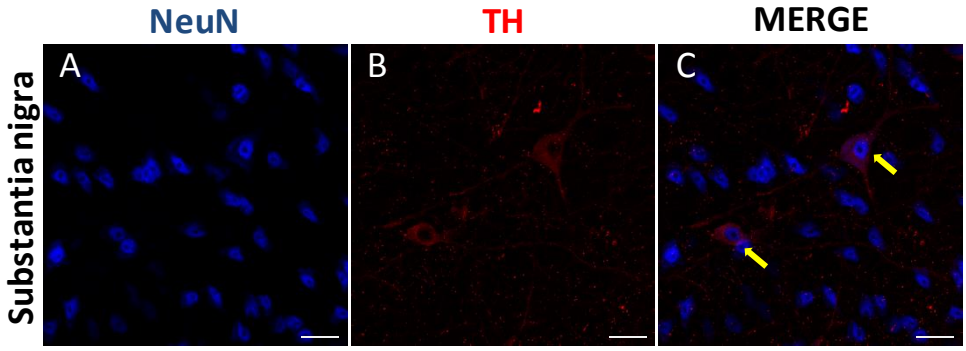

Figure 9. Positive control for TH. Endogenous populations of TH-ir cells were detected (red) in the SN. Yellow arrows: co-localization of NeuN/TH. Scale bar 20µm.

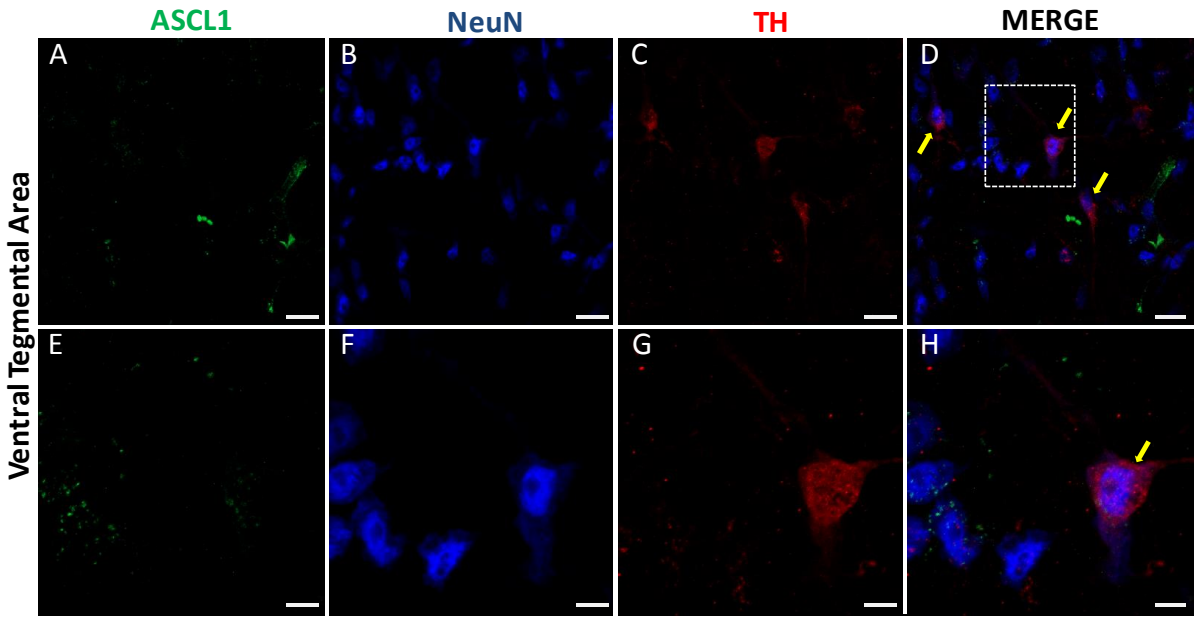

Figure 10. Analysis of TH expression in the VTA. Triple IF against Ascl1, NeuN and TH in the VTA of the mice brain. Ascl1 IF was to confirm the absence of endogenous Ascl1-ir cells in the VTA. Arrow = TH-ir / NeuN-ir. Scale bar low magnification 20µm (A-D) and high magnification 5µm (E-H).

B.2.3 No TH-ir cells were detected in animals that received the AAV8.hGFAL.hAL vector 3 wpi

The animals injected with AAV8.hGFAP.hAL (group 3) were analyzed three wpi. The following analysis were performed: 1) determination of the expression of the TFs in mice striatum, 2) determination of the co-localization of the TFs with the astroglial marker GFAP, 3) determination of the co-localization of the TFs with the pan-neuronal marker NeuN, 4) analysis of the expression of the dopaminergic neurons marker TH.

Following the methodology described above, triple IFs were performed in order to determine the co-localization of Ascl1-ir cells with either GFAP (**Figure 11B**) or NeuN (**Figure 11F**). 4.9% of GFAP-ir cells expressed Ascl1, however, the majority of Ascl1 expressing cells (82%) were GFAP negative, those cells were also NeuN (**Figure 11D, H**). More experiments must be performed to determine the nature of those cells. No TH-ir cells were detected when we analyzed the group carrying only the AL TFs (group 3) three wpi (**Figure 11C, G**).

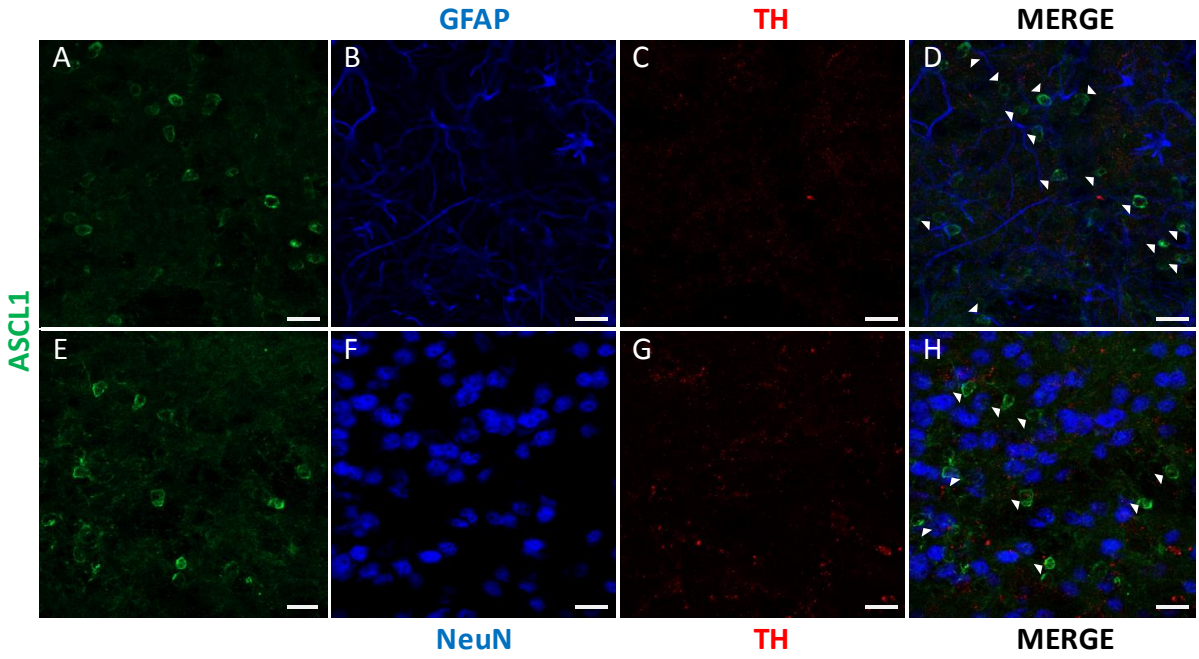

Figure 11. Analysis of Ascl1 expressin in the striatum of mice receiving AAV8.hGFAP.hAL. Triple IF for Ascl1 (green), GFAP (blue) and TH (red). Scale bars: 20µm.

B.2.4 TH-ir cells are detected 3 wpi in the striatum of animals receiving AAV.hGFAP.hNurr1

Animals receiving AAV8.hGFAP.hNurr1 were sacrificed 3 wpi and Nurr1 expression was detected in mice striatum. Interestingly, 5,8% of the cells expressing Nurr1 were TH-ir (**Figure 12C)** however no co-localization with GFAP or NeuN was detected (**Figure 12D´-13D´**). The morphology of TH/Nurr1-ir cells is similar to the one of an immature neuron. The expression of additional markers needs to be analyzed to determine if they express neuronal precursors markers.

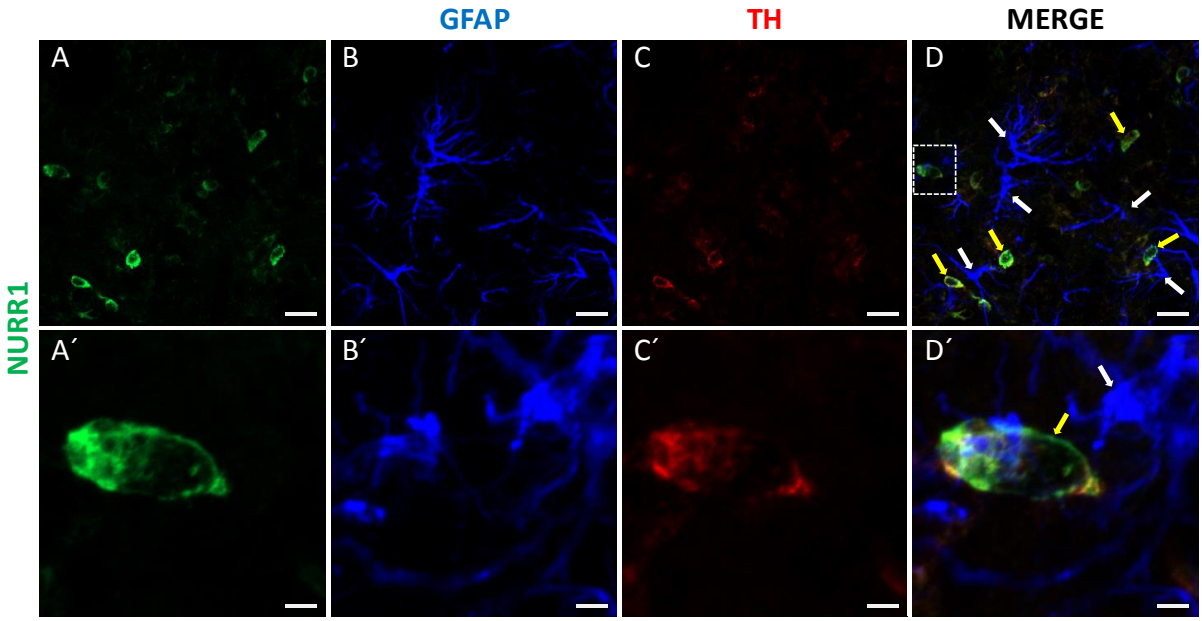

Figure 12. Expression of TH was co-detected with Nurr1 but not with GFAP in group 4. Immunoreactive cells for Nurr1 (green) and TH (red) did not co-localize with GFAP (blue). Arrows: yellow = Nurr1-ir /TH-ir, white = GFAP-ir. Scale bars: 20µm (A-D) and 5µm (A´-D´).

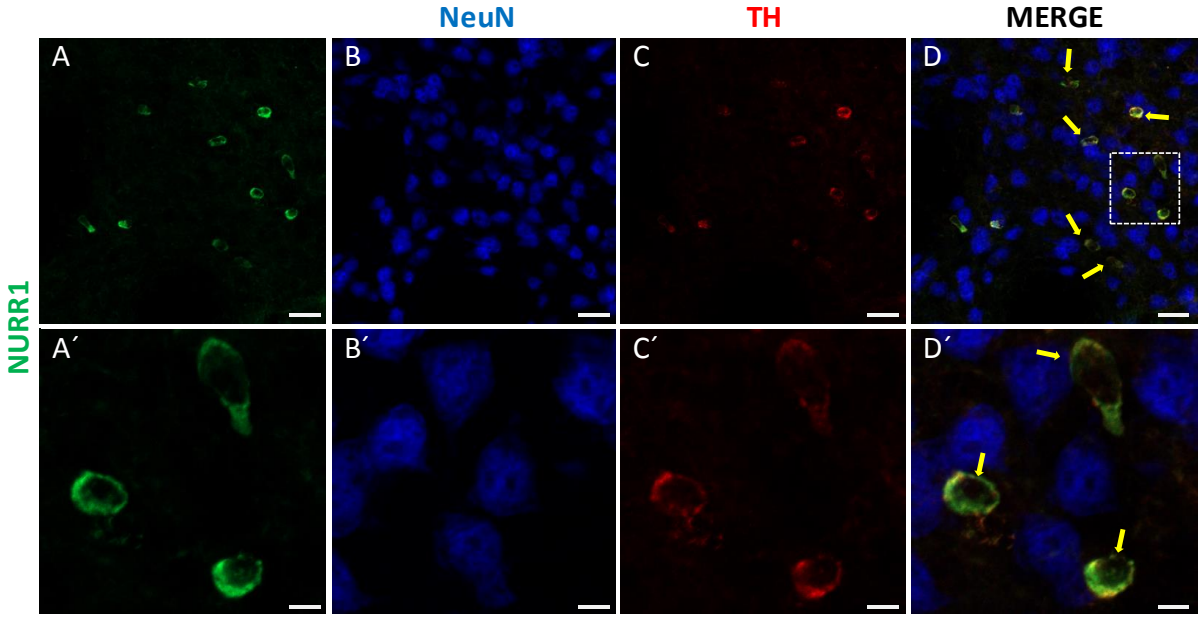

Figure 13. Expression of TH was co-detected with Nurr1 but not with NeuN in group 4. Immunoreactive cells for Nurr1 (green) and TH (red) did not co-localize with NeuN (blue). Arrows: yellow = Nurr1-ir /TH-ir. Scale bars: 20µm (A-D) and 5µm (A´-D´).

B.2.5 Co-localization of Ascl1, Lmx1A and Nurr1 with GFAP when the two vectors are injected at 3 wpi

The animals injected with AAV8.hGFAP.hAL and AAV8.hGFAP.N1 (groups 5, 6 and 7) were sacrificed three, eight and twelve wpi. To determine the co-localization of the TFs with GFAP or NeuN we performed different triple IFs combining Ascl1, Lmx1A or Nurr1 with NeuN or GFAP markers. The triple IF combining Ascl1, NeuN and GFAP showed Ascl1 expression in GFAP-ir cells (**Figure 14A-D**) and the absence of Ascl1 expression in the NeuN-ir population of the striatum (**Figure 14A-D**). A higher magnification of this area is shown in **Figure 14A´-D´**. Ascl1 was detected in the nucleus of GFAP-ir cells.

The analysis of the tissue labeled with the triple IF for Lmx1A, NeuN and GFAP, showed a similar expression pattern. Lmx1A-ir cells co-localized with GFAP and not with NeuN (**Figure 14E-H**). A high magnification of those cells showed also a nuclear localization of Lmx1A in GFAP-ir cells (**Figure 14E'- H'**). Immunoreaction for Lmx1A with NeuN was not possible due to the incopatibilty of antibodies (**Figure 14F- F'**).

The analysis of Nurr1 expression was also performed. As described for Ascl1 and Lmx1A, few Nurr1-ir cells co-localized with GFAP but no co-localization with NeuN was detected (**Figure 14I-L**). Similarly to Ascl1 (**Figure 14A´- D´**) and Lmx1A (**Figure 14E´- H´**), Nurr1 expression showed also a nuclear localization in GFAP-ir cells (**Figure 14I´- L´**).

The contralateral striatum did not show immunoreactivity for any of the three TFs, Ascl1, Lmx1A or Nurr1 (as described for the groups 1, 3 and 4).

TH-expression analysis revealed the presence of TH-ir cells in the striatum of mice injected with both vectors. TH-ir cells express Ascl1 (**Figure 15D**), Lmx1A (**Figure 15H**) and Nurr1 (**Figure 15L**). However, no co-localization with NeuN was detected for Ascl1 (**Figure 15D**) or Nurr1 (**Figure 15L**). It is important to highlight that TH-ir cells presented a triangular (**Figure 15C**) or rounded shape (**Figure 15G, K**) which is not the common morphology of the endogenous TH-ir mature neurons from the SN in the adult brain.

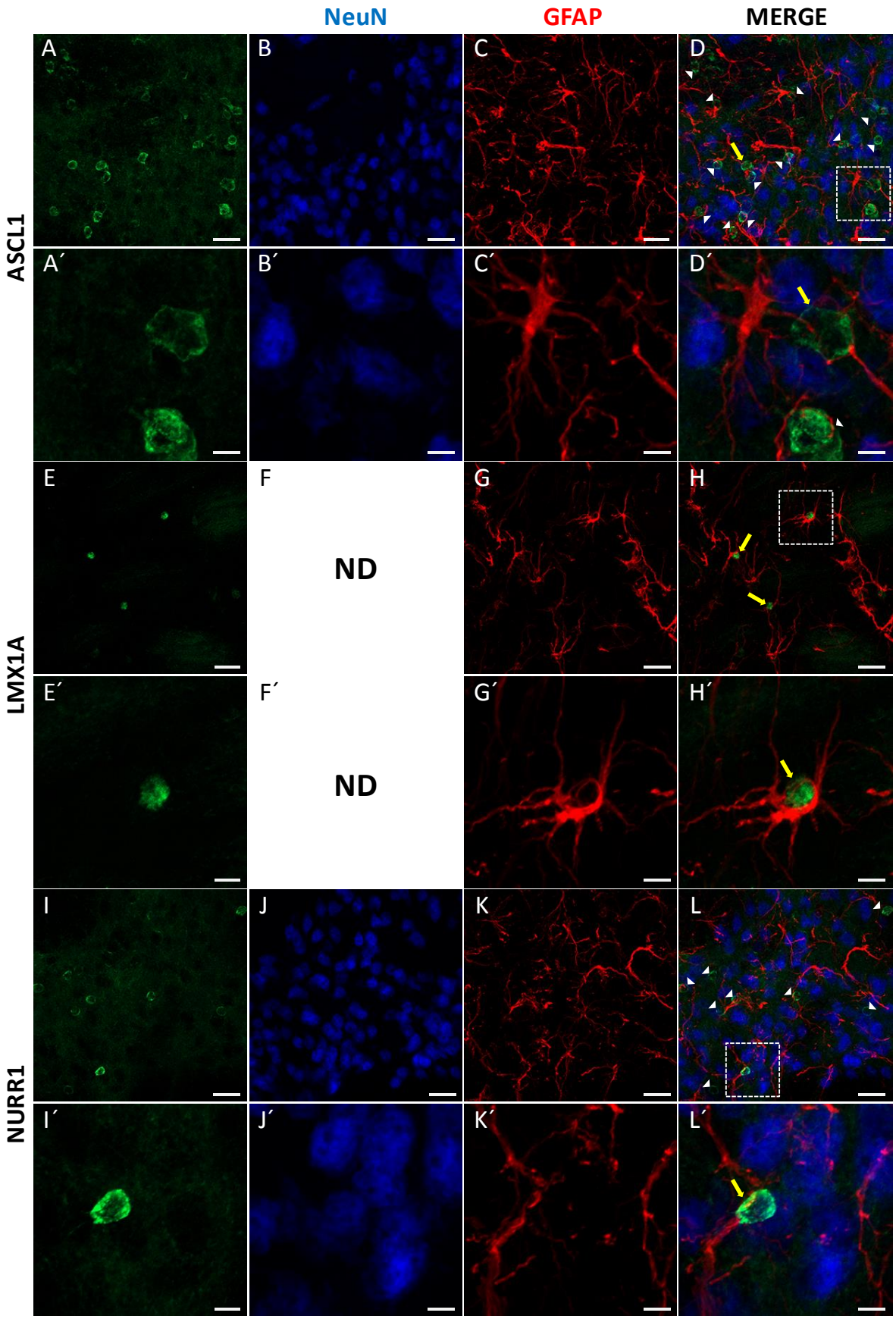

Figure 14. Analysis of TFs expression together with NeuN or GFAP. Arrows: Yellow = ASCL1-ir/GFAP-ir; LMX1A-ir/GFAP-ir; NURR1-ir/GFAP-ir, white = ASCL1-ir, LMX1A-ir or NURR1-ir. Scale bar low magnification 20µm (A-L) and high magnification 5µm (A´-L´). ND: Not determined.

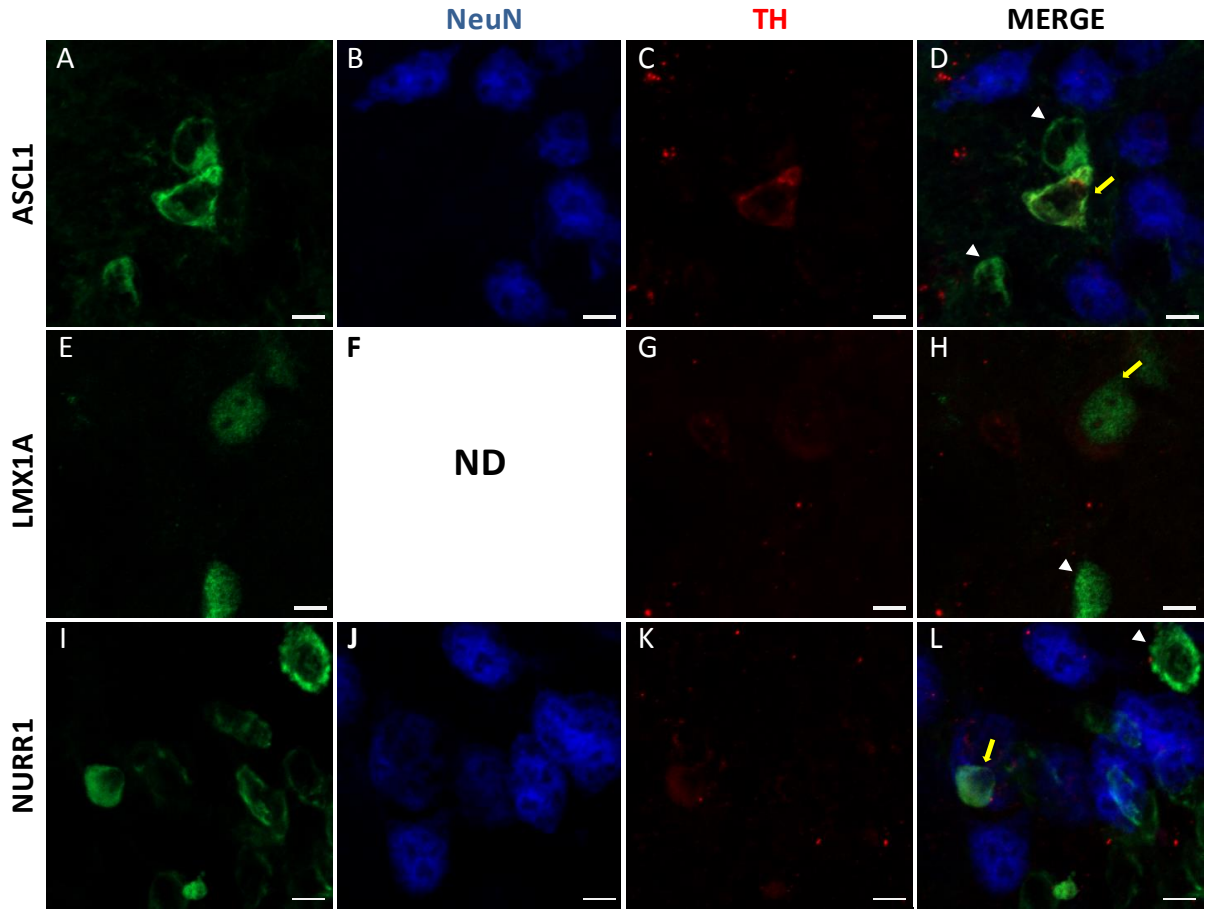

Figure 15. TH positive cells co-expressing the TFs in mice striatum. Mice injected with both AAV vectors were sacrificed and brain sections were labeled with anti-Ascl1 (green; upper panels); anti-Lmx1A (green; middle panels); or anti-Nurr1 (green; lower panels). TH immunoreactive cells (D, H, L) and Ascl1, Lmx1A or Nurr1, showed co-expression (arrows: yellow = TF-ir/TH-ir, white = TF$^-$ir cell) while NeuN cells did not co-localize with any TF or TH. Immunoreaction for Lmx1A with NeuN was not possible due to the incopatibilty of antibodies (F). Scale bars: 5µm. ND: Not determined.

B.2.6 Transduced cells show a neuronal phenotype after 8 and 12 wpi

To determine if the reprogramming efficiency could be improved by giving the transduced cells more time, mice were sacrificed 8 and 12 wpi of both vectors.Coronal brain sections were stained for double or triple IFs to detect co-expression of the TFs with TH or NeuN/GFAP. Eight wpi Ascl1 or Nurr1 expression was detected in NeuN-ir cells while no co-localization with GFAP expression was detected. Remarkably, from 8 wpi cells co-expressing either Ascl1 with NeuN (**Figure 16E-H**) or Nurr1 with NeuN (**Figure 17E-H**) showed neuronal morphology. At 12 wpi, similar results were obtained, but the neuronal morphology detected was more complex and defined in cells co-expressing either Ascl1 with NeuN (**Figure 16I-L**) or Nurr1 with NeuN (**Figure 17I-L**). In order to better understand the different expression profiles observed after the injection of the two AAV vectors along the three time

points, quantification of the number of cells co-expressing either Ascl1 with GFAP or NeuN was performed (**Table 18A, B**). Quantification of the number of cells co-expressing Nurr1 with GFAP or NeuN was also done (**Figure 18C, D**). Ir-cells co-expressing Ascl1 with GFAP were only observed at 3 wpi (8.9%) (Ascl1-ir + GFAP-ir/Total GFAP-ir) (**Figure 18A**). Ir-cells co-expressing Nurr1 with GFAP were principally observed at 3 wpi (7.3%) (Nurr1-ir + GFAP-ir/Total GFAP-ir) (**Figure 18C**). No Ascl1 or Nurr1 cells GFAP-ir were detected 8 or 12 wpi when the expression of both TFs was detected in NeuN-ir cells. The number of cells co-expressing the TFs Ascl1 and NeuN increased from 1.23% at 8 wpi to 2.73% at 12 wpi (Ascl1-ir + NeuN-ir/Total NeuN-ir) (**Figure 18B**). A similar pattern was observed for cells co-expressing Nurr1 and NeuN (1% to 4.22%)(Nurr1-ir + NeuN-ir/Total NeuN-ir) (**Figure 18D**).

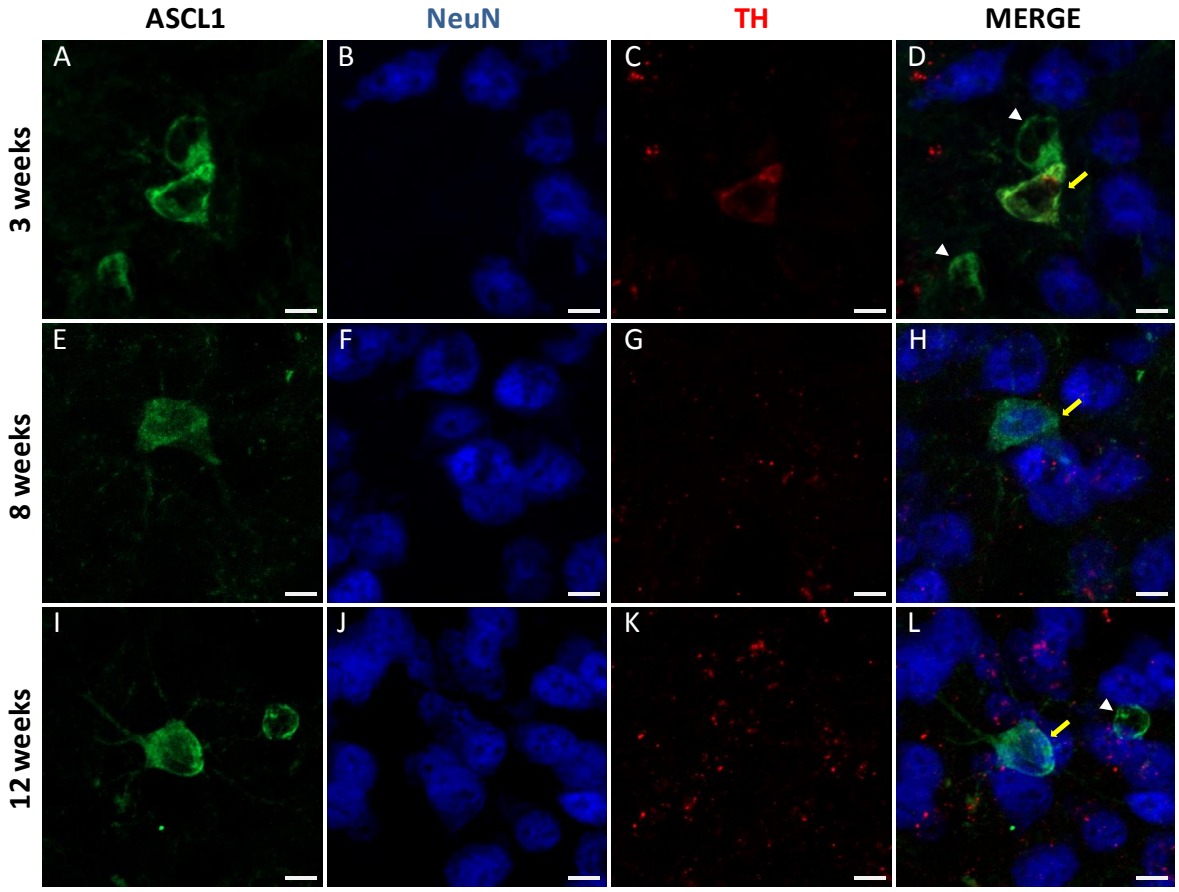

Figure 16. Changes in cells expressing Ascl1 along the 3 time points. Three wpi (upper panels) Ascl1-ir/TH-ir cells did not co-localize with NeuN. Eight wpi (middle panels) Mash1-ir/TH-ir cells start to co-localize with NeuN and present a more neuronal morphology, although no TH^+ cells were detected. Twelve wpi (lower panels) more Ascl1-ir/TH-ir cells were observed with a neuronal phenotype but again, no TH-ir cells were detected. Arrows: white = Ascl1-ir/TH-ir; yellow = Ascl1-ir/NeuN-ir. Scale bars: 5µm (A-L)

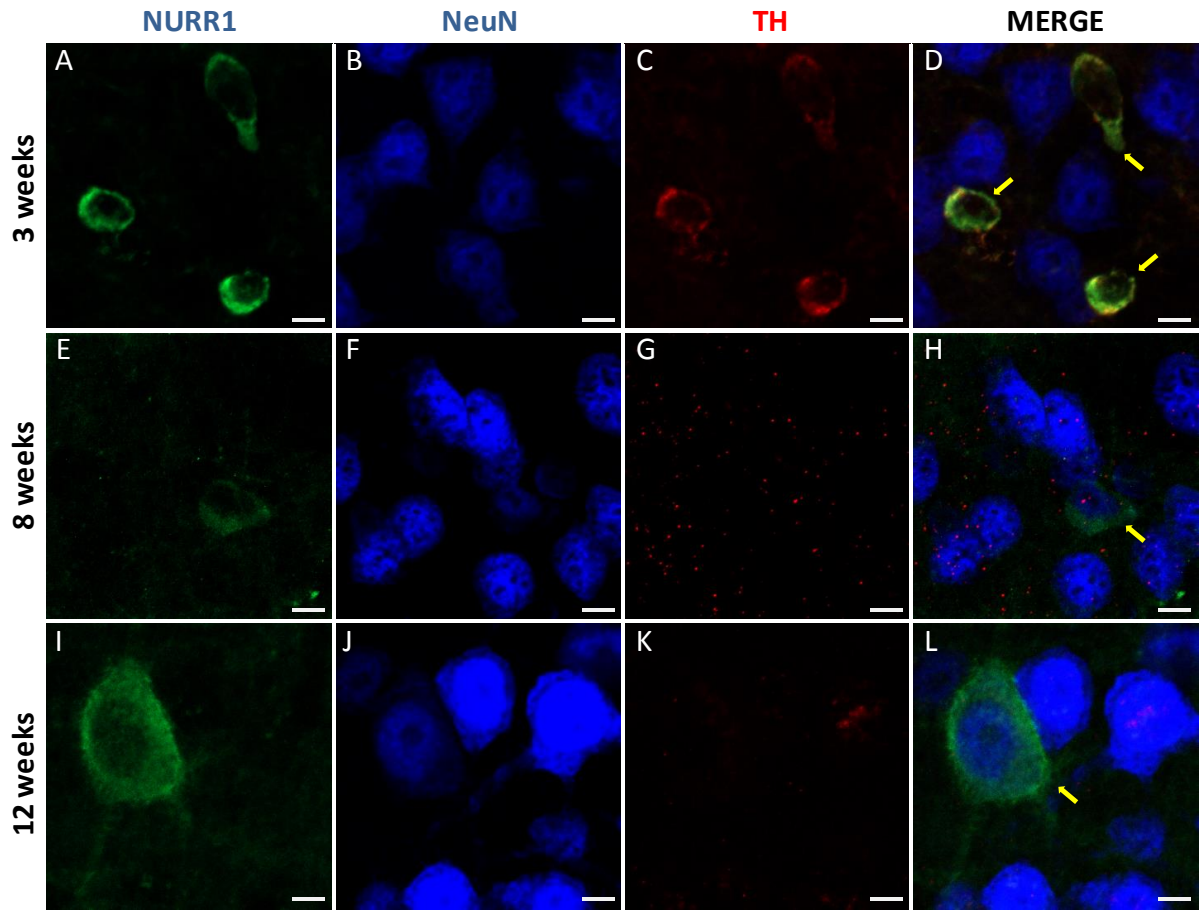

Figure 17. Changes in cells expressing Nurr1 along the 3 time points. Three wpi (upper pannels) Nurr1-ir/Th-ir cells did not co-localize with NeuN. Eight wpi (middle pannels) Nurr1-ir/TH-ir cells start to co-localize with NeuN and present a more neuronal morphology, although no TH-ir cells were detected. Twelve wpi (lowe pannels) more Nurr1-ir/TH-ir cells were observed with a neuronal phenotype but again, no TH-ir cells were detected. Arrows: white=Nurr1-ir/TH-ir; yellow=Nurr1-ir/NeuN-ir. Scale bars: 5 µm (A-H).10 µm (I-L).

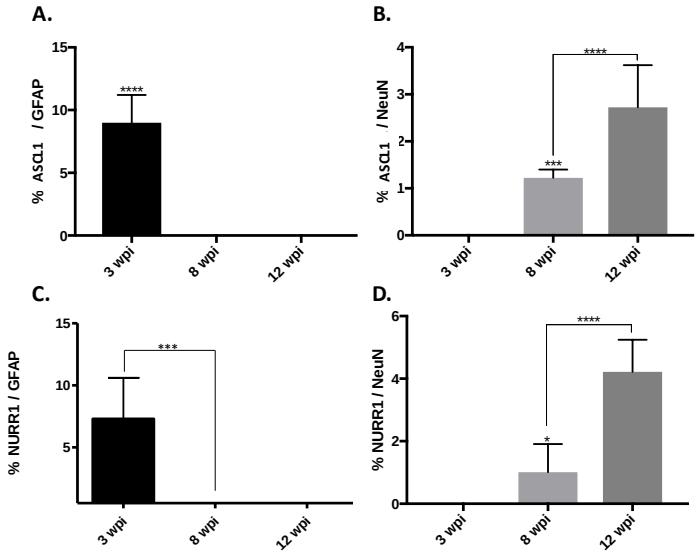

Figure 18. Quantification of Ascl1 or Nurr1 in GFAP-ir cells or NeuN-ir cells after different time points after vector injection. Differences in the percentage of Ascl1-ir or Nurr1-ir cells were statistically evaluated by One-way ANOVA. Results were considered $p<0.05$ and levels of significance was set to ***$p<0.001$.

Discussion

Parkinson disease is characterized by a dramatic lost of dopaminergic neurons (TH-ir) from the substantia nigra pars compacta (SNpc) resulting in low levels of dopamine reaching the striatum. For this reason current treatment for PD patients are based on the administration of dopamine. The objective of the present work was the development of dopamine producing cells in the striatum as a source of dopamine to treat PD.

For this purpose an *in vivo* reprogramming strategy was used. Expression of TFs associated with the development of miDA neurons in non-neuronal cells was used to transform those cells into dopamine producing cells. The genes encoding the TFs Ascl1, Lmx1A and Nurr1 were delivered into the mice striatum.

In order to achieve a successful delivery and expression of the TFs, selection of both the delivery vehicle and the optimal expression cassette is essential, especially if more than one gene need to be expressed. As delivery vehicle we choose the adeno-associated viral vector (AAV) because they are widely described in the literature as an effective tool to transduce the CNS (Liu et al., 2015; Guo et al., 2014; Torper et al., 2015; Niu et al., 2013; Lentz et al., 2012). Specifically, the AAV serotype 8 was chosen because we formerly demonstrated that using this vector we can deliver genes to different cells in the mice striatum including astrocytes (Pignataro et al., 2017).

We took advantage of the fact that astrocytes are the most abundant cell type in the vertebrate CNS (Sofroniew et al., 2010). Our strategy was based on the expression of the TFs specifically on astrocytes using a reduced version of the human astrocyte specific promoter hGFAP (Lee et al., 2008; Liu et al., 2015; Niu et al., 2013; Pignataro et al., 2017).

Since one of the main limitations of AAV-vectors lies in its reduced cloning capacity (Kyostio-Morre et al., 2016; Lai et al., 2010), the use of a small GFAP promoter allowed us to generate AAV cassettes containing larger inserts. Two vectors were constructed due to the impossibility of expressing the three TFs from a single AAV vector, AAV8.hGFAP.hAL expressing Ascl1 and Lmx1A and AAV8.hGFAP.hNurr1 expressing Nurr1.

In the present study using AAV vector, we found expression of Ascl1 and Lmx1A in GFAP positive and negative cells after injecting the AAV8.hGFAP.hAL alone but no co-localization with NeuN was observed. More analysis need to be performed to clarify which are the cells expressing the TF that are GFAP negative. It remains unclear if such Ascl1-ir cells were a subtype of neurons similar to the one described by Liu et al., (2015) that were obtained from astrocytes after Ascl1 expression. Ascl1 has been shown to reprogram astrocytes to non-dopaminergic neurons in the absence of additional factors (Chanda et al., 2014). Although the morphology of those cells observed do not correspond with the one of a mature neuron in the striatum. The size of the cells was smaller than a dopaminergic or GABAergic neuron. More over, no cytoplasm extension was observed. Longer evaluation times must be pursue to determine more consistently its effect when Ascl1 and Lmx1A are expressed in striatal astrocytes. Not a single TH-ir cell was detected in the striatum of mice receiving AAV8.hGFAP.hAL however in mice injected with AAV.hGFAP.Nurr1 alone, a significant number of TH-ir cells were detected. Interestingly those TH-ir cells express Nurr1 but no NeuN or GFAP. This result suggests that Nurr1 expression by itself induce the development of dopaminergic neuronal progenitors and are able to express TH, and is in concordance with previous studies (Kim et al., 2003; Rodríguez-Traver et al., 2015). Morever, the morphology observed for these TH-ir cells were rounded and simple with few cytoplasm extensions. It is important to indicate that TH-ir cells are not detected in mice injected with a control vector or saline or in the contralateral un-injected site.

When the two vectors were injected together, AAV8.hGFAP.hAL and AAV8.hGFAP.Nurr1, the situation is slightly different that the one observed after the injection of each vector separately. Nurr1 expression is detected in few GFAP-ir cells and TH-ir cells expressing Ascl1 or Nurr1 were detected 3 wpi. TH-ir cells detected in mice injected with both vectors did not co-localize with either NeuN or GFAP, as previously observed after the expression of Nurr1 alone. More over, TH-expressing cells did not show a classical neuronal-like morphology, they showed a triangular/rounded shape. Although, further studies must be performed in order to determine if the TH-ir cells were co-transduced by both vectors. Also, attempts to detect “uncleaved” forms of the polyprotein AL must be performed and if they could have a direct effect on cell survival. Cell death could be triggered by the presence of the polyprotein as describe in the work of Theodorou et al., (2015).

Previous studies have described the generation of neurons expressing features of miDA neurons *in vitro* using Nurr1 alone or in combination with other TFs such as Ngn2, Ascl1, Foxa2, Pitx3 (Kim et al., 2002; Park et al., 2006; Oh et al., 2014; Martinat et al., 2006; Caiazzo et al., 2011; Carrey et al., 2009; Addis et al., 2011). In neurosphere cultures transduced with a retrovirus it has been shown that expression of Nurr1 alone was sufficient to generate TH expressing cells. These cells present an immature morphology and do not express any additional marker of DA neurons. It was necessary the co-expression of more TFs such as Ngn2 to generate morphologically mature TH-expressing neurons that also express additional mesencephalic markers (Andersson et al., 2007). Furthermore it has been described that Nurr1 expression in combination with Ascl1 in neuronal progenitor cells from rats (Park et al., 2006) or with Ascl1 and Lmx1a (Caiazzo et al., 2011) induces the development of mature reprogrammed cells expressing miDA neuronal markers *in vitro*. The proof of principle that parenchymal astrocytes can be converted into neurons *in vivo* was obtained by Torper et al., (2013) and from oligodendrocyte precursor cells (NG2 glia cells) in 2015. Similar to our findings, the obtention of miDA neurons was not possible and the reprogramming efficiency into a different neuronal subtype was low. (Torper et al., 2015).

In the literature, it has been described four small populations of TH-ir interneurons in the mice striatum. They are likely to play a potential role in compensation for dopamine loss in PD (Ibañez-Sandoval et al., 2010; Xenias et al., 2015). These TH interneurons exhibit a small, transient but significant increase in number after unilateral destruction of the nigrostriatal dopaminergic pathway. Although none of them were found to project to striatal output structures. Such striatal interneurons manifest great electrophysiological, neurochemical and morphological diversity and expressing pan-neuronal markers like NeuN (Ünal et al., 2015). In contrats, the TH-ir cells detected by us at 3 wpi did not co-localize with NeuN suggesting an intermediate phenotype between an adult neuron or a neuronal progenitor cell. More over, these TH-ir cells co-localized with the TFs Ascl1 or Nurr1. Thus TH/Ascl1 or TH/Nurr1-ir cells should not be considered as false positive but more experiments should be performed in order to determine their final cell fate.

The direct reprogramming of resident astrocytes into neuronal progrenitors has been described previously with a single TF (Niu et al., 2015). They describe that SOX2-driven *in*

vivo reprogramming of adult astrocytes passes through a sequence of distinct cell states, which mimics aspects of endogenous neurogenesis. To determine if the TH-positive cells observed at 3 wpi of both vectors might correspond to cells in an intermediate cell state of neurogenesis, mice striatum was analyzed at longer time points post vector injection (8 and 12 wpi). Eight and 12 wpi of both vectors no TH-ir cells were further detected in the mouse striatum. However, Ascl1 or Nurr1 expression was detected in NeuN-ir cells. More interestingly, Ascl1/NeuN-ir or Nurr1/NeuN-ir cells showed a neuronal-like morphology at 8 wpi. Later on (12 wpi), this tendency was maintained and the numbers of Ascl1/NeuN and Nurr1/NeuN-ir cells increased and they showed a more typical neuronal-like morphology. Importantly, we are fully aware that the results we are obtaining in these groups (8 and 12 wpi) might be just the result of overexpressing Nurr1, and additional experiments are currently being performed to clarify this open question.

At this point we do not have an explanation for the transient presence of TH-ir cells detected 3 weeks after Nurr1 expression. It is possible that TH-ir cells are disappearing or they are transformed to the NeuN-ir cells detected at later time points. Moreover, the cellular and molecular mechanisms sustaining their viability should be addressed. In comparison with the previus i*n vitro* studies that demonstrate the capacity of ALN to convert fibroblast into DA neurons, we must considerate the tremendous differences between *in vitro* and *in vivo* reprogramming. Conditions like cell density, nutritional environment, oxygen levels and many other factors can be manipulated *in vitro*. *In vivo* the environment is complex and there is considerable tissue-dependent gene expression and the efficiency is influenced by several factors that cannot be controlled or even predicted (Ming et al., 2011; Theodorou et al., 2015; Grande et al., 2013). DA neurons interact with each other, as wells as with several cell types in their original environment in the brain. These features could be apparently necessary for their full functionality. In consequence, several extracellular factors could be mediating its transient appearance.

Taken together, our data provide evidence that neuronal conversion can take place *in vivo* by gene delivery of specific TFs. The ability to convert astrocytes into neurons *in vivo* point towards the feasibility of using direct conversion of endogenous cells in the brain for different strategies. More experiments will be needed to determine the reasons involving

the transient appearance of TH-ir cells. Moreover, we must determine if the *in vivo* conversion of astrocytes into neurons is caused only by the presence of Nurr1 or by the three TFs and if those cells are functional and might have disease modifying effects. Finally, it is clear that a deeper understanding of the extrinsic and intrinsic cues instructing the induction of DA neurons will be critical for the refinement of this reprogramming approach before start considering the implementation of similar experiments both in the NHP model of PD as well as in early stages of clinical trials engaging PD patients.

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Supplementary data 1

Mus musculus achaete-scute complex homolog 1 (Drosophila) (ASCL1), mRNA.

MESSGKMESGAGQQPQPPQPFLPPAACFFATAAAAAAAAAAAAQSAQQQQPQAPPQQAPQLSPVA
DSQPSGGGHKSAAKQVKRQRSSSPELMRCKRRLNFSGFGYSLPQQQPAAVARRNERERNRVKLVNLGF
ATLREHVPNGAANKKMSKVETLRSAVEYIRALQQLLDEHDAVSAAFQAGVLSPTISPNYSNDLNSMAGSP
VSSYSSDEGSYDPLSPEEQELLDFTNWF

PREDICTED: achaete-scute homolog 1 (ASCL1) [Macaca fascicularis]

MESSAKMESGGAGQQPQPQPQPQPQQPFLPPAACFFATAAAAAAXAAAAAAQSAQQQQQQQQQQQ
APQLRPAADGQPSGGGHKSAPKQVKRQRSSSPELMRCKRRLNFSGFGYSLPQQQPAAVARRNERERNR
VKLVNLGFATLREHVPNGAANKKMSKVETLRSAVEYIRALQQLLDEHDAVSAAFQAGVLSPTISPNYSND
LNSMAGSPVSSYSSDEGSYDPLSPEEQELLDFTNWF

>gi|55743094|ref|NP_004307.2| achaete-scute homolog 1 (ASCL1) [Homo sapiens]

MESSAKMESGGAGQQPQPQPQQPFLPPAACFFATAAAAAAAAAAAAAQSAQQQQQQQQQQQQQAP
QLRPAADGQPSGGGHKSAPKQVKRQRSSSPELMRCKRRLNFSGFGYSLPQQQPAAVARRNERERNRVK
LVNLGFATLREHVPNGAANKKMSKVETLRSAVEYIRALQQLLDEHDAVSAAFQAGVLSPTISPNYSNDLN
SMAGSPVSSYSSDEGSYDPLSPEEQELLDFTNWF

PREDICTED: Mus musculus LIM homeobox transcription factor 1 alpha (LMX1A), transcript variant X2, mRNA.

MLDGLKMEENFQSAIETSASFSSLLGRAVSPKSVCEGCQRVISDRFLLRLNDSFWHEQCVQCASCKEPLET
TCFYRDKKLYCKYHYEKLFAVKCGGCFEAIAPNEFVMRAQKSVYHLSCFCCCVCERQLQKGDEFVLKEGQL
LCKGDYEKERELLSLVSPAASDSGKSDDEESLCKSAHGAGKGASEDGKDHKRPKRPRTILTTQQRRAFKAS
FEVSSKPCRKVRETLAAETGLSVRVVQVWFQNQRAKMKKLARRQQQQQQDQQNTQRLTSAQTNGSG
NAGMEGIMNPYTTLPTPQQLLAIEQSVYNSDPFRQGLTPPQMPGDHMHPYGAEPLFHDLDSDDTSLSN
LGDCFLATSEAGPLQSRVGNPIDHLYSMQNSYFTS

>gi|544398040|ref|XM_005539841.1| PREDICTED: Macaca fascicularis LIM homeobox transcription factor 1, alpha (LMX1A), transcript variant X3, mRNA

MLDGLKMEENFQSAIDTSASFSSLLGRAVSPKSVCEGCQRVILDRFLLRLNDSFWHEQCVQCASCKEPLET
TCFYRDKKLYCKYDYEKLFAVKCGGCFEAIAPNEFVMRAQKSVYHLSCFCCCVCERQLQKGDEFVLKEGQL
LCKGDYEKERELLSLVSPAASDSGKSDDEESLCKSAHGAGKGTAEEGKDHKRPKRPRTILTTQQRRAFKAS

FEVSSKPCRKVRETLAAETGLSVRVVQVWFQNQRAKMKKLARRQQQQQQDQQNTQRLSSAQTNGGG
SAGMEGIMNPYTALPTPQQLLAIEQSVYSSDPFRQGLTPPQMPGDHMHPYGAEPLFHDLDSDDTSLSNL
GDCFLATSEAGPLQSRVGNPIDHLYSMQNSYFTS

>gi|28893581|ref|NP_796372.1| LIM homeobox transcription factor 1-alpha (LMX1A) [Homo sapiens]

MLDGLKMEENFQSAIDTSASFSSLLGRAVSPKSVCEGCQRVILDRFLLRLNDSFWHEQCVQCASCKEPLET
TCFYRDKKLYCKYDYEKLFAVKCGGCFEAIAPNEFVMRAQKSVYHLSCFCCCVCERQLQKGDEFVLKEGQL
LCKGDYEKERELLSLVSPAASDSGKSDDEESLCKSAHGAGKGTAEEGKDHKRPKRPRTILTTQQRRAFKAS
FEVSSKPCRKVRETLAAETGLSVRVVQVWFQNQRAKMKKLARRQQQQQQDQQNTQRLSSAQTNGGG
SAGMEGIMNPYTALPTPQQLLAIEQSVYSSDPFRQGLTPPQMPGDHMHPYGAEPLFHDLDSDDTSLSNL
GDCFLATSEAGPLQSRVGNPIDHLYSMQNSYFTS

Mus musculus nuclear receptor subfamily 4, group A, member 2 (NURR1), transcript variant 2, mRNA

MPCVQAQYGSSPQGASPASQSYSYHSSGEYSSDFLTPEFVKFSMDLTNTEITATTSLPSFSTFMDNYSTGY
DVKPPCLYQMPLSGQQSSIKVEDIQMHNYQQHSHLPPQSEEMMPHSGSVYYKPSSPPTPSTPSFQVQH
SPMWDDPGSLHNFHQNYVATTHMIEQRKTPVSRLSLFSFKQSPPGTPVSSCQMRFDGPLHVPMNPEP
AGSHHVVDGQTFAVPNPIRKPASMGFPGLQIGHASQLLDTQVPSPPSRGSPSNEGLCAVCGDNAACQH
YGVRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRRNRCQYCRFQKCLAVGMVKEVVRTDSLKGRR
GRLPSKPKSPQDPSPPSPPVSLISALVRAHVDSNPAMTSLDYSRFQANPDYQMSGDDTQHIQQFYDLLT
GSMEIIRGWAEKIPGFADLPKADQDLLFESAFLELFVLRLAYRSNPVEGKLIFCNGVVLHRLQCVRGFGEWI
DSIVEFSSNLQNMNIDISAFSCIAALAMVTERHGLKEPKRVEELQNKIVNCLKDHVTFNNGGLNRPNYLSK
LLGKLPELRTLCTQGLQRIFYLKLEDLVPPPAIIDKLFLDTLPF

Macaca fascicularis nuclear receptor subfamily 4, group A, member 2 (NURR1), transcript variant X3, mRNA.

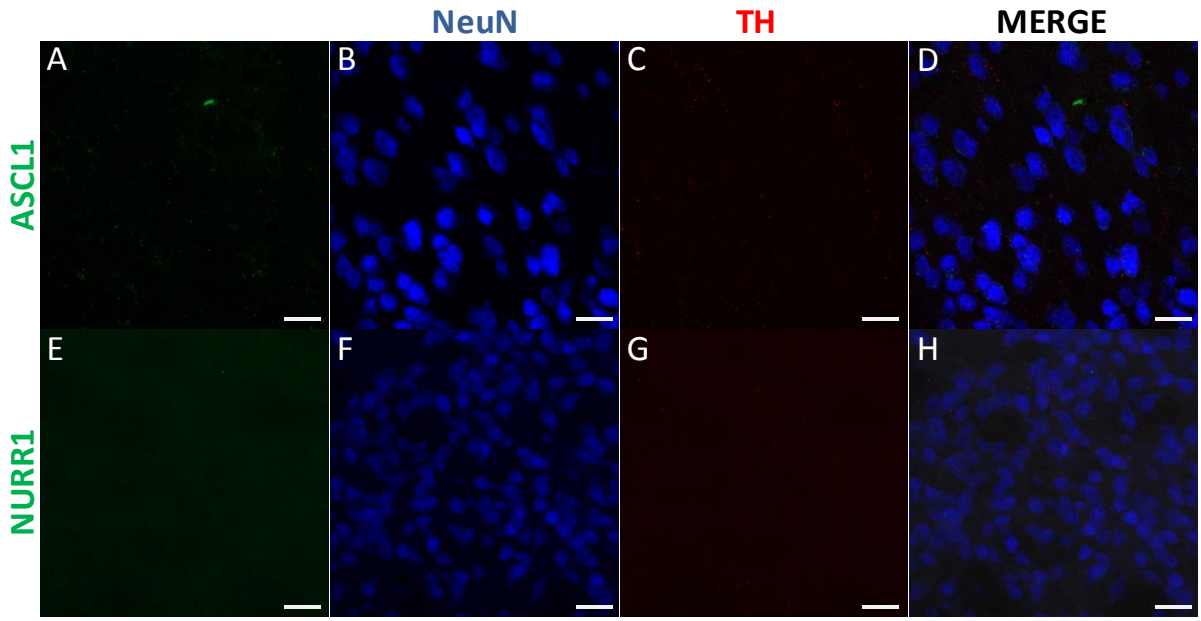
MPCVQAQYGSSPQGASPASQSYSYHSSGEYSSDFLTPEFVKFSMDLTNTEITATTSLPSFSTFMDNYSTGY
DVKPPCLYQMPLSGQQSSIKVEDIQMHNYQQHSHLPPQSEEMMPHSGSVYYKPSSPPTPTTPGFQVQH
SPMWDDPGSLHNFHQNYVATTHMIEQRKTPVSRLSLFSFKQSPPGTPVSSCQMRFDGPLHVPMNPEP
ASSHHVVDGQTFAVPNPIRKPASMGFPGLQIGHASQLLDTQVPSPPSRGSPSNEGLCAVCGDNAACQH

YGVRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRRNRCQYCRFQKCLAVGMVKEVVRTDSLKGRR
GRLPSKPKSPQEPSPPSPPVSLISALVRAHVDSNPAMTSLDYSRFQANPDYQMSGDDTQHIQQFYDLLTG
SMEIIRGWAEKIPGFADLPKADQDLLFESAFLELFVLRLAYRSNPVEGKLIFCNGVVLHRLQCVRGFGEWID
SIVEFSSNLQNMNIDISAFSCIAALAMVTERHGLKEPKRVEELQNKIVNCLKDHVTFNNGGLNRPNYLSKLL
GKLPELRTLCTQGLQRIFYLKLEDLVPPPAIIDKLFLDTLPF

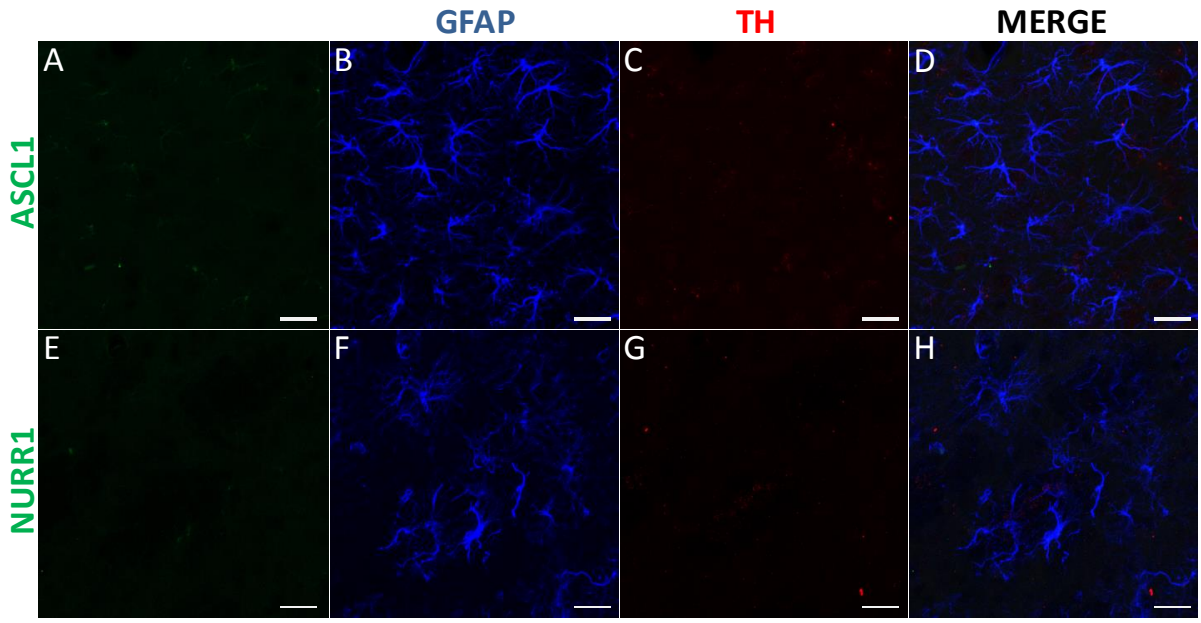
>gi|530370485|ref|XP_005246678.1| PREDICTED: nuclear receptor subfamily 4 group A member 2 isoform X1 (NURR1) [Homo sapiens]

MNEDRRGELLTMPCVQAQYGSSPQGASPASQSYSYHSSGEYSSDFLTPEFVKFSMDLTNTEITATTSLPSF
STFMDNYSTGYDVKPPCLYQMPLSGQQSSIKVEDIQMHNYQQHSHLPPQSEEMMPHSGSVYYKPSSPP
TPTTPGFQVQHSPMWDDPGSLHNFHQNYVATTHMIEQRKTPVSRLSLFSFKQSPPGTPVSSCQMRFDG
PLHVPMNPEPAGSHHVVDGQTFAVPNPIRKPASMGFPGLQIGHASQLLDTQVPSPPSRGSPSNEGLCA
VCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRRNRCQYCRFQKCLAVGMVKE
VVRTDSLKGRRGRLPSKPKSPQEPSPPSPPVSLISALVRAHVDSNPAMTSLDYSRFQANPDYQMSGDDT
QHIQQFYDLLTGSMEIIRGWAEKIPGFADLPKADQDLLFESAFLELFVLRLAYRSNPVEGKLIFCNGVVLHR
LQCVRGFGEWIDSIVEFSSNLQNMNIDISAFSCIAALAMVTERHGLKEPKRVEELQNKIVNCLKDHVTFNN
GGLNRPNYLSKLLGKLPELRTLCTQGLQRIFYLKLEDLVPPPAIIDKLFLDTLPF

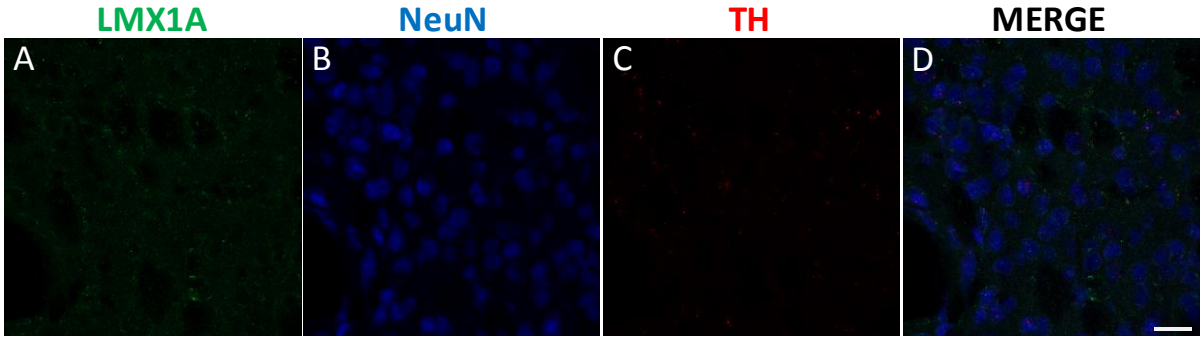
Supplementary data 2A. Negative controls for ASCL1/NURR1/TH in the contralateral striatum of mice injected with AAV8.hGFAP.eGFP. Triple IF agains ASCL1/NURR1 (green), NeuN (blue) and TH (red). Images were taken on the contralateral of the injection site. Scale bars 20µm (A-H).



Supplementary data 2B. Negative controls for ASCL1/NURR1/TH in the contralateral striatum of mice injected with AAV8.hGFAP.eGFP. Triple IF agains ASCL1/NURR1 (green), GFAP (blue) and TH (red). Images were taken on the contralateral of the injection site. Scale bars 20µm (A-H).



Supplementary data 3. Negative controls for Lmx1A in mice injected with saline. Triple IF agains Lmx1A (green), NeuN (blue) and TH (red). Images were taken along the injection site. Scale bars 20µm (A-H).



GENERAL DISCUSSION

General discussion

Parkinson Disease (PD) is a neurodegenerative disorder that affects 1.5% of the global population over 65 years of age (reviewed by Hernandez et al., 2016). Bearing in mind that in most of PD patients the cause of the disease is unknown, except for the rare case of specific genetic inheritance (Kalia and Lang, 2015b), the development of new therapies represent a huge challenge. In the last decade, a number of advances have being made, which have changed the former thinking on the development of more plausible strategies to treat central nervous system (CNS) and neurodegenerative diseases (Ruitenberg et al., 2002; Bartus et al., 2013; Marks et al., 2016). Although it may be argued that we are facing quite negative scenario, it seems that there will be some chances for the development of cell and gene therapy-based approaches.

As explained in the introductory section of this work, PD is a progressive, long-lasting neurodegenerative disease that raises numerous challenges for pharmacological treatments; for instance L-3,4-dihydroxyphenylalanine (L-dopa) is the most effective drug available for the treatment of PD, it provides great benefit for reducing the motor symptoms but after certain time patients develop wearing-off symptoms and dyskinesias (reviewed by Salat et al., 2016). The need for the development of new strategies is urgent, gene therapy might offer an alternative to make the current treatments more efficient and with less side effects than the current pharmacological and surgical treatments. Among gene therapy-based therapies, those based on *in vivo* reprogramming have emerged as an appealing manouver to convert endogenous cells (either neurons or glia cells) as an unlimited autologous source of new neurons with the desired phenotype and without the development of rejection phenomena. On this regard, the resident glial cells are the most abundant cell type in the CNS and they probably have the most widely recognized form of cell plasticity in the CNS, moreover they keep features of progenitors (reviewed by Srivastava and DeWuitt, 2016). Such glial cells become active, acquire proliferative and scar-forming capacity in response to injury or neurodegeneration. These local populations become hypertrophic and present changes in their gene expression profile and become proliferative (Gabel et al., 2015). Hence, they may be a promising cell type for therapies aimed at initiating a cellular repair (Niu et al., 2015). Direct reprogramming of these activated cells was demonstrated

previously as a new strategy to recover their functionality in a disease context like PD (Gao et al., 2016; Guo et al., 2014; Niu et al., 2015; Mosteiro et al., 2016). If changes within these cells vary according the nature and severity of the insult, we may be able to modulate such insult in favor of obtaining a potential active population "more predisposed" to be reprogrammed.

For these reasons the goal of the experimental work presented in this thesis was the development of a tool to reprograming astrocytes to DA neurons, to subsequently develop a new therapeutic option to treat PD in non-human primates (NHP). The final goal of the present study will be offer a therapy to PD patients, but before considering the potential translation of *in vivo* reprogramming to humans, extensive testing should be carried out in NHP models of Parkinsonism, in an attempt to properly bridging the gap between basic science and clinical translation.

The strategy we have used in the study is based on the AAV-mediated gene delivery of three transcription factors (TFs) driven by an astrocyte specific promoter to target the glial cells in the striatum. We have designed two AAV8 vectors, one carrying the transcription factors Ascl1 and Lmx1A (pAAV8.hGFAP.hAL) as a bicistronic protein and another vector carrying the Nurr1 (AAV8.hGFAP.hN1) TF. AAV serotype 8 has previously shown high efficiency transducing cells of the CNS and also infects larger areas than other serotypes (Aschauer et al., 2013; Watakabe et al., 2015). Although, it has also been described that AAV5 and AAV1 are superior to AAV8 for gene delivery to the NHP striatum (Dodiya et al., 2010). These data may suggest that we did not choose the most appropriate serotype for our study but is not the case. AAV serotypes can be easily changed in our laboratory conditions buy manufacturing a new batch of viral vectors. We must clarify that we chose AAV8 because in our hands it gave us satisfactory results when it was tested in our previous study and it was the ideal candidate for our proof of concept approach.

Although not described here in too much detail, we have performed several attempts to obtain the best constructs targeting astrocytes and further inducing its reprogramming to DA neurons in the mice striatum as a proof of principle study. In order to achieve a successful delivery and expression of the TFs, selection of both delivery vehicle and an optimal

expression cassette is essential, especially if more than one gene should be expressed. As delivery vehicle we chose the viral vector AAV because of its safety profile and efficiency in transducing a wide range of cell types. Moreover, a notable advance has been described for preclinical and clinic research on AAV-based CNS therapy (Hocquemiller et al., 2016), demonstrating not only long-term *in vivo* expression (Hadaczek et al., 2010) but also safety. These results are in fact encouraging even when multiple injections tracks into the brain parenchyma are necessary (Leone et al., 2012). Unfortunately, due to its small size one of its limitations is its cloning capacity. Thus we were forced to minimize the elements to be cloned on the AAV backbone and use two different vectors instead of one.

The use of minimal specific promoters facilitates the expression of larger genes or co-expression of more than one gene from the same vector. For instance, in studies of axonal regeneration it is useful to express a candidate regeneration-associated gene together with a fluorescent marker to label axons of transduced neurons in order to quantify the regeneration in the specific population of axons due to viral vector-transduced neurons (Williams et al., 2012; Fagoe et al., 2014). It is worth noting that AAVs can infect different cell types, but the expression of a given transgene can be directed to a particular cell type by choosing a specific promoter. In this regard, astrocyte-selective targeting was accomplished using a reduced version of the GFAP promoter (murine of human). As described in the first chapter, three glia-specific AAV vectors were generated using two versions of the human promoter for GFAP as well as a murine version of the GFAP promoter. Such study allowed us to choose the hGFAP promoter for the *in vivo* reprogramming approach as described on the second chapter. Although both, murine and human GFAP promoters showed a high transduction rate, we chose the human version of the promoter because as we stated earlier the final goal of the present study is to provide new tools to develop a human therapy.

The injection of the two vectors into the mouse striatum was capable of inducing newly reprogrammed neurons 8 weeks post injection (wpi) in the mice striatum. However, additional experiments are needed to clarify the necessity of the three TFs for the effect we are observing. In a similar way, as explained above regarding the serotype election, one of the main factors that could be mediating the low efficiency in the induction of new neurons is the fact that we have used the hGFAP promoter in a murine model. A priori we knew that

this election could affect the efficiency of the TFs expression. In fact, we have previously described how the efficiency of the transgene expression can be modulated if we use the murine GFAP promoter in the murine model compared with the human GFAP promoter. Following this logic, we decided to move forward and develop the AAV vectors carrying the hGFAP promoter. In practice it would be much easier for us to change the serotype rather than the promoter for new assays.

Although we were able to observe TH-positive cells after three weeks, the reprogramming efficiency was still low and the few TH-positive cells obtained disappear between the 3 and 8 wpi without showing a clear evidence of being some neuronal type. Contrary, the highest efficiency reached (90%) was reported with TF Neuro-D1 (Guo et al., 2014) but most of the published work shows an efficiency of no more than 50% or even less than 20% (Torper et al., 2013, 2015; Liu et al., 2015; Guo et al., 2014), although none of them have achieved TH-reprogrammed neurons *in vivo.*

In our attempt to reprogram astrocytes into DA neurons, we were able to obtain newly reprogrammed neurons from astrocytes. Most of the Ascl1 or Nurr1 positive cells in the striatum at 3 weeks showed non-mature neuron morphology. In fact, none of them co-localized neither with NeuN nor GFAP. On the other hand, it seems that between 3 and 8 wpi cells expressing either Ascl1 or Nurr1 they were beginning to obtain morphology neuron-like. In deed, and in contrast to what we have observed at 3 week post-injection, such cells co-localized with NeuN at 8 wpi. Moreover, this pattern was also observed in mice sacrificed at 12 wpi. These results suggest that those cells that we observed at 3 wpi may be in a transition from glia to a neuronal phenotype. NeuN is a marker for mature neurons and it is detected exclusively in post-mitotic neurons and it is not observed during development (reviewed by Duan et al., 2016). Otherwise, the lack of TH positive cells after 8 wpi open new questions regarding the transient appearance of these cells. Different experiments *in vitro*, showed how to convert glia or fibroblast in neurons (Heinrich et al., 2010; Chanda et al. 2014; Hu et al., 2015; Cheng et al., 2015) using different TF; although in some cases they also used small molecules to be able to reprogramming into full mature neurons (Hu et al., 2015; Cheng et al., 2015). In this case, we must consider that we are forcing the presence of a highly specialized neuron in a niche and time that is not his. Endogenous populations of

miDA neurons develop during the embryogenesis and it does not occur in the striatum. Here we are trying to obtain mDA neurons in the striatum of adult mice and the disappearance of these TH positive cells can be due to a simple natural selection. In between these extrinsic factors, we must consider that we are forcing the expression of Nurr1 in glial cells, which it has recently described as not physiologic (Oh et al., 2016). Hence, a deeper study to rule out potential adverse effect caused by non-physiological expression of Nurr1 is needed.

Even though it was not our first aim, this result opens new possibilities for the treatment of diseases where the neuronal loss is the main concern (not necessarily DA neurons). It would be ideal if we could determine which neuronal subtype we are obtaining in order to direct the efforts towards a specific therapy.

On the other hand, since AAVs are able to transduce neurons and induce stable, long-term gene expression in the absence of inflammation and/or toxicity, it was also our interest characterize neurons-specific small promoters in the context of the AAV8. The aim of this part of the project was to enable the development of not only astrocyte-specific vectors, but also neuronal-specific vectors for future therapeutic applications in neurodegenerative diseases besides PD. Robust and specific neuronal transgene expression was achieved when we used these minimal CHNB2 and BM88 promoters in the mice striatum. Moreover, the small size of these promoters allows the expression of larger genes or more than one gene in neurons. Diseases caused by the deficiency of large genes are not uncommon among the spectrum of neurological disorders, such as for instance in autism spectrum disorders, intellectual disability or Dravet syndrome, in which mutations in several genes are involved. Thus, development of vectors allowing the insertion of multiple genes would be of paramount importance for the adequate development of gene therapy approaches when dealing with these diseases.

CONCLUSIONS

CONCLUSIONS

1. Five AAV vectors have been developed and characterized carrying minimal promoters for the specific expression of the transgene in neurons but not in astrocytes (BM88 and NeuN) , and in astrocytes but not in neurons (GFAP derived promoters) that allowed the expression of large genes or several genes.

2. The delivery of the transcription factor Nurr1 to astrocytes in the mouse striatum using AAV vectors induces the development of TH positive cells in this region of the brain 3 weeks after vector injection.

3. The injection of AAVs expressing Ascl1/Mash1, Lmx1A and Nurr1 is associated with the development of NeuN positive cells expressing the transcription factors Ascl1 and/or Nurr1 8 and 12 weeks after vector injection. Those cells show neuronal morphology.

4. A deeper understanding of the intrinsic and extrinsic cues instructing the induction of dopaminergic neurons is critical for the refinement of the development of strategies for *in vivo* reprogramming.

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Related publication

Published in the Journal of Neural Transmission

doi: 10.1007/s00702-017-1681-3.

“Gene therapy approaches in the non-human primate model of Parkinson´s disease”

Gene therapy approaches in the non-human primate model of Parkinson's disease

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Running title: Gene therapy for PD

25 pages typescript, 3 figures (including 3 color figures) and 1 supplementary figure.

Keywords: Basal ganglia, dopamine, dyskinesia, viral vectors, neurodegeneration, macaques

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Acknowledgements:

Supported by the European Research Council (ERC Advanced Grant number 340527 ReproPARK), Spanish Ministry of Economy and Competitiveness (grant number BFU2012-27907), CiberNed (2014/01) and Fundación La Marató TV3 (grant number 20141331). Salary for Diego Pignataro is partially supported by a grant from Jon Zarandona. The plasmids, maps and sequences of hRheb-S16H are a generous gift from Drs. R.E. Burke and N. Kholodilov from the Department of Neurology, Columbia University.

ABSTRACT

The field of gene therapy has recently witnessed a number of major conceptual changes. Besides the traditional thinking that comprises the use of viral vectors for the delivery of a given therapeutic gene, a number of original approaches have been recently envisaged, focused on using vectors carrying genes to further modify basal ganglia circuits of interest. It is expected that these approaches will ultimately induce a therapeutic potential being sustained by gene-induced changes in brain circuits. Among others, at present it is technically feasible to use viral vectors to (i) achieve a controlled release of neurotrophic factors, (ii) conduct either a transient or permanent silencing of any given basal ganglia circuit of interest, (iii) perform an in vivo cellular reprogramming by promoting the conversion of resident cells into dopaminergic-like neurons and (iv) improving levodopa efficacy over time by targeting aromatic L-amino acid decarboxylase. Furthermore, extensive research efforts based on viral vectors are currently ongoing in an attempt to better replicate the dopaminergic neurodegeneration phenomena inherent to the progressive intraneuronal aggregation of alpha-synuclein. Finally, a number of incoming strategies will soon emerge over the horizon, these being sustained by the underlying goal of promoting alpha-synuclein clearance such as for instance gene therapy initiatives based on increasing the activity of glucocerebrosidase. In order to provide adequate proof-of-concept on safety and efficacy and to push forward true translational initiatives based on these different types of gene therapies before entering into clinical trials, the use of non-human primate models undoubtedly plays an instrumental role.

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1. Introduction

Parkinson's disease (PD) is, by large, an idiopathic neurodegenerative disorder of basal ganglia origin sustained by the progressive loss of midbrain dopaminergic neurons located in the substantia nigra pars compacta (SNc). Although only a minimal fraction of cases can be attributed to a Mendelian inheritance due to several genetic mutations, there is an overall consensus in considering the etiology of PD as a mixed scenario comprising genetic susceptibility together with environmental factors including insecticides, pesticides and herbicides. The so-called "genetic susceptibility" stands on a quickly-increasing list of involved genes such as the ones coding for alpha-synuclein (α-SYN), parkin, leucine-rich repeat kinase 2 (LRRK2; also known as dardarin), PTEN-induced putative kinase 1 (PINK-1), DJ-1 and ATP13A2 (reviewed in Klein and Westenberger, 2003). Moreover, mutations in the gene coding for glucocerebrosidase (known as GBA1 gene) deserve special attention since a tight relationship between homo- and heterozygous-GBA1 mutations and the appearance of PD has been only recently uncovered (Sidransky, 2005).

Bearing in mind that PD in mostly diagnosed people lacks a specific known cause (at least without specific genetic inheritance) it seems there will be little chances for the development of gene therapy-based approaches. Although it might be argued that we are facing quite a negative scenario, a number advances being made in the last few years have completely changed the former thinking into the development of more plausible strategies that so far can be broadly categorized into five main groups. First category is represented by the controlled release of neurotrophic factors through viral vectors, mostly focused on glial cell-derived neurotrophic factor (GDNF) and neurturin (NRTN; a close relative of the GDNF family). A second group is made up of a number of gene therapy-based initiatives using genes with the aim of modifying basal ganglia circuits (DREADD-related systems, tract-targeting approaches and Rheb-induced nigrostriatal reconstruction). Next, in vivo reprogramming has also recently emerged as an appealing strategy. A fourth group of initiatives are directed towards targeting neurotransmitters such as glutamic acid decarboxylase and aromatic L-amino acid decarboxylase. Finally, a fifth group comprises options made available in the last few years following the identification of key downstream targets within pathogenic pathways implicated in PD pathophysiology. Among others,

extensive research is currently being conducted with genes implicated in autophagy and mitochondria, lysosomal impairment and endoplasmic reticulum (ER) stress. Furthermore, it is also worth noting that gene therapy might also be a very helpful choice for modeling PD in experimental animals by promoting the progressive aggregation of alpha-synuclein (see accompanying manuscript by Jeff Kordower, this issue). Moreover, it is worth noting that gene therapy with glucocerebrosidase has been appointed as a feasible approach for conducting alpha-synuclein clearance (Rocha et al., 2015). Although it is practically impossible to deal with all the currently available gene therapy tools for PD, here we will try to summarize the most popular ones in an attempt to provide potential readers with a landscape view of what's going on in the field.

2. Different types of viral vectors for different purposes

2.1. *The vector matters: Selection of the most appropriate viral vector*

To bypass the blood brain barrier (BBB), viral vectors are directly injected into the brain parenchyma through stereotaxic surgery, a strategy termed "*in vivo* gene therapy". Up to a dozen viral families have been the focus of intensive research for CNS application (Nassi et al., 2015), each family with his own properties for packaging capacity (i.e., cargo), tropism, transduction efficacy and safety concerns. Besides earlier studies that were conducted with retroviral and herpes viral vectors, the most popular and broadly used choices for CNS research in animal models and in clinical trials are currently represented by lentiviruses and adeno-associated viruses (AAVs). Lentiviruses have a cargo capacity of approximately 9 Kb (8.5 Kb between the LTRs) and are able to infect non-dividing cells. In most cases, the original envelope is replaced by the vesicular stomatitis viral glycoprotein to further generate pseudotyped lentiviral vectors. By contrast, AAVs have a smaller cargo capacity (approximately 4.7 Kb) and infect both dividing and non-dividing cells. Upon intraparenchymal stereotaxic delivery, the diffusion of lentiviral vectors if often limited to few millimeters away from the injection site (Linterman et al., 2011), whereas for AAVs the transduced area largely depends on the AAV serotype, production and purification (Aschauer et al., 2013; Cearley et al., 2006; Ayuso et al., 2010). Bearing in mind that when considering non-human primates (NHPs) increasing the transfected area as much as possible

often is a desirable need, the so-called "convection-enhanced delivery" is a highly recommended choice (Richardson et al., 2011; San Sebastian et al., 2012). Alternatively, a new generation of pseudotyped lentiviruses (Kato et al., 2014) as well several AAVs serotypes can be used for the retrograde spread of therapeutic genes, thus representing new choices for selective viral infection of projection neurons. In other words, brain circuits can be used for long-distance gene delivery without the need of directly injecting the viral vector into the brain area of interest. Finally, AAV serotype 9 vectors can bypass the BBB quite efficiently, particularly when engineered through the so-called Cre recombination-based AAV targeted evolution (CREATE; see Deverman et al., 2016). When customized this way, the vector transduces both neurons and astrocytes in the CNS after systemic delivery, without the need of performing stereotaxic surgery for viral vector administration. A number of modifications of this AAV-based system are expected in the near future to drive cell-specificity of the transduction (i.e., neurons vs. glial cells) as well as specificity for a given neuronal phenotype (for instance, dopaminergic neurons).

2.2. *The promoter matters: selection of the most appropriate AAV promoter*

The AAV vector is a Parvovirus that belongs to the genus Dependovirus. The 4.7-kilobase (kb) single-stranded wild-type AAV genome is composed of three open reading frames (ORF), flanked by two 145 nucleotide inverted terminal repeat sequences (ITRs). The *rep* ORF encodes four proteins essential for replication, packaging, transcriptional regulation of viral promoters and site-specific integration. The *cap* ORF acts as a template for the production of three structural proteins that only differ in their N-terminus: VP1, VP2 and VP3 proteins; these proteins form the capsid at a ration of 1:1:10, respectively. An alternative ORF nested in *cap* encodes for an assembly activating protein (AAP), which interacts with the capsid proteins VP1, 2 and 3 and is necessary for viral assembly (Murlidharan et al., 2014; Ojala et al., 2015). To construct a recombinant AAV vector both the *cap* and *rep* genes are replaced by a cassette containing the transgene of interest and the regulatory sequences needed for the transgene expression such as the promoter and the poliA sequence (Flotte and Cater, 1995; Carter and Samulski, 2000; Gaj et al., 2016). AAVs transduce both dividing and non-dividing cells and are capable of long-term, stable gene expression without noticeable inflammation and toxicity phenomena. Bearing in mind that neurons are post-mitotic cells, the ability of AAVs for transducing non-dividing cells is of paramount importance within the

context of neurodegenerative disorders (Bartlett et al., 1998, 1999). The selection of the most appropriate promoter is an essential factor when considering the design of gene therapy tool (Gray et al., 2011), and this also includes defining the choice between a cell-specific or region-specific promoter (Papadakis et al., 2004). In other words, although AAVs can infect different cell types, the expression of a given transgene can be directed to a particular cell type by choosing a specific promoter. Considering that AAVs have a limited cloning capacity due to the small genome, the use of small promoters facilitates the expression of large genes or even the co-expression of more than one gene from the same vector. In this regard, astrocyte-selective targeting can be accomplished using a reduced version of the GFAP promoter named gfaABC$_1$D (hGFAP pr; Lee et al., 2008). Different versions of this astrocyte-specific promoter have been designed, such as one in which the D sequence of the promoter was removed (hGFAPΔD pr; Besnard et al., 1991; Lee et al., 2008) as well as a murine version of the human gfaABC$_1$D promoter (Pignataro et al., 2016). Considering oligodendrocytes, specific promoters have also been made available elsewhere (Chen et al., 1999; McIver et al., 2005; Lawlor et al., 2009; von Jonquieres et al., 2013; Kagiava et al., 2014). Furthermore, when dealing with the design of neuron-specific promoters, there are at hand a number of available choices. The most popular one is represented by the use of the synapsin I promoter (Kügler et al., 2003), a neuronal protein localized in the surface of synaptic vesicles (Thiel et al., 1991). It has been demonstrated that the regulatory element of synapsin I promotes neuron-specific gene expression (Schoch et al., 1996). Furthermore, the mouse calcium-calmodulin kinase-2a promoter (CaMKIIa) also is a feasible alternative to synapsin I for achieving specific neuronal transgene expression (Gerits et al., 2015; Watakabe et al., 2015). When constructed this way, only neurons and not glial cells became specifically transduced with the gene of interest. Illustrative examples of neuron-specific promoters are provided in Figure 1.

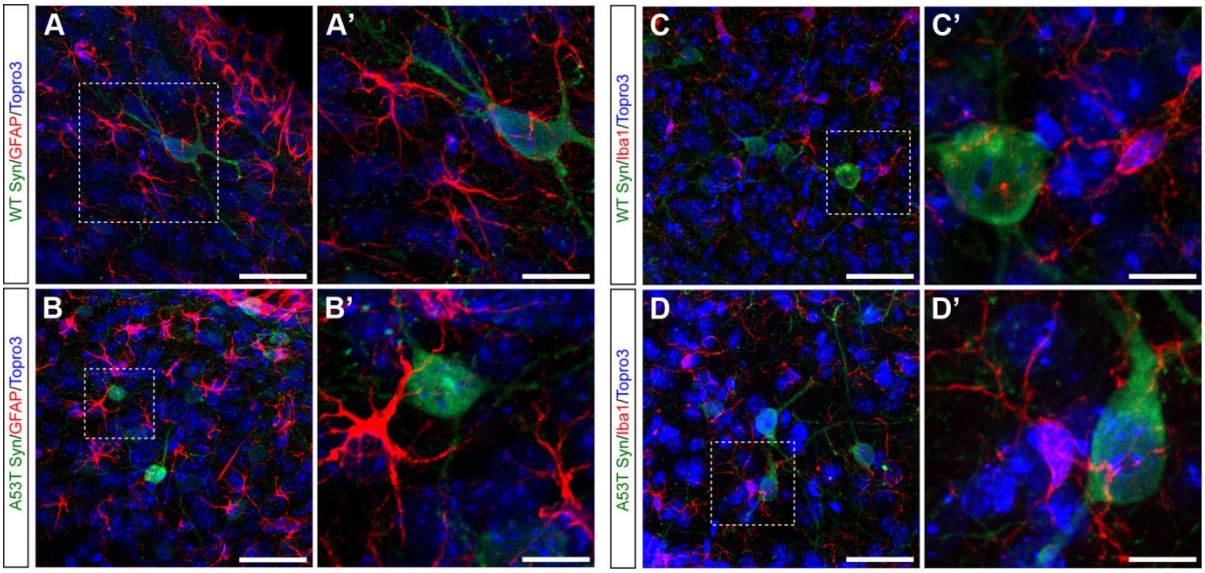

Figure 1. Neurospecific gene transfer with an AAV9 vector encoding either the wild type or the mutated form of alpha-synuclein using a hybrid synapsin I promoter. Following the delivery of the vector into the rat striatum, only striatal neurons became infected (green). Both astrocytes and microglial cells completely lacked transgene expression (red channel). Scale bars 20 lm (A, B, C, D); 35 lm (A'); 10 lm (B', C', D').

3. Viral vectors for the delivery of neurotrophic factors

Neurotrophic factors (NTFs) are naturally-occurring proteins acting on a number of intracellular signaling pathways. Among the NTFs with a proven dopaminotrophic effect, glial cell line-derived neurotrophic factor (GDNF) and neurturin (NRTN) have been extensively tested in animal models of PD and indeed translated to early phases of clinical trials (for review see Kelly et al., 2015). NTFs act on membrane receptors (GFR and Ret) before activating downstream intracellular pathways in dopaminergic neurons (Jing et al., 1997; Walker et al., 1998; Quartu et al., 2007). Although it has long been known that intracerebral infusion of the naked GDNF recombinant protein promoted functional and structural improvements in MPTP-treated macaques (Gash et al., 1996; Gerhardt et al., 1999; Iravani et al., 2001; Grondin et al., 2002), the initially encouraging results gathered from two different phase I clinical trials (the "Bristol" and "Kentucky" studies, see Gill et al., 2003; Slevin et al., 2005) were not confirmed in a phase II randomized, placebo-controlled trial (Lang et al., 2006). The conducted research pushing forward intracerebral infusion of NRTN mimicked -at least to some extent- former experiences with GDNF. Although the chronic infusion of NRTN ameliorated motor scores in MPTP-treated NHPs (Grondin et al., 2008), the discouraging results gathered from randomized GDNF clinical trials likely prevented pushing NRTN

infusion towards more translational initiatives. Moreover, it is also worth noting that reduced or impaired axonal transport has also been appointed as a potential cause of the failure of these trials. In this regard, NRTN retrograde transport from the putamen to the substantia nigra was found to be reduced in advanced stages of the disease (Bartus et al., 2011, 2015).

Besides a number of safety concerns, the limited diffusion of GDNF and NRTN proteins has often been claimed to play a key role in clinical trials failure. In an attempt to overcome these limitations and to further obtain a more widespread distribution of NTFs, viral-mediated gene transfer has emerged as a feasible choice. Both lentiviruses and AAVs have been used for the controlled release of GDNF in MPTP-treated macaques (Kordower et al., 2000; Eslamboli et al., 2005), whereas for NRTN, AAVs serotype 2 (known as Cere-120) have been administered to both MPTP-treated and aged macaques showing high efficacy without safety concerns in pre-clinical testing (Kordower et al., 2006; Herzog et al., 2007; 2008). These encouraging results sustained the translation of Cere-120 in an open-label phase I clinical trial (Marks et al., 2008), then followed by a double-blind randomized, controlled phase II trial (Marks et al., 2010). Furthermore, it is worth noting that important differences were observed when comparing biodistribution of Cere-120 in macaques and patients (the latter from post-mortem analysis conducted on two patients), thus further emphasizing the need for achieving a broader brain distribution of delivered AAV vectors. In this regard, Kystoff Bankiewicz and co-workers have long been pushing forward the so-called convection enhanced delivery (CED) for the administration of AAV2-GDNF. When going through this way, remarkable improvements in motor performance and striatal dopaminergic function have been reported (Eberling et al., 2009; Johnston et al., 2009; Kells et al., 2009, 2010).

4. **Use of viral vectors for silencing basal ganglia circuits**

Most of our current understanding of basal ganglia function and dysfunction stands on the so-called “classic basal ganglia model” (Albin et al., 1989; DeLong, 1990). Although modified and amplified by the emergence of new data, much of the model has remained (for review, see Lanciego et al., 2012). Most importantly, the classical model settled the basis for the renaissance of functional neurosurgery for movement disorders, best exemplified by

high-frequency deep brain stimulation (DBS) approaches targeting hyperactive basal ganglia-related nuclei such as the internal division of the globus pallidus (GPi) and the subthalamic nucleus (STN). Indeed, lesioning strategies targeting these nuclei in NHPs (Guridi et al., 1994; 1996) paved the way for the translational development of DBS to individuals suffering from PD in an attempt to tune down this hyperactivity back to baseline levels. Within the field of gene therapy, a number of approaches have recently been made available for conducting either a transient or a permanent silencing of hyperactive brain circuits (DREADD-based systems and neuroanatomical tract-targeting, respectively).

4.1. **DREADD-based approaches**

DREADD is the acronym for Designer Receptors Exclusively Activated by Designer Drugs, a chemogenetic tool well suited for the transient manipulation of a given brain circuit. Different DREADD systems, for different purposes, have been added to the current technical portfolio at a breathtaking speed. Briefly, DREADDs are useful tools to modulate GPCR signaling, either by activation or inhibition of a given neuronal circuit. For the purposes of performing a transient silencing of a given brain circuit, the most conventional way is to use an AAV coding for a modified human muscarinic receptor (hM4D). Once injected into the brain area of interest, the hM4D is expressed in AAV-infected neurons. Bearing in mind that the hM4D receptor is exclusively activated by a pharmacologically inert compound known as clozapine-N-oxide (CNO; a metabolite of the atypical antipsychotic drug clozapine), the peripheral administration of CNO will result in hM4D-mediated neuronal hyperpolarization through a G protein related activation of inward-rectifying potassium channels (Armbruster et al., 2007). In other words, this approach will ultimately result in a transient silencing of a brain circuit being sustained by CNO-mediated hyperpolarization of the parent neurons giving rise to such a brain circuit. As the CNO serum levels decrease, the hM4D-AAV-infected neurons returned back to the normal state of polarization.

Within the basal ganglia field, DREADDs have been used to modulate the activity of striatal projection neurons in rodents (Ferguson et al., 2013; Farrell et al., 2013; Bellochio et al., 2016; López-Huerta et al., 2016). Moreover, this technique has also been shown useful for the dissection of corticolimbic networks being recruited in awake vs. anesthetized conditions at the level of the shell of the nucleus accumbens (Michaelides et al., 2013).

Similar approaches were used to activate mesolimbic dopaminergic projections arising from the ventral tegmental area and innervating neurons of the nucleus accumbens (Boender et al., 2014). Furthermore, the role of astrocytes from the nucleus accumbens core in the glutamatergic-mediated reinstatement of cocaine seeking was recently untangled using an hM3D DREADD-coding AAV under the control of a GFAP promoter (Scofield et al., 2015). Besides using DREADDs for dissecting brain circuits noninvasively, there is also a role for DREADDs as therapeutic adjuncts improving dopaminergic cell replacement strategies (Dell Anno et al., 2014; see also Vazey and Aston-Jones, 2014). Considering NHPs, a reversible and repeated disconnection of the orbitofrontal and rhinal cortices was achieved with an hM4Di-coding lentivirus under a neuron-specific promoter (Eldridge et al., 2016). In summary, DREADDs are chemogenetic tools with a broad range of applications in neuroscience research. When thinking on the potential translational use of these techniques in humans, the pharmacologically inert compound most likely to be used is perlapine instead of CNO since the latter has a proved long history of safety in humans. Regarding the viral vector of choice, AAVs seem to be the most natural choice. Potential readers interested in going on deeper in this technique are referred to reviews made recently available by Urban and Roth (2015) and Roth (2016).

4.2. **Pseudotyped lentiviruses for tract-targeting**

When dealing with gene therapy approaches for CNS diseases, in most cases stereotaxic administration of the viral vector is the only choice at hand. Moreover, the way in which the target area is approached also plays a fundamental role. As pointed out above, although AAVs are the most commonly used platforms for gene delivery, there is often a need for delivering the AAVs directly into the desired brain area of interest in an attempt to improve the spread of transduction. Although certain AAV serotypes can be transported retrogradely (Towne et al., 2010, 2011), both AAVs and lentiviruses have been more broadly used at large. The overall concept stands on the idea of using brain circuits for the retrograde spread and selective expression of the transgene of interest. In this regard, upon delivery in a given brain area, the viral vector is taken up by axon terminals and retrogradely transported back to the parent cell body of neurons innervating the injected area. In other words, long-distance access of any given gene can be achieved by means of the retrograde spread of the viral vector, thus circumventing the need of directly approaching the designated brain area

of interest. Moreover, reaching access to brain territories that are non-approachable by direct stereotaxic surgery can be secured in a very specific way.

For retrograde transduction, lentiviruses are better choices than AAVs (Kato et al., 2013b; Oguchi et al., 2015), particularly when lentiviruses are pseudotyped, e.g., by replacement of the original lentiviral envelope glycoprotein with the rabies glycoprotein or with fusions of the extracellular domain of the rabies glycoprotein and the intracellular domain of the vesicular stomatitis virus glycoprotein (Kato et al., 2013a,b). When constructed this way, the pseudotyped lentiviral vector known as NeuRet showed a very high efficiency of gene transfer through retrograde transport (Kato et al., 2011a). Furthermore, enhanced efficiency of retrograde gene delivery with the NeuRet vector has been achieved by optimizing the junction between the rabies virus glycoprotein and the vesicular stomatitis virus glycoprotein in fusion glycoproteins in their membrane-proximal region (Kato et al., 2014). Based on these retrogradely-transported pseudotyped lentiviruses, Kazuto Kobayashi and co-workers developed the concept of immunotoxin-mediated tract-targeting (Kobayashi et al., 1995; Kato et al., 2012), a tool with a proven efficacy for the selective elimination of brain circuits. Briefly, the lentiviral vector was designed to encode the human interleukin 2 receptor α subunit (IL-2Rα), a receptor molecule for a recombinant immunotoxin, anti-Tac(FV)-PE40. Once the lentiviral vector was retrogradely transported to the first-order projection neuron, infected neurons started expressing the IL-2Rα receptor. In a second surgical step, local administration of the recombinant immunotoxin leads to a selective elimination of neurons expressing the IL-2Rα receptor. By going this way, a selective elimination of the thalamostriatal pathway in rats has been efficiently achieved, firstly by injecting the pseudotyped lentivirus into dorsal striatal territories, later followed by imunotoxin delivery within the parafascicular nucleus (Kato et al., 2011b). In NHPs, the delivery of the lentiviral vector into the subthalamic nucleus, later followed by immunotoxin injections in the supplementary motor area resulted in a selective removal of the cortico-subthalamic pathway (Inoue et al., 2012). Furthermore, we have also tested the efficacy of the immunotoxin-mediated tract-targeting for the alleviation of levodopa-induced dyskinesia in macaques. For this purpose, a lentivirus coding for both the IL-2Rα receptor and for enhanced green fluorescent protein (eGFP) was injected in the ventral anterior/ventral lateral thalamic nuclei into the left thalamus, whereas a lentivirus

only-carrying the GFP gene was injected into similar locations in the right thalamus for control purposes. Next, the recombinant immunotoxin was delivered bilaterally into the internal divisions of the left and right globus pallidus (GPi). Most of the pallidothalamic-projecting neurons from the left GPi were eliminated, whereas eGFP-only expressing neurons from the right GPi remained unaffected from the immunotoxin. Preliminary results showed an almost complete alleviation of the contralateral levodopa-induced dyskinesia (see Figure 2).

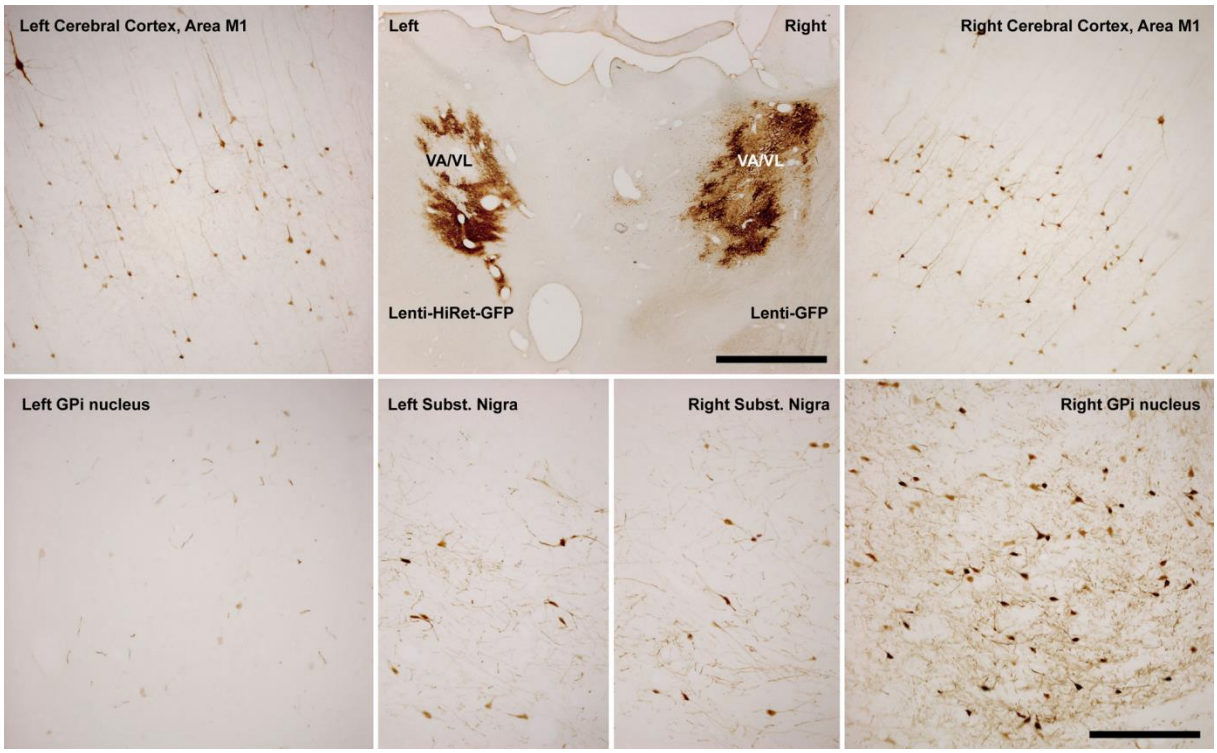

Figure 2. Selective elimination of the left pallidothalamic pathway in dyskinetic NHPs with immunotoxin-mediated tract-targeting. A lentiviral pseudotyped vector encoding the IL-2Ra receptor (HiRet vector) and GFP was delivered into the left VA/VL thalamic nuclei, whereas for control purposes, a lentiviral vector carrying the GFP gene only was injected into similar locations in the right thalamus. After adequate transgene expression (3 weeks post-lentiviral delivery), the recombinant immunotoxin was injected into both the left and right GPi nuclei. The immunohistochemical detection of GFP revealed that most of the GPi neurons giving rise to the Left pallidothalamic pathway were damaged following immunotoxin treatment. Similar treatment conducted in the right GPi revealed no changes in neuron survival. This procedure allows eliminating the left pallidothalamic pathway in a very specific way, since corticothalamic and nigrothalamic projections in the left brain hemisphere remained unaffected. As expected, a marked alleviation of the levodopainduced dyskinetic syndrome was observed in the side of the body contralateral to the removal of the left pallidothalamic pathway. Scale bar is 3000 µm for the panel showing the injection sites and 1000 µm for the remaining panels.

5. Viral vector-mediated reconstruction of the nigrostriatal pathway

Although Parkinson's disease cannot be merely seen as the result of dopaminergic neuronal cell loss in the substantia nigra pars compacta, achieving a reconstruction of the damaged nigrostriatal pathway has often been considered as a long, unmet need. A number of attempts based on gene therapy tools, even alone or in combination with other procedures have been made in the past few years. Initial experiments showed that the intrastriatal delivery of GDNF potentiates the growth of dopaminergic axons through the medial forebrain bundle after the implantation of dopaminergic cell grafts into the substantia nigra (Wang et al., 1996; Wilby et al., 1999).This concept was further expanded by Thomson et al. (2009) who injected an AAV vector coding for GDNF under the control of a constitutive promoter (chicken beta actin) into the striatum to enhance dopaminergic axonal outgrowth arising from intranigral cell grafting. When stimulated this way, the "calling effect" exerted by AAV-mediated over-expression of GDNF in the host striatum largely improved the sprouting of dopaminergic fibers from cells being implanted in the substantia nigra.

For the purposes of reconstructing the nigrostriatal dopaminergic pathway, a different approach was undertaken by Robert E. Burke and colleagues (Kim et al., 2011, 2012) by taking advantage of the inheritance properties of the mammalian target of rapamycin (mTOR) kinase as mediator in many aspects including axon growth, axon number per neurons, branching, caliber and growth cone dynamics. They analyzed the effects of activation of the mTOR complex 1 (mTORC1) by its immediate upstream regulator, the GTPase ras homolog enriched in brain (Rheb) and its constitutively active form (hRheb.S16H). These authors explored the ability of AAVs for transducing dopaminergic nigral neurons with hRheb-S16H to further induce the regrowth of axons from dopaminergic neurons after they have been partially destroyed by the intrastriatal delivery of 6-hydroxidopamine (6-OHDA). Upon AAV-hRheb(S16H)-mediated activation of intrinsic cellular programs regulated by mTor, an impressive regrowth of dopaminergic axons coming from surviving nigral dopaminergic neurons was observed (Kim et al., 2011). In mice treated with AAV1-hRheb(S16H), the extent of striatal reinnervation was 44.6% compared to the contralateral control, reflecting a 71% of increase in the number of tyrosine hydroxylase-positive axons observed in the medial forebrain bundle ipsilateral to the AAV-hRheb(S16H)-

injected substantia nigra (Kim et al., 2012). Furthermore, it is worth noting that the induced reinnervation apparently was functionally adequate, as shown by the marked reduction in contralateral turning behavior observed in AAV-hRheb(S16H)-treated animals when compared with the control cases being injected with a GFP-coding AAV. Accordingly, here we have conducted few exploratory preliminary experiments testing whether this approach would also be useful for MPTP-treated macaques. hRheb(S16H)-FLAG-coding AAV serotype 5 vectors driven by a constitutive hybrid promoter composed of the CMV immediate-early enhancer fused to chicken beta-actin promoter (CBA promoter) were prepared in our in-house facilities from the plasmids, maps and sequences generously provided by R.E. Burke and N. Kholodilov. Two macaques with a severe MPTP-induced parkinsonism (87% of dopaminergic depletion on average, as estimated with ^{11}C-dihydrotetrabenazine PET scans) were injected with hRheb(S16H)-AAV5 into lateral territories of the substantia nigra pars compacta, unilaterally. Follow-up lasted for 6 months and included regular assessment of motor symptoms with an UPDRS clinical-rating scale as well as regular PET scanning. Both animals were sacrificed 6 months post-AAV delivery. The conducted neuropathological studies revealed a very few number of FLAG+ neurons being infected with the AAV in the substantia nigra (less than a dozen dopaminergic neurons per animal, see Supplementary Figure 1). Nevertheless, few FLAG+ axons were seen travelling through the medial forebrain bundle (Supplementary Figure 1), ultimately ending into the putamen as few discrete patches of TH+ terminals distributed throughout a rostrocaudal extent of 4.5 mm comprising both the pre- and the post-commissural putamen (as shown in Figure 3). Obtained patterns of reinnervation closely mimicked the known morphology of arborization for single nigrostriatal axons (Matsuda et al., 2009; see also Bolam and Pissadaki, 2012). These results suggested that the nigrostriatal pathway can be reconstructed -albeit to a very limited extent- in macaques with severe dopaminergic damage. Although the obtained results represent an appealing proof-of-concept, it is worth noting that Rheb is known to be a potent oncogen (Shaw et al., 2006) and therefore the potential therapeutic translation of these findings obviously deserves a very careful consideration.

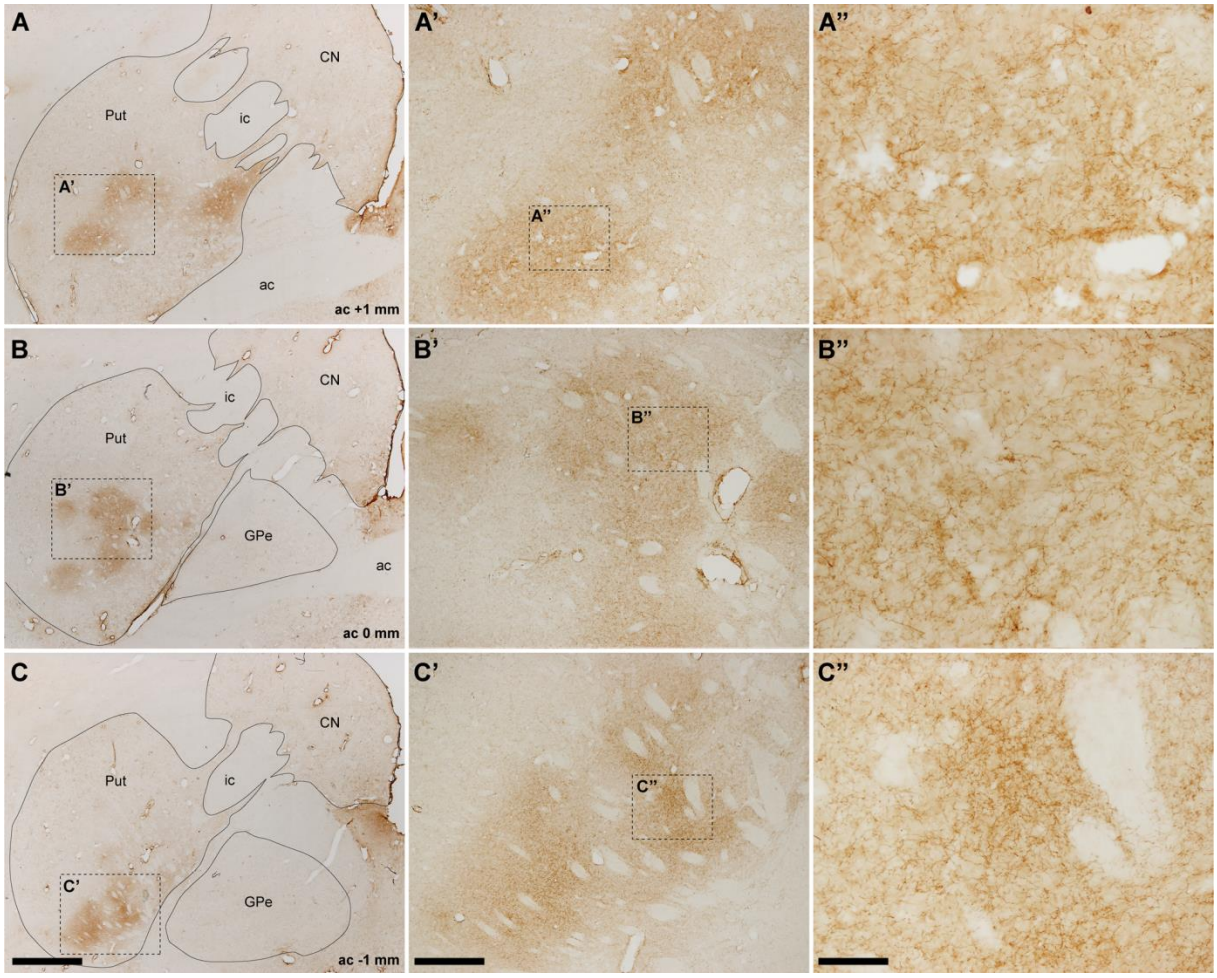

Figure 3. Partial reconstruction of the nigrostriatal pathway in macaques with hRheb-carrying AAV5. Following the stereotaxic delivery of hRheb(S16H)-AAV5 into lateral territories of the substantia nigra in two macaques showing a severe parkinsonian syndrome (estimated dopaminergic depletion of 87% on average), less than a dozen of neurons became infected with the vector. After a follow-up of 6 months, the immunohistochemical analysis revealed the presence of tyrosine-positive axons coursing rostrally through the medial forebrain bundle, ultimately forming discrete patches of axon terminals through the anteroposterior axis of the putamen. In keeping with the known pattern of axonal arborization of dopaminergic neurons (Matsuda et al. 2009), these patches likely belong to Rheb-infected nigral neurons. Scale bars 2000 µm (A–C), 200 lm (A'–C') and 100 µm (A''–C'').

6. **AAV-mediated delivery of glutamic acid decarboxylase**

According to the basal ganglia model, under circumstances of dopaminergic depletion there is a well-known hyperactivity of the subthalamic nucleus (STN) driven by reduced GABAergic innervation coming from the external division of the globus pallidus (for review, see Obeso et al., 2000). Besides paving the way for the implementation of high-frequency DBS neurosurgical procedures targeting the STN nucleus, such hyperactivity opened new avenues for gene therapy approaches. In this regard, Emborg and colleagues (2007) developed a gene therapy approach in NHPs by targeting STN neurons with the gene coding

for glutamic acid decarboxylase (GAD) through an AAV2 vector under the control of a ubiquitous promoter (CBA). Six animals were injected with AAV-GAD into the right STN, whereas in another cohort of six macaques, GFP-coding AAVs were delivered into the STN for control purposes. After a follow-up of 55 weeks, animals treated with AAV-GAD showed significant improvements in bradykinesia, gross motor skills and tremor. These findings correlated with improvements observed in FDG-PET neuroimage studies. Throughout the follow-up period, AAV-GAD proved safe and without adverse events. These promising pre-clinical evidences prompted to the implementation of an open-label, non-randomized phase I clinical trial (Kaplitt et al., 2007). The ultimate goal was to tune down STN hyperactivity back to baseline levels in parkinsonian patients (12 individuals, Hoehn and Yahr stage 3 or greater). Although this trial was not intended to assess efficacy of the treatment, substantial improvements in the off and on states were found. The procedure was found to be safe and well-tolerated, without substantial adverse effects.

7. **Cellular reprogramming with viral vectors**

The concept of in vivo reprogramming is based on the idea of using endogenous cells (either neurons or glial cells) as an unlimited autologous source for the generation of new neurons with the desired phenotype and without the appearance of immunorejection phenomena. This type of “phenotypic switch” is termed in vivo reprogramming. The first successful demonstration of this approach in adult animals was carried out in the pancreas, by reprogramming pancreatic exocrine cells into insulin-secreting beta cells using a combination of three transcription factors (Zhou et al., 2008). Similar approaches -to some extent- have been carried out in other organs, such as the heart and liver (Qian et al., 2012; Song et al., 2012, 2015, 2016; Rezvani et al., 2016). Within the CNS, a priori it sounds reasonable to focus on astrocytes instead of in neurons for in vivo reprogramming purposes, thus directly converting astrocytes into different types of neuronal-like phenotypes. Among others, the genes coding for a number of transcription factors such as neurogenin 2 (Ngn2), NeuroD1, Sox2, Ascl1, Lmx1a/b, Nurr1, Bcl2, FGF2 (or combinations herein) have been mounted into different viral vectors to promote the in vivo reprogramming of astrocytes in the CNS with low to high reprogramming efficiency. Furthermore, instead of directly reprogramming astrocytes into neurons, other approaches have tested the feasibility of firstly converting glial cells into neuroblasts, the latter being further converted into neurons

(Su et al., 2014; Niu et al., 2015). Moreover, dedicated genes have also been used for the conversion of olygodendrocyte precursors (NG2 glia) into neurons in the adult mouse brain (Torper et al., 2015). Finally, a given type of neuron can also be converted into a neuron with a different phenotype, particularly at embryonic or early postnatal stages (for an in-depth review, see Li and Chen, 2016). Besides the huge potential use of these approaches for clinical application, a number of challenges still remain, such as for instance achieving best functional reintegration of reprogrammed cells within the local scenario to further restore the formerly lost brain function. Before considering the potential translation of in vivo reprogramming, extensive testing should be carried out in non-human primate models of parkinsonism, in an attempt to properly bridging the gap between studies in mice and clinical testing in humans.

8. **Strategies focused on aromatic L-amino acid decarboxylase**

Levodopa is the gold-standard pharmacological approach for early stages of PD. Aromatic L-amino acid decarboxylase (AADC) is the enzyme in charge of converting levodopa to dopamine. With disease progression the waning therapeutic effect of levodopa is probably due to declining striatal levels of AADC (Nagatsu and Sawada, 2007). This has prompted to viral-mediated approaches trying to increase AADC levels. In NHPs, initial attempts were conducted by Krystof Bankiewic's group using an AAV serotype 2 coding for AADC under the control of a CMV promoter. These experiments resulted in a long-term improvement in clinical rating scores, together with reduced levodopa requirements and persistent AADC activity as seen with PET neuroimage studies (Bankiewicz et al., 2006). Furthermore, a linear relationship between vector dosage and AADC enzymatic activity was noticed (Forsayeth et al., 2006). Obtained preclinical evidence on the effectiveness of AAV2-AADC in parkinsonian NHPs sustained the implementation of clinical trials. In a first clinical trial (NCT002229736), 10 patients received bilateral intraputaminal infusions of AAV2-AADC through convection-enhanced delivery and results were reported with a follow-up of 6 and 12 months (Eberling et al., 2008; Christine et al., 2009; Mittermeyer et al., 2012). Throughout the evaluated period, UPDRS scores showed significant improvements both in the on and off periods, consistent with elevated AADC expression levels as seen with PET neuroimage scans. Very recently, Voyager Therapeutics, Inc (Cambridge, Mass.) published a

press release reporting positive results from a phase 1b trial of VY-AADC01 (AAV2-AADC vector; trial identifier NCT01973543) comprising two cohorts of patients begin treated with low and high doses of VY-AADC01(cohorts #1 and #2, respectively) and followed-up for 6 and 12 months. Although important ameliorations of the parkinsonian syndrome were found in both cohorts, best positive results were particularly observed in cohort #2 (the one in which patients were treated with the highest dose of VY-AADC01). These positive data include substantial improvement in UPDRS scales in on and off medication states, reduction in the daily off-time and lower requirements for daily doses of levodopa. In parallel, a good safety profile and a lack of serious adverse events was also reported. According to the information provided within the press release, this company is planning to enroll five more patients within a new cohort, to be treated with a three-fold higher total dose than cohort #2. By the end of 2017 the start of a placebo-controlled trial is planned. Taken together, these data hold great promise for gene therapy strategies targeting AADC and indeed very good results were also reported by another trial with the same focus on AAV2-AADC that has been conducted in Japan by an independent research group (Muramatsu et al., 2010).

At this point, it is also worth commenting on a recently-available strategy known as "ProSavin®" that also shares a similar rationale as AAV2-AADC, at least to some extent. ProSavin® is based on initial experiments carried out in the non-human primate model of Parkinson's disease. To the aim of achieving a natural production of dopamine in a continuous manner, Jarraya et al. (2009) have designed a lentiviral vector coding for three genes needed for dopamine synthesis within a tricistronic cassette (tyrosine hydroxylase, AADC and guanosine 5'-triphosphate cyclohydrolase 1). Upon delivery of the lentiviral vector into the post-commissural putamen of MPTP-treated macaques, a safe restoration of extracellular dopamine levels is noticed, together with a marked improvement in motor deficits throughout a follow-up of 12 months. Furthermore, lentiviral-driven observed increases in extracellular dopamine levels did not resulted in the appearance of off-induced dyskinesia. Such appealing data gathered from experimental testing in NHPs motivated the design of an open-label phase 1/2 clinical trial enrolling a cohort of 15 patients. Recruited patients received a bilateral injection of ProSavin® into the post-commissural putamen (low, med and high doses were tested) and safety and tolerability was assessed with a follow-up of 12 months. UPDRS motor scores off medication improved significantly both at 6 and 12

months after ProSavin® administration, together with the lack of serious adverse effects. Long-term tolerability and clinical benefit was maintained up to 4 years after treatment. Similarly to AAV2-AADC-based trials, patients being treated with the highest doses of ProSavin® showed the greatest motor alleviation, the highest reduction in dopaminergic replacement medication and the best neuroimage improvements (for more information, see Palfi et al., 2014).

9. **New arrivals for the near future**

The use of gene therapy methodologies for achieving a symptomatic and/or disease-modifying effect in Parkinson's disease has overall a great promise. An impressive amount of new approaches are under current implementation by targeting a broad range of biological pathways. Within the past two decades and in keeping with the increased knowledge of the pathophysiology of Parkinson's disease, including genetic susceptibilities, several targets have been continuously appointed. Although at this stage it would be hard to anticipate which are going to be the most successful candidates for gene therapy, several choices will surely be tested, most of them with the underlying denominator of targeting downstream biological pathways related to alpha-synuclein intracellular processing and aggregation.

9.1. *Gene therapy-based models of alpha-synuclein aggregation*

Although the MPTP-based model of Parkinson's disease in non-human macaques has settled most of our current understanding of basal ganglia function and dysfunction (DeLong, 1990), it is also worth recognizing a number of inherent limitations to neurotoxin-based models. For instance, the natural course of the disease is not fully replicated even when achieving a chronic, long-term treatment of the macaques with very low doses of MPTP. Furthermore, the main neuropathological hallmark of the human disease, represented by the aggregation of alpha-synuclein in the form of Lewy bodies is not properly mimicked. At present, there is a marked tendency for the preparation a new generation of animal models better reproducing the progressive dopaminergic neuronal degeneration as a result of alpha-synuclein aggregation.

In this regard, the use of viral vectors (mostly AAVs) carrying the alpha-synuclein gene(s) for modeling Parkinson's disease has gained increased acceptance by the scientific community at large. Pioneer studies using AAVs coding for either the wild-type form of alpha-synuclein (WT-Syn) or the mutated form (A53T-Syn) showed a sustained transduction of alpha-synuclein in dopaminergic neurons from the substantia nigra pars compacta in rodents (Kirik et al., 2002; Klein et al., 2002). When infected this way, a substantial death of dopaminergic neurons was induced, correlated with striatal denervation and the appearance of motor defects together with behavioral abnormalities like rotational behavior upon administration of apomorphine and amphetamine (for review, see Volpicelly-Daley et al., 2016). Furthermore, it is worth noting that rodent AAV-syn animal models accurately recapitulate most of the pro-inflammatory phenomena typically observed in human Parkinson's disease (Allen Reish and Standaert, 2015). However, there still is a need for reaching a consensus on a number of issues critical for the standardization of rodent AAV-Syn models of parkinsonism, such as the best type of AAV serotype, the most efficient promoter as well as on the most adequate species and strains of the rodents. Furthermore, we strongly believe that "upgrading" the model to NHPs would undoubtedly represent a major step forward in pushing ahead these initiatives and further entering in a completely new scenario. Considering NHPs, alpha-synuclein-induced dopaminergic degeneration was carried out in marmosets by using both AAV5-mediated overexpression of either wild type or mutated forms of alpha-synuclein (Eslamboli et al., 2007). When injected directly into the substantia nigra, cell loss was more pronounced for mutated forms of alpha-synuclein when compared to the wild type form. Most importantly, this model recapitulates most of the behavioral, motor and histological disturbances that typically characterize Parkinson's disease. For those scientists interested on going deeper into these initiatives, please see the accompanying manuscript of Jeff Kordower and co-workers.

9.2. *Gene therapy with glucocerebrosidase for alpha-synuclein clearance*

Glucocerebrosidase (GBA) is a lysosomal enzyme involved into the conversion of glucosylceramide into glucose and ceramide. Homocygotic mutations of the gene coding for GBA (GBA1 gene) leads to the development of Gaucher's disease, the most frequent lysosomal storage disease. Most importantly, it has only been recently uncovered a tight link between Gaucher's and Parkinson's diseases. It has been recently proven that the presence

of homo- or heterozygous GBA1 mutations confer a 20- to 30-fold increase in the risk of suffering from Parkinson's disease (reviewed in Sidransky and Lopez, 2012). Such finding shook the field and GBA is currently viewed as a very hot target for Parkinson's disease. Although the exact mechanism through which GBA and alpha-synuclein talk to each other still remains to be fully elucidated, there is a kind of bidirectional loop sustaining loss-of-function of mutated GBA following augmented alpha-synuclein neuropathology, and indeed it seems that increases in alpha-synuclein aggregation results in a reduced expression of GBA (see Blanz and Saftig., 2016; Migdalska-Richards and Schapira, 2016). Furthermore, a natural increase in alpha-synuclein oligomerization leading to reduced expression of GBA activity has been reported in aged monkey brains (Liu et al., 2015). Accordingly, a number of initiatives under the common ground of increasing GBA activity to conduct a clearance of aggregated forms of alpha-synuclein are currently under development. For instance, small molecular chaperones under current use for Gaucher's disease (ambroxol and isofagomine) are also being tested in an attempt to reduce alpha-synuclein burden (reviewed in Migdalska-Richards and Schapira, 2016). Within the field of gene therapy and Parkinson's disease, Rocha et al. (2015) have recently shown that the co-injection into the substantia nigra of two different AAVs (one coding for A53T alpha-synuclein, the other one coding for GBA) exerts a substantial neuroprotective effect on dopaminergic neurons. It is expected that AAV-mediated transfection of GBA into dopaminergic neurons in macaque model of synucleinopathies will soon start being tested.

10. **The added value of non-human primates for gene therapy-based therapies**

Non-human primate models of Parkinson's disease have been -and surely will continue to be- instrumental in advancing our understanding of Parkinson's disease. Gene therapy tools in NHPs open new appealing research avenues not only under a translational perspective, but also for boosting the existing know-how on the fundamental mechanisms underlying of the pathophysiology of PD. We guess that in the next few years currently available models of Parkinson's disease in NHPs by chronic MPTP intoxication will be gradually superseded by models based on dopaminergic cell death sustained by the progressive aggregation of alpha-synuclein. Models based on alpha-synuclein aggregation in most cases are likely to be generated with different types of viral vectors. Nevertheless and before these new arrivals became a truly available alternative, it is worth recognizing that

the MPTP model of Parkinson's disease in NHPs will continue to be -at least for the next years- the gold-standard choice that best recapitulates the main cardinal symptoms of the disease. Indeed, it is worth stressing the fact that most of the clinical trials mentioned here, together with few more ones under current development, have been finally implemented as a result of preclinical evidence gathered from experiments conducted in MPTP-treated NHPs. Finally, when modeling PD in NHPs, it should be taken into consideration that most of the patients to be enrolled in clinical trials will probably be suffering from advanced stages of PD and therefore preclinical research to be conducted in NHPs should be based on animal models properly mimicking both disease progression and severity. To what extent this can be achieved with either neurotoxins like MPTP or with models based on alpha-synuclein aggregation still is an open question requiring a properly-balanced debate in an attempt to reach a final consensus.

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