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Biodistribution Analysis of Striatal and Cerebellar Administration of Modified Adeno-Associated Viral Vectors in Normal Mice

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Mestrado em Ciências Biomédicas - Mecanismo de Doenças

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coorientação de Professor Doutor Clévio Nóbrega

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Declaro ser o(a) autor(a) deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Inês Torquato Afonso)

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Resumo

Os vírus adeno-associados (AAV) têm 25 nm de diâmetro e transportam uma molécula de DNA de cadeia simples. O seu genoma está flanqueado por duas repetições terminais invertidas e codifica para proteínas de replicação, proteínas da cápside e para a proteína AAP. É um vírus cuja replicação depende da co-infeção com outro vírus, como por exemplo o adenovírus ou o herpes simplex vírus, pois não possui a capacidade de se replicar por si só. Até hoje foram descritos 13 serótipos de AAV e não existe na literatura doenças associadas à sua infeção. Os vetores AAV demonstraram ser sistemas de entrega seguros e fiáveis para entrega de transgenes terapêuticos no sistema nervoso central, mas também noutros órgãos e tecidos. O sistema nervoso central possui um caráter imuno privilegiado, uma vez que, está protegido pelas meninges, pela barreira hemato-encefálica e pelo líquido cefalorraquidiano, que em conjunto previnem a passagem de agentes nocivos exógenos, mas também de agentes terapêuticos. É, portanto, urgente encontrar novos métodos para que o transgene terapêutico atinja as células alvo no tratamento ou na cura de doenças neurológicas. O presente estudo pretende investigar as propriedades do novo vetor mosaico AAV1/AAV2 injetado no estriado e nos núcleos profundos do cerebelo (DCN), comparando-o com os serótipos parentais AAV1 e AAV2. Os critérios avaliados foram a biodistribuição do vetor pelo cérebro, as regiões do cérebro que é capaz de transduzir, o tipo de células que infeta e a toxicidade inerente à sua administração.

O novo vetor mosaico AAV1/AAV2 é um AAV recombinante que transporta uma cadeia de DNA simples constituído por as repetições terminais repetidas do serótipo AAV2, o gene que codifica para a proteína *green flourescent protein* (GFP) e pelo promotor do citomegalovírus. A particularidade que diferencia este vetor dos já existentes, é a constituição da sua cápside, uma vez que a mesma é constituída por proteínas da cápside do serótipo AAV1 e do serótipo AAV2, originando-se por isso uma cápside tipo mosaico, daí o nome, vetor mosaico AAV1/AAV2. Ao possuir estes dois serótipos como constituintes da sua cápside, é possível que o vetor mosaico contenha características das cápside dos vetores parentais tais como, a capacidade de transduzir as células neuronais, característica dos AAV1 e ao mesmo tempo seja capaz de reconhecer o recetor de heparina que é comumente conhecido por ser o recetor primário do AAV2. Este recetor é por isso utilizado para o processo de purificação dos vetores recombinantes do AAV2, através de cromatografia por afinidade à heparina. Desta forma, há a possibilidade do vetor mosaico

AAV1/AAV2 poder ser purificado pelo mesmo método. O método da tripla transfecção foi utilizado para a produção dos vetores virais. Este consiste na transfecção de uma linha celular de *packaging* (HEK293) com três plasmídeos: um plasmídeo *helper*, um plasmídeo com o transgene de interesse e um plasmídeo que codifica para as proteínas da cápside; que, neste caso específico, são dois plasmídeos, um codificante para as proteínas da cápside do serótipo do AAV1 e outro codificante para as proteínas da cápside do serótipo AAV2. Do mesmo, resulta a produção de um novo vetor com aproximadamente 50% de proteínas da cápside do serótipo AAV1 e os restantes 50% do serótipo AAV2. Numa primeira experiência, murganhos C57BL/6 foram injetados no estriado com 3×10^9 genomas virais (vg) do vetor mosaico AAV1/AAV2 ou com um dos vetores dos serótipos parentais, AAV1 ou AAV2. Numa segunda experiência, os murganhos foram injetados nos núcleos profundos do cerebelo com 5×10^9 vg do vetor mosaico AAV1/AAV2 ou com os vetores dos serótipos parentais. Um mês após a injeção, os animais foram sacrificados e o cérebro foi separado, um hemisfério para análise molecular e outro dos hemisférios para análise histológica.

Os resultados sugerem que o vetor mosaico AAV1/AAV2 tem uma melhor biodistribuição e transdução comparativamente aos vetores dos serótipos parentais, aquando da sua injeção no estriado. No estriado, os vetores parentais e o vetor mosaico AAV1/AAV2 foram capazes de se espalhar desde a região da injeção para a região mais dorsal do cérebro. No cerebelo os vetores distribuíram-se desde os núcleos profundos do cerebelo para todo o cerebelo, assim como para as regiões que o cerebelo recebe e envia projeções. No estriado, o vetor mosaico AAV1/AAV2 foi o que obteve melhores resultados de transdução e biodistribuição, sendo capaz de transduzir o córtex, o estriado, tálamo, os núcleos endopiriformes, núcleos sub-talâmicos e o *globus pallidus;* enquanto que, aquando a injecção nos núcleos profundos do cerebelo, o vetor do serótipo AAV1 teve melhor transdução e biodistribuição, sendo capaz de transduzir o cerebelo, o tálamo, o mesencéfalo, o núcleo central da amígdala, a medula e a ponte. Tanto no estriado, como nos núcleos produndos do cerebelo não foi possível inferir o tropismo celular dos vetores parentais e o vetor mosaico AAV1/AAV2, pois nenhum co-localizou com os marcadores utilizados, NeuN, GFAP e Iba-1 que marcam respetivamente, os neurónios, astrócitos e microglia. No entanto, é possível verificar a transdução das células de Purkinje por microscopia, uma vez que, a região onde estas se localizam se encontra a expressar GFP. Os testes toxicológicos revelaram que após a administração dos

vetores parentais e o vetor mosaico AAV1/AAV2 no estriado, não houve perda de marcador neuronal.

Em conclusão, o vetor mosaico AAV1/AAV2 demonstrou ser um vetor seguro, com resultados que indiciam ser um bom candidato para transdução de células do sistema nervoso central. Este foi capaz de manter o tropismo celular do serótipo parental AAV1 e, simultaneamente, ser purificado pelo método de purificação utilizado para o AAV2, i.e. a cromatografia de afinidade à heparina. Aquando da injeção do vetor mosaico AAV1/AAV2 no cerebelo, a biodistribuição parece estar diminuída em relação ao serótipo AAV1, o que levanta a questão quanto ao contributo do serótipo AAV2 para o modo de dispersão do vetor nesta região.

No futuro são necessários testes complementares para determinar se a utilização do vetor mosaico AAV1/AAV2 é superior relativamente aos serótipos parentais e ainda determinar a população neuronal especifica traduzida pelo vetor de interesse. Seria muito interessante que o vetor AAV1/AAV2 fosse empacotado com um gene terapêutico para ser testado, por exemplo, num modelo animal de uma doença neurodegenerativa. Caso os resultados demonstrassem que a utilização do vetor mosaico AAV1/AAV2 fossem vantajosos em relação aos vetores conhecidos, poderia vir a ser utilizado em ensaios clínicos para o tratamento ou cura de doenças neurodegenerativas.

Palavras-chave: Vírus Adeno-Associado, Sistema de Entrega, vetor mosaico AAV1/AAV2, Estriado, Cerebelo, Núcleos Profundos do Cerebelo

Abstract

Adeno-associated viral (AAV) vectors have demonstrated to be safe and reliable gene delivery systems to the central nervous system (CNS). Due its immune privileged status, it is difficult to deliver the therapeutical treatment to the target cell and treat neurological disorders of the CNS. This study investigates the spreading, transduction, cell tropism, and toxicity of a novel mosaic AAV, the AAV1/AAV2 delivery system, upon intraparenchymal injection in the striatum and in the deep cerebellar nuclei (DCN) of normal mice.

The mosaic AAV1/AAV2 vector was produced using the "triple" transfection method, however, the packing cell was transduced by 2 plasmids encoding for the AAV1 and AAV2 serotype capsid proteins instead of one. The first cohort of C57BL/6 mice was bilaterally injected with 3×10^9 vg in the striatum with mosaic AAV1/AAV2 or parental serotypes, AAV1 and AAV2. The second cohort was bilaterally injected in the DCN region of the cerebellum with 5×10^9 vg of mosaic AAV1/AAV2 or parental serotype.

The results indicate that in the striatal injection, the mosaic AAV1/AAV2 vector had better spreading and transduce more brain regions than the parental serotypes. The mosaic AAV1/AAV2 and parental vector tropism was unable to be detected with the used antibodies, NeuN, GFAP and Iba-1, which detect neurons, astrocytes, and microglia, respectively, in both striatum and DCN. None of the viral vectors exhibited toxicity in the striatal injection. In the DCN injection, the AAV1 serotype had better spreading and higher transduction levels than the other vectors.

In conclusion, the mosaic AAV1/AAV2 is a safe delivery vehicle for CNS with wide transduction results upon intraparenchymal injection. It is capable of sustaining the AAV1 neuronal tropism, as well as to be purified by the AAV2 standard technique, i.e. heparin affinity chromatography. However, further investigation is necessary to increase the sample size and determine the vector cell tropism.

Keywords: Adeno-Associated Virus, Delivery System, Mosaic AAV1/AAV2 vector, Striatum, Cerebellum, Deep Cerebellar Nuclei.

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List of abbreviations, Acronyms and Symbols

AADC – Aromatic L-amino Acid Decarboxylase

AAP – Assembly Activation Protein

AAV – Adeno-associated virus

AAVS1 – AAV integration site 1

Ab – Antibody

AD – Alzheimer's disease

ALS – Amyotrophic Lateral Sclerosis

ANOVA -Analysis of Variance

APCs – Antigen Presenting cells

ATMPs – Advanced Therapy Medical Products

BBB – Brain-Blood Barrier

BCSFB – Blood-Cerebral Spinal Fluid Barrier

Cap – Capsid

cDNA – complementary DNA

CFTR – Cystic Fibrosis Transmembrane Conductance Receptor

cGMP – current Good Manufacturing Practice

CLIG/GEEC – Clathrin-Independent Carriers / GPI-anchored-enriched endosome compartment

CMT1A – Charcot-Marie-Tooth Neuropathy Type 1A

CMV – Cytomegalovirus

CNS – Central Nervous System

CREATE – Cre Recombinant-based AAV-targeted Evolution

CRISPR – Clustered Regulatory Interspace Short Palindromic Repeat

CSF – Cerebrospinal Fluid

CTE – Chronic Traumatic Encephalopathy

DC – Dendritic Cell

DCN – Deep Cerebellum Nuclei

DMEM - Dulbecco's Modified Eagle Medium

dsDNA – double stranded DNA

dsDNA – double stranded DNA

EGFR-PTK – Epidermal Growth Factor Receptor Protein Tyrosine Kinase

EMA – European Medicine Agency

FDA – Food and Drug Administration

FELASA - Federation of Laboratory Animal Science Association

FGFR1 – Fibroblast Growth Factor 1 Receptor

GAL – Galactose

GARD – Genetic and Rare Diseases Information Center

GFAP – Glial Fibrillary Acidic Protein

GFP – Green Fluorescent Protein

HEK293 – Human Embryonic Kidney 293

HGFR – Hepatocyte Growth Factor Receptor

HSPG – Heparin Sulfate Proteoglycan

HSPG – Heparin Sulfate Proteoglycan

IC – Intrathecal

ICV – Intracerebroventricular

IEC – Ion Exchange Chromatography

IM – Intramuscular

IP – Intraparenchymal

ITR – Inverted Terminal Repeat

IV – Intravenous

LamR – Laminin Receptor

LDL – Lipoprotein Lipase

LPLD – Lipoprotein Lipase Disorder

miRNA – micro RNA

MMP – Metalloproteases

MS – Multiple Sclerosis

NAbs – Neutralizing Antibodies

NGS – Normal Goat Serum

NHP – Non-Human Primate

NSE – Neuron Specific Enolase

O.T.C. – Optimal Cutting Temperature

ORF – Open Reading Frame

pAAV-RC – plasmid AAV – Replication and Capsid

PBS – Phosphate-buffered Saline

PCR – Polymerase Chain Reaction

PDGFR - Platelet-Derived Growth Factor Receptor

PFA – Paraformaldehyde

PolyA – Polyadenylation

rAAV – recombinant AAV

RBE – Rep Binding Elements

rc- Replication Competent

Rep – replication

RPE65 – Retinal Pigment Epithelium-specific 65

scAAV – self-complementary AAV

SCA – Spinocerebellar Ataxia

SEM – Standard Error of the Mean

shRNA – short-hairpin RNA

SIA – Sialic Acid

siRNA – small interference

SMA – Spinal Muscular Atrophy

SMN1 – Survival Motor Neuron 1

ssDNA – single stranded DNA

ssRNA – single stranded RNA

SV40 – Simian Virus 40

TALEN – Transcription Activator-like Effector Nucleases

ZFN – Zinc-finger

Chapter 1 – Introduction

1.1. Central Nervous System

The nervous system is complex and responsible for cognition, learning, memory, behaviour, and movement (Artusi *et al.*, 2018). With 100 billion neurons, the brain is considered the most complex organ in the human body (Pacitti *et al.*, 2019). Together, the brain and the spinal cord comprise the central nervous system (CNS) and is where information analysis occurs (John Laterra, 1999). The CNS is protected by the blood brain barrier that restrains the cerebral spinal fluid and CNS cells from infections spread in the blood (Koyuncu *et al.*, 2013).

Neurodegenerative disorders are typically difficult to treat due to various factors, late diagnosis, complexity of the nervous system and physical barriers that limit the entry of systemic drug administration (Choong *et al.*, 2016). Given the circumstances the brain is considerate a prospective target for gene therapy of neurological disorders (Sanftner *et al.*, 2004). Herein, an overview of the CNS and its immune privileged status is going to be made, and finally propose a novel strategy for neurological disorders vehicle.

1.1.1 CNS barriers

The CNS is an immune privileged organ (Rua and McGavern, 2018), in other words it is protected from exogenous neurotoxic or pathologic molecules via blood stream. Thusly, three different protective mechanisms exist to maintain the environment and protect from injury and disease: 1) the meninges, which are a three-layered system that surrounds and supports the brainstem and spinal cord, conferring a physical protection, 2) the Blood-cerebral spinal fluid barrier (BCSFB) that is located at the choroid plexus epithelium of the 4 ventricles, and 3) the Blood-brain barrier (BBB) that seals the vascular system in the brain conferring anatomical and physical protection revised by Pacher and his collaborators (Pachter *et al.*, 2003).

1.1.1.1 Meninges

The meninges are composed by three cellular layers: the dura mater (meaning “hard mother”), arachnoid mater and pia mater (Rua and McGavern, 2018). Located adjacent to the skull, the dura mater is a dense and tough, collagenous membrane, where the blood and lymph vessels are located and is highly enervated. The middle layer is the arachnoid mater, where cells are

connected by tight junctions that regulate the molecules passage from the blood. Those cells also protect spiderlike processes that extend until the third layer called trabeculae where the cerebrospinal fluid flows. The inner layer is the pia mater composed by a thin layer of cells that coats the brain and the spinal cord parenchyma.

1.1.1.2 Blood-Cerebral Spinal Fluid Barrier (BCSFB)

The BCSFB is formed by the choroid plexus which are in the cerebral ventricular system and the arachnoid membrane (Erb *et al.*, 2020; Ghersi-Egea *et al.*, 2018). The choroid plexus functional unit is a monolayer of cuboidal ependymal cells with plentiful villi, highly vascularised by fenestrated endothelium, that contrary from the BBB are not tightly joint by tight junction; therefore are permeable to molecules up to ~800 kDa (Ghersi-Egea *et al.*, 2018). The choroid plexus epithelium prevents toxic and infectious molecules from entering the CNS (Reinhold and Rittner, 2017), producing a large portion of the cerebrospinal fluid (CSF) that is later reabsorbed at the subarachnoid space. The CSF is responsible for protecting the brain from injury by creating the buoyancy effect, hydrating the brain and maintaining the appropriate intracranial pressure, transport of nutrients, regulations of the stem cells niche by the secretion of regenerative factors and lastly, work as a neuroimmune gateway for the immune cells in the CNS (Laterra J, 1999).

1.1.1.3 Blood-Brain Barrier

The Blood Brain Barrier (BBB) is a physiological barrier which limits the exchange of potential neurotoxic components from the plasma, blood cells and pathogens to the CNS (Siegenthaler *et al.*, 2013). It acts also as an enzymatic degradation barrier, allowing however the passive diffusion of gas molecules and small lipidic molecules (<400Da) (Banks, 2016). The cells that comprise the neurovascular unit of the BBB are vascular cells (endothelium, pericytes and smooth muscle cells), neurons and neuroglial cells, (Iadecola, 2017). Altogether, the neural cells are needed for the functional barrier-genesis and regulation of the molecular exchange from the blood to the brain and vice-versa (Daneman *et al.*, 2010; Siegenthaler *et al.*, 2013). The BBB is organized by a monolayer of tightly-sealed endothelial cells that display low paracellular and transcellular permeability, through molecular junctions between each other and pericytes (Armulik *et al.*, 2010; Daneman *et al.*, 2010), through tight junctions, gap junctions and adherents junctions

(Sweeney *et al.*, 2018). Also, the endothelial and pericytes membrane have specific efflux and influx transports, receptors and channels that allow the transcytosis (Banks, 2016; Reinhold and Rittner, 2017).

New insights about the BBB physiology at the molecular and cellular level have raised attention about how impaired function can lead to neurodegenerative diseases (Duarte Lobo *et al.*, 2020; Sweeney *et al.*, 2019), such as Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis (ALS) and multiples sclerosis (MS), HIV-1-associated dementia and chronic traumatic encephalopathy (CTE) (Iadecola, 2017; Reinhold and Rittner, 2017; Sweeney *et al.*, 2018).

1.1.2 Cell types and organization

Neuroanatomists classify the tissue composing the nervous system in three groups, such as: neuron, neuroglia or glial cells and ependymal cells (Morest and Silver, 2003). This distinction exists due to the structural and morphological difference between these cell types (Morest and Silver, 2003). Researchers have determined the glia:neuron ratio to be less than 1:1 in the human brain (von Bartheld *et al.*, 2016).

1.1.2.1 Neuron or Nerve Cell

The neuron is known to be the basic unit of the brain and it was discovered in the late 19th century by Santiago Ramon y Cajal. Neurons have an ectodermal, neuroepithelium origin and differ according to their functional properties in structure and features. However, all have in common the cell organization in tree distinct regions: dendrites, soma or neural cell body and axon, confining a specific region for organelles and subcellular components granting a specific feature for each region (Morest and Silver, 2003). Neurons can be classified into sensory, motor or interneurons, but all share the same function, receive a signal, integrate the incoming signal and communicate signal to the target cell.

1.1.2.2 Neuroglia or Glia Cells

Neuroglia cells are supportive cells that confer the maintenance of the homeostasis and protect CNS cells. The neuroglial cells have different precursor origins, microglial cells have mesodermal/neuroepithelial origin and macroglia cells have ectodermal/myeloid origin. Macroglia cells also include astrocytes and oligodendrocytes (Verkhratsky *et al.*, 2019).

Microglia

Microglia cells are the only cells from the nervous system cells derived from an erythromyeloid progenitor cells (Barres, 2008). Microglia cells are known as brain macrophages and have recently been shown to play a key role in various stages of nervous system development and activity, such as neural survival and neuron phagocytosis: thereby microglia refine CNS formation and function (Allen and Lyons, 2018; Crotti and Ransohoff, 2016).

Microglia most used antibodies are against HLA-DR, Iba1 and CD68; however, Iba1 is used in structural studies while the two others are used as neuropathological markers. Iba1 is a cytoplasmic protein with F-actin binding and actin-cross-linking activity (Hendrickx *et al.*, 2017).

Astrocytes or Astroglia

Astrocytes are specific cells of the brain and spinal cord and its morphology can vary between stellate-shaped protoplasmic and fibrous (Liddelow and Barres, 2017; Miller and Raff, 1984). The protoplasmic astrocytes are predominantly located in the grey matter and function by prolonging its extensions to the spine clefts, creating an appropriate chemical environment for the neural signalling. Fibrous astrocyte are frequently found in the white matter and are typically smaller, their main function is to clothe the blood vessels in the nervous system to prevent the passage of harmful compounds (Barres, 2008; Kohler *et al.*, 2019; Miller and Raff, 1984).

Astrocyte has been targeted to combat neurodegenerative diseases (Kery *et al.*, 2020; Siracusa *et al.*, 2019) since their dysfunction may lead to an active inflammatory state of the astrocytes, induction and progression of inflammation and astrocyte senescence which can lead neuro-pathologies, such as Alzheimer's disease and Huntington Disease (Siracusa *et al.*, 2019).

Mature astrocytes markers are antibodies against to glial fibrillary acidic protein (GFAP), present in the cell processes (Blackburn *et al.*, 2009)

Oligodendrocytes or Oligodendroglia and Schwann cells

Oligodendrocytes are specialised cells from de CNS that generate myelin sheaths, thereby augmenting nerve impulse conduction and providing metabolic support to axons from different neurons (Allen and Lyons, 2018). The equivalent of oligodendrocytes in the peripheral nervous system are the Schwann cells; however, Schwann cells can only myelinate one neuron while oligodendrocytes can myelinate more than one neuron (Philips and Rothstein, 2017).

Typical oligodendrocytes marker is antibody against oligodendrocyte-specific protein (Doorbar *et al.*) (Michalski *et al.*, 2018) know as a protein present in the myelin sheaths and is also involved with proteins responsible for tight junction formation (Bronstein *et al.*, 1997).

1.1.2.3 Ependymal cells

The ependymal cells raise from the neuroepithelium located in the ventricular zone in the fetal cerebrum and spinal cord (Lehman, 2008). In the adult brain, ependymal cells are located within hypothalamic region of the third ventricle in the specialized ependyma of the circumventricular organs. Ependymal cells are highly linked by tight junctions and their function is to maintain the fluid balance in the brain and support the neurogenesis in subventricular zones (Lehman, 2008). The apical pole of the cell has cilia extensions that play critical role in the development and maintenance of the nervous system by beating in a specific direction.

1.1.3 Neural transduction

Transduction is a generic term used for the transferring of genetic material from one cell to the other. It was firstly described in bacteria, where researchers noticed that genetic material could be transferred during the mating process (Tatum and Lederberg, 1947). Later, other ways of genetic material transferring were observed between drug-resistant mutants of salmonella and wild-type form. This happened because of the presence of a bacteriophage that carried the nucleic acid from one bacterium to another, this process was termed as transduction. A few years later it was

uncovered the existence of a virus able to transduce eukaryotic cells and, in 1994, occurred the first successful adeno-associated (AAV)-based long term gene expression transduction in murine brains (Kaplitt *et al.*, 1994).

Nowadays, many therapies have used viruses as vectors to deliver nucleic acid to the target cells. Studies using viral vectors in the CNS have demonstrated that viruses behave differently in infecting neurons (Koyuncu *et al.*, 2013).

1.2. Delivery Systems for Gene Therapy

According to the Genetic and Rare Diseases Information Center (GARD) and Global Genes, about 80% out of the total 7.136 existing diseases are genetic, and only 500 are treatable with the 10 000 drugs available (Goswami *et al.*, 2019). Such drugs offer temporary relief of the symptoms and increase the life expectancy, but never cure the disease. Therefore, it is necessary to find long lasting strategies that ideally would slow or stop the disease progression for patients and avoid the emerge of symptoms in eventual patients (Lykken *et al.*, 2018).

Gene therapy is a burgeoning field with the far beyond potential to cure genetic diseases, and with 2500 approved clinical trials worldwide (Anguela and High, 2019; Wirth *et al.*, 2013). Gene therapy is described by the European Medicine Agency (EMA) as a "biological medicinal product that contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to humans to regulate, repair, replace, add or delete genetic sequences and its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence" (Goswami *et al.*, 2019). Gene therapy has a broad application, such as: gene addiction, gene editing, gene silencing, gene modification/engineering and non-coding RNA modulation (Valdmanis and Kay, 2017) and can be used as a *in vivo* or *ex vivo* method (Choong *et al.*, 2016).

Gene therapy appears simple in principle, but in reality, delivery of nucleic acid to the target cell has many obstacles. It has been estimated that ten minutes following intravenous injection in mice of plasmid DNA (pDNA), the endonucleases present in physiological fluids and the extracellular space degrade it (Yin *et al.*, 2014). For this reason, it is urgent to find suitable carriers for gene therapy.

1.2.1 Ideal Features of a Delivery System

A delivery system for a gene therapy strategy must take in consideration several factors. For instance, it is important to know if the therapeutic strategy is *in vivo* or *ex vivo*, target dividing or non-dividing cells, target cell/organ location (surface or deep), duration (Kaestner *et al.*, 2015), integrative vector and trigger immune reaction.

Safe and effective vectors have been the fundamental challenge for gene therapy. It is important for a gene vehicle to have packing capacity and protection of an effective gene, trigger

low immune response and long circulation time, targeted tropism, effective endocytosis, effective endosomal escape and effective nucleic acid delivery to the nucleus (Athanasopoulos *et al.*, 2017; Chen *et al.*, 2017).

Due to the CNS complexity, ideal vectors for neurological applications would be able of crossing the BBB upon intravenous administration and demonstrate adequate viral spread or overall CNS transductions (Lykken *et al.*, 2018).

1.2.2 Vectors

Vectors can be classified as non-viral (Celec and Gardlik, 2017; Pahle and Walther, 2016) and viral vectors (Sung and Kim, 2019). Both viral and non-viral vectors present advantages and disadvantages and have been tested for neurological disorders in pre-clinical and clinical trials (see Table 1.1).

Table 1. 1 Advantages and disadvantages between non-viral and viral vectors.

	Non-Viral vectors	**Viral Vectors**
Advantages	Safe and stable Potentially unlimited packing capacity Inexpensive to manufacture at GMP Biocompatible Low immunogenicity response Eligible re-administration High production yields	Broad tropism Higher transduction efficiency Stable transduction
Disadvantages	Low delivery efficiency Cytotoxicity	Limited DNA packaging capacity Difficulty of vector production Advised against re-administration

1.2.2.1 Non-Viral Vectors

Non-viral delivery systems for therapeutic DNA delivery comprise two major groups: “physical methods”, which are less applicable in systemic delivery, and “chemical methods” that can be applied both locally our systemically (Pahle and Walther, 2016; Yin *et al.*, 2014).

Physical Delivery System

Over the last decade, physical gene transfer methods have significantly improved the efficiency on delivering genetic material, such as naked DNA or RNA, by applying mechanical, electrical, ultrasonic, or hydrodynamic energy to transiently disrupt the cell membrane for nucleic acid entry without adding chemical agents (Slivac *et al.*, 2017; Yin *et al.*, 2014). This includes needle injection of naked DNA, gene gun, electroporation, hydrodynamic delivery, sonoporation, magnetofection and laser radiation (see Table 1.2) (Pahle and Walther, 2016; Yin *et al.*, 2014).

Table 1. 2 Summary of physical non-viral delivery systems.

Non-viral vectors	**Scientific principle**	**Genetic material:**	**Method**	**Targeted diseases:**	**References:**
Physical vectors					
Needle injection	Microneedle injection	Naked DNA	The DNA uptake occurs due the pressure caused by solution injection.	Tumor	(Mellott *et al.*, 2013; Pahle and Walther, 2016; Slivac *et al.*, 2017)
Gene gun	Mechanical pressure	DNA and RNA coated nanoparticle	This mechanism forces the intracellular nucleic acid transfer by high voltage electronic discharge or helium discharge.	Acute lymphoblastic leukemia; skin cancer.	
Jet- injection	Mechanical compression	Naked or complex nucleic acid	The genetic material is placed in a small jet and released at high speed directly on the target tissue.	Malignant melanoma	
Electroporation	Electric field	pDNA	Two electrodes needles are placed equidistant from the center of the circle, where the nucleic acid was injected, to transiently form pores in the cells membrane and enable the nucleic acid diffusion.	Malaria; viral infections.	

Hydro-poration	Hydrodynamic pressure	pDNA	This method utilizes hydrodynamic pressure by injection of a large volume and flow of pDNA into the intracellular space.	Cancer; hematological disorders; metabolic disease; infectious disease; muscle disease; malignant diseases.	
Sonoporation	High intensity ultrasound waves	DNA and RNA	This method transiently permeabilize of cell membrane with high intensity ultrasound waves for nucleic acid entry.		
Magnetofection	Magnetic field	DNA carried by magnetic nanoparticle	When a magnetic field is applied, the nanoparticles disintegrates, releasing the DNA or drug.	Cancer	
Photoporation	Laser pulse	DNA	This method relays on a laser pulse to create pores to permeabilize cell membrane and enable entry of exogenous DNA		

Chemical Delivery System and Bacterial Vectors

Chemical non-viral delivery systems represent powerful methods for successful and efficient gene transfer in *in vivo* and *in vitro* studies and in clinical approaches (Pahle and Walther, 2016). It is classified into peptide-based, polymer or lipid-based vectors and inorganic vectors (see Table 1.3) (Pahle and Walther, 2016; Sung and Kim, 2019). The chemical carriers form condensed complexes by binding to charge dependently to nucleic acid, sheltering the molecules against cellular mechanisms of degradation and denaturation (Slivac *et al.*, 2017).

This system faces many challenges in gene delivery such as extracellular stability, specific cell targeting, internalization, endosomal escape, nuclear envelope entry, nucleic acid release, and genomic integration (Pahle and Walther, 2016). However, alternatives such as peptides and cell-derived vesicular structures can mimic natural transfection particles, plus, redesigning the composition can improve its transfection efficiency (Slivac *et al.*, 2017).

Bactofection is the genetic material transfer into eukaryotic cells from bacterial vectors (Celec and Gardlik, 2017). Some of the bacteria used as a gene delivery vehicle are Salmonella, Escherichia, Listeria, Clostridium and Bifidobacterium (Cronin *et al.*, 2012). Bacterial vectors have been used in clinical research for cancer gene therapy treatment (Cronin *et al.*, 2012) and immunotherapy (Le Gouellec *et al.*, 2012). Bacterial vectors have an unlimited capacity of cargo, tropism to specific tissues and inexpensive production and amplification (Celec and Gardlik, 2017).

Table 1. 3 Summary of non-viral vectors as delivery systems- chemical delivery and bacterial vectors.

Non-viral Vector	**Carrier**	**Genetic material**	**Mechanism:**	**References:**
Chemical vectors				
Peptide-based Vectors	Cationic amino acid monomers linked by peptide bonds	DNA	The peptide-based vector complex easily with nucleic acid, shielding it from degradation and allowing endosomal escape.	(Amreddy *et al.*, 2017; Chen *et al.*, 2017; Labatut and Mattheolabakis, 2018; Nowakowski *et al.*, 2013)
Polymer based vectors	Cationic polymers	DNA and RNA	The cationic polymers escape the endosomal membrane to deliver the nucleic acid to the cytoplasm.	
Lipid-based Vector	Liposomes	DNA and RNA	The liposome is a bilayer lipid membrane able to fuse with the plasma membrane and via endocytosis the genetic material is released into the cytoplasm.	
Inorganic vectors	Inorganic nanoparticles	DNA and RNA	The inorganic nanoparticles are successfully up taken by the cells and degrade releasing the nucleic acid content.	
Bactofection	Bacterium	DNA and RNA	This method has two approaches: i)insertion of the therapeutic material in a bacterial vector, which, after the entry in the target eukaryotic cell undergoes lysis with consequent transmission of the therapeutic gene(s) into the target cell, ii)insertion of genetically modified bacterial vectors in the target tissue for direct in situ production of therapeutic molecule.	(Celec and Gardlik, 2017; Cronin *et al.*, 2012)

1.2.2.2 Viral Vectors

Viruses are known for their natural ability to transfer nuclei acids to a cell (Blessing and Deglon, 2016). The question is "why not to use them as vehicles to deliver a treatment?". The most used viruses as gene delivery vehicles are retroviruses, herpes simplex viruses, adenoviruses and adeno-associated viruses (see Table 1.4) (David and Doherty, 2017; Sung and Kim, 2019). Viral vectors vary in size, capacity, tropism, transfection efficiency, ability of transfecting dividing and non-dividing cells and triggering immune response.

For the CNS research and applications, the adeno-associated virus vectors and lentivirus vectors are the most used (Cappella *et al.*, 2019) (Blessing and Deglon, 2016).

Table 1. 4 Summary of viral vectors as delivery systems.

Virus	**Gammaretroviral**	**Lentiviral**	**Herpes Simplex Virus**	**Adenoviral**	**Adeno-Associated Virus**
Family	*Retroviridae*	*Retroviridae*	*Herpesvirus*	*Adenoviridae*	*Parvoviridae*
Viral Genome	ssRNA	ssRNA	dsDNA	dsDNA	ssDNA
Packing Size	9 - 12 kb	9 - 12 kb	120 - 240 kb	7.5 kb - 30 kb	4.7 kb
Cell Transduction	Dividing	Dividing and Non-dividing	Dividing and non-dividing	Dividing and non-dividing	Dividing and non-dividing
Integrative	Yes	Yes	No	No	No
Immunogenicity	Low*	Low*	Low	High	Low
Expression	Stable	Stable	Transient	Transient	Transient or Stable

ssRNA- single stranded DNA; dsDNA – double stranded DNA; ssDNA – single stranded DNA; kb – kilo base-pairs; *Low immunogenicity in integration-deficient lentiviral vectors (Athanasopoulos *et al.*, 2017); References:(Artusi *et al.*, 2018; Chen and Lee, 2014; Goswami *et al.*, 2019; Karasneh and Shukla, 2011; Maes *et al.*, 2019; Nowakowski *et al.*, 2013; Somia and Verma, 2000)

1.2.3 State of The Art – Clinical Trials in Gene Therapy

The recent commercialization approval of gene therapeutic agents in the European Union was a milestone in the gene therapy history and gave hope for many patients suffering from orphan diseases and other common illnesses (Kaufmann *et al.*, 2013). To increase the patient number in clinical trials and obtain more informative and reliable data, multicenter studies and gene therapy consortia studies are preferred (Kaufmann *et al.*, 2013).

According to the *The Journal of Gene Medicine - Clinical Trial site*, currently 70.2% of the clinical trials use viral vectors as gene delivery systems and a total of 244 gene therapy clinical trials (7.9%) make use of AAV as delivery vectors, (see Figure 1.1) (Medicine, 17/02/2020).

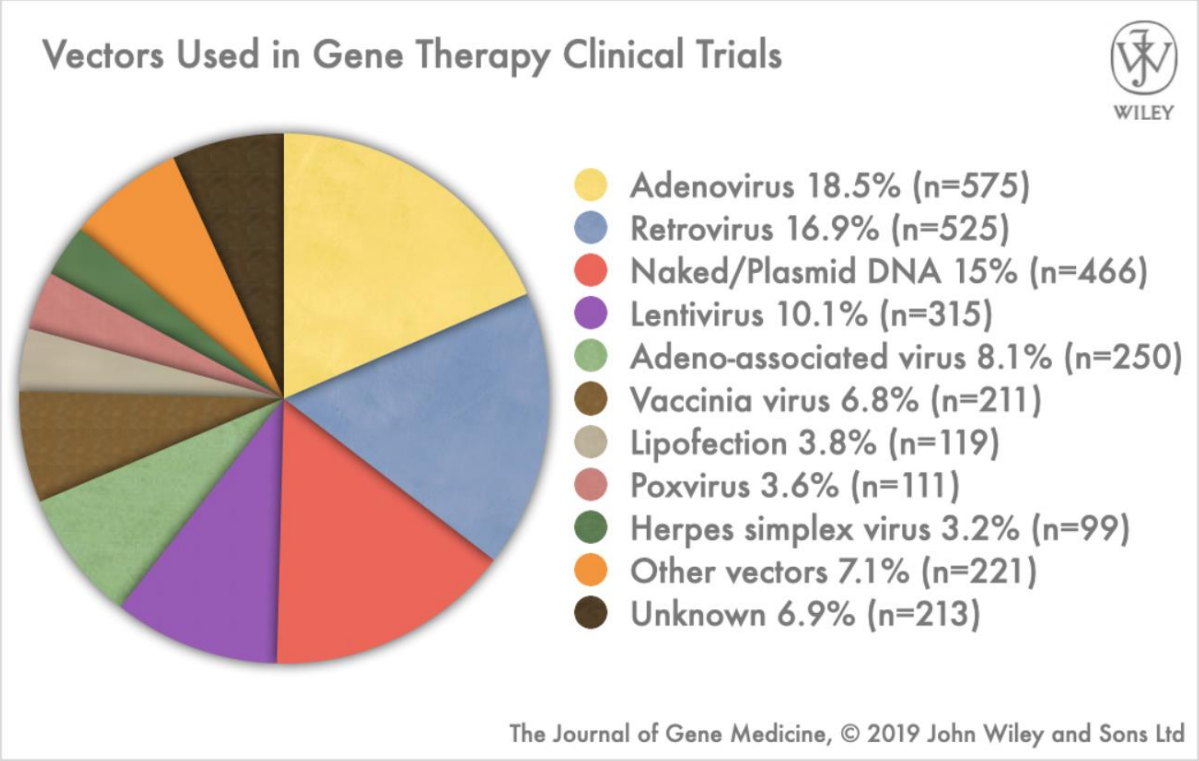

Figure 1. 1 Summary of vectors user in gene therapy clinical trials. Adapted from ABEDIA.

Gene therapy delivery to the CNS have demonstrated to be safe and well tolerated in initial trials (O'Connor and Boulis, 2015). The clinical trial website on the 17th of February,2020, also indicates that, from the 3001 ongoing clinical trials, there are 53 clinical trials for neurological diseases of which 22 use AAV vectors as delivery systems. The diseases that are being clinically tested aimed to treat Parkinson's Disease, Sanfilippo A syndrome, aromatic L-amino acid decarboxylase (AADC) deficiency, Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease

(AD), Intractable Temporal Lobe Epilepsy and Charcot-Marie-Tooth Neuropathy Type 1A (CMT1A)(Trial, 2020/02/17).

1.3. Adeno-Associated Virus (AAV)

AAV is a small non-enveloped virus classified as a *Dependoparvovirus* genus from the *Parvoviridae* family, with an icosahedral shaped capsid of approximately 25 nm in diameter (Saraiva *et al.*, 2016). AAV is an helper dependent virus, thus it needs a cellular coinfection with other virus, that encode for essential proteins to complete the AAV lifecycle and avoid cell injury or death (Maes *et al.*, 2019). The AAV virions are very resistant particles, tolerant to wide temperatures and pH variations with little or no lost loss in activity (Naso *et al.*, 2017).

AAV are capable of transducing dividing and non-dividing cells (Linden *et al.*, 1996) *in vitro* and *in vivo* and no disease has so far been associated with its infection. Together, these characteristics infer AAVs as excellent delivery vectors for gene therapy, making them widely used in preclinical and clinical trials to target monogenic disorders or complex diseases (i.e. glaucoma (Rodriguez-Estevez *et al.*, 2019)).

1.3.1 Genome

AAV holds a linear ssDNA of both positive and negative polarities (Srivastava, 2016a), which comprises two T-shaped inverted terminal repeats and an open reading frame (ORF) encoding for Rep (Replication), Cap (Capsid) and AAP (assembly activating protein) proteins genes (see Figure 1.2) (Saraiva *et al.*, 2016). The Rep open reading frame (ORF) is controlled by the p5 and p19 promoters, while Cap and APP ORF is regulated by p40 promoter (Balakrishnan and Jayandharan, 2014). The ITRs are important for genome replication and packing. The Rep proteins Rep40, Rep52, Rep68, and Rep78, are involved in protein replication, transcription integration and encapsulation. Cap proteins are important structural proteins for the AAV icosahedral capsid rigid architecture, and to mediate cell binding and internalization (Buning and Srivastava, 2019; Naso *et al.*, 2017; Saraiva *et al.*, 2016). The AAP protein is essential for nucleolar localization of VP proteins and capsid assembly (Hori *et al.*, 2019; Naso *et al.*, 2017). AAP subnuclear localization varies among different AAV serotypes and a study indicates it is a nonessential in AAV4, AAV5 and AAV11 (Earley *et al.*, 2017).

Each capsid is comprised of 60 protein monomers (VP1, VP2 and VP3 proteins), in a ratio of 1:1:10, respectively (Xie *et al.*, 2002). VP proteins vary in molecular size, (VP1 = 87kDa, VP2 = 72kDa and VP3 = 62kDa), but share a common C-terminal domain. The VP1 and VP2 N-terminal domains have an important function protecting the virus. During the packing process the N-terminal domain reside inside the capsid. However when the endosomal vesicle coats the virus the N-terminal domains externalize exposing its regions, of which phospholipase A_2 region important for endosomal escape as well as others essential regions for the nuclear trafficking (Lee *et al.*, 2018).

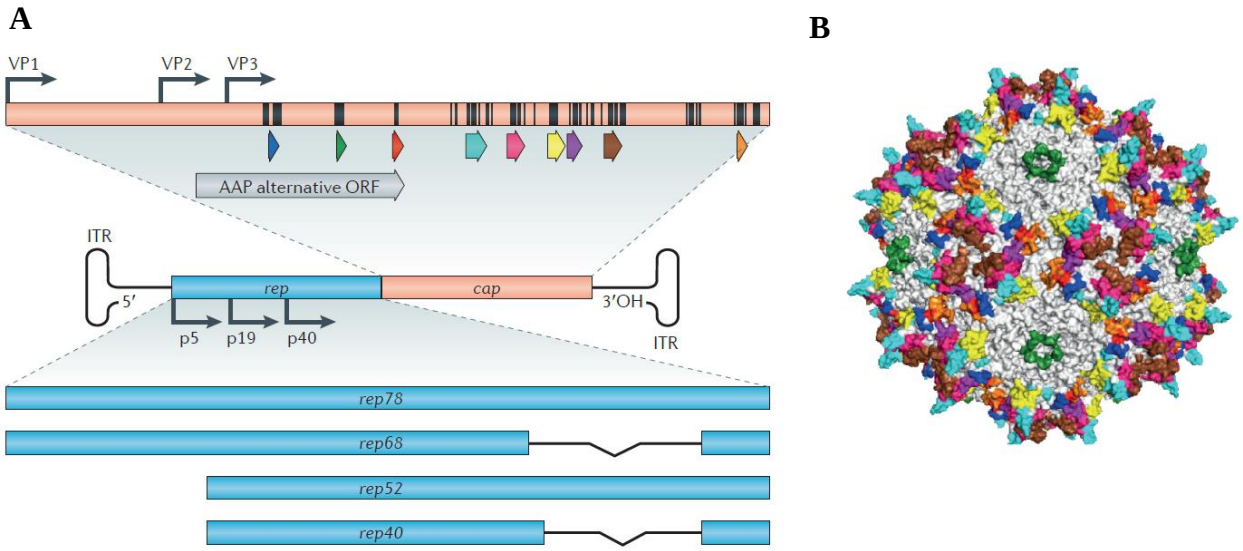

Figure 1. 2 AAV capsid and genome schematic image. **A** Represents the wild-type AAV 7.4 kb genome which encodes for rep (replication), cap (capsid) and AAP (assembly activating protein) genes between two ITRs (inverted terminal repeats) which form T-shaped hairpin ends. The rep genes rep78 and rep68 are controlled by p5 promoter; The rep52 and rep40 are controlled by the p19 promoter and the VP and AAP genes are controlled by the p40 promoter. **B** AAV capsid shown in a crystal structure, where the hypervariable regions of VP3 gene are colored to match the corresponding region on the capsid. Adapted from Kotterman *et al*, 2014.

AAV was firstly described as an integrative virus with a site-specific on human chromosome 19 locus q13.3, also known as AAVS1 (Kotin *et al.*, 1990; Linden *et al.*, 1996). A study demonstrated for instance that the presence of Rep78 expression cassette targets the integrations at the AAVS1 locus and, in combination with the AAV ITRs, it augments the integration frequency in the cellular genome (Balagúe *et al.*, 1997; Surosky *et al.*, 1997). However, upon the generation of recombinant AAVs, it was demonstrated that AAVs are persisting in tissues mainly as circular episomes (Schnepp *et al.*, 2005), which limits the risk of insertional mutagenesis.

1.3.2 Serotypes and Tropism

Knowing the panoply of existing AAV capsids allows researchers to choose the best serotype for each application, as well as the best delivery route and dose. (Keeler and Flotte, 2019).

Currently, it has been identified 13 natural occurrences of AAV serotypes, that differ in its capsid composition and cell/tissue tropism (see Table 1.5) (Hori *et al.*, 2019; Naumer *et al.*, 2012; Srivastava, 2016b). The serotype is determined by the capsid protein motifs that are recognized by specific neutralizing antibodies (Guo *et al.*, 2019). Attached to cell surface glycans, thus setting the AAV tropism (Srivastava, 2016b). However, over a hundred genomic variants have been discovered in humans and non-human primates samples (Keeler and Flotte, 2019; Saraiva *et al.*, 2016).

AAVs can be classified into three large groups, according to the glycan receptors on the cell surface that it binds to. These groups are: group i) AAV serotypes that bind to Sialic acid (AAV1, AAV4, AAV6 and AAV6), group ii) AAV serotypes that bind to heparin sulfate proteoglycans (HSPG) (AAV2, AAV3, AAV6 and AAV13) and iii) AAV serotypes that bind to galactose (GAL) (AAV9) (Huang *et al.*, 2016; Srivastava, 2016b).

The AAVR is considered the universal AAV receptor for infections of some variants (Srivastava, 2016b). The AAVR originally known as KIAAA0319L, is an uncharacterized type I transmembrane protein which is involved in the rapid endocytosis process and trafficking to the *trans*-Golgi network, *in vitro* and *in vivo.* Studies using wildtype AAV1, 2, 3, 5, 6, 8, and 9 described AAVR as an essential receptor for AAV infection (Pillay *et al.*, 2016). AAV 4 is an exception because uses an AAVR-independent pathway for internalization (Lee *et al.*, 2018).

Table 1. 5 Summary of AAV serotypes and their features.

Serotype	**Origin**	**Receptors**		**Homology to AAV2**	**Tissue Tropism**	**CNS cells**	**Cross the BBB**
		Primary Receptors	**Co-receptor**				
AAV1	NHP	α2-3/α2-6 N-linked SIA	AAVR	83%	Muscle, CNS, Heart	Neurons, Astrocytes, Oligodendrocytes; Primary Neurons	Yes (neonatal mice)
AAV2	Human	HSPG	FGFR1; integrins αVβ5 and α5β1; HGFR; LamR; AAVR	100%	Liver, CNS, Muscle	Neurons; Astrocytes	No
AAV3	Human	HSPG	LamR; HGPG; AAVR	87%	Muscle, Stem cells		
AAV4	NHP	α2-3 O-linked SIA	AAVR	62%	Eye, CNS	ependymal cells	No
AAV5	Human	α2-3 N-linked SIA	PDGFR; AAVR	51%	CNS, Lung, Eye	ependymal cells; Neurons, Astrocytes, Oligodendrocytes; Primary neurons	No

AAV6	Human	HSPG; α2-3/α2-6 N-linked SIA	EGFR; AAVR	57%	Muscle, CNS, Heart; Lung	Neurons, Motor neurons, Glia	
AAV7	NHP		AAVR	84%	Muscle, CNS	Phrenic motor neurons	No
AAV8	NHP		LamR; AAVR	83%	Liver, Muscle, Pancreas, CNS	Astrocytes (mouse) Neurons, Astrocytes, Oligodendrocytes; Primary neurons	Yes (neonatal mice)
AAV9	Human	N-linked galactose	terminal N-linked GAL of SAI; LamR; AAVR	82%	Every Tissue	Cortical Neurons (mouse)/ Neurons, Astrocytes, Oligodendrocytes; Primary neurons	Yes
AAV10	NHP			84%	Muscle	Neurons, Glia and dorsal root ganglia; Primary neurons	Yes (neonatal mice)
AAV11	NHP			65%			
AAV12	NHP				Nasal		
AAV13/AAV(VR-942)	NHP		HSPG;	88%			

References:	(Hori *et al.*, 2019; Schmidt *et al.*, 2008; Wu *et al.*, 2006)	(Srivastava, 2016b)	(Hardcastle *et al.*, 2018; Schmidt *et al.*, 2008)	(Li and Samulski, 2020)	(Aschauer *et al.*, 2013; Haery *et al.*, 2019; Li and Samulski, 2020)	Hardcastle, Boulis, & Federici, 2018;

NHP- non-human primates; H – human; SIA-Sialic Acid ; HSPG-heparin sulfate proteoglycan ;GAL - galactose; HGFR - hepatocyte growth factor receptor; PDGFR- platelet-derived growth factor receptor; EGFR- epidermal growth factor receptor; LamR- laminin receptor; CNS – central nervous system; Yes – can cross the BBB; No – Cannot cross the BBB.

1.3.3 Infection and Life Cycle

Since AAV is a non-enveloped DNA virus, it is crucial for its survival to have a very stable capsid, capable of resisting degradation at the extracellular and cellular level, i.e., proteasome, and simultaneously release its content to the target cell. A study was performed to evaluate the changes in infectivity of rAAV1, rAAV2, rAAV8 and rAAV9 in the exposure to various eluents and environmental conditions. This study concluded that all serotype infectivity was weakened when exposed to UV radiation, NaOH and NaCIO (Tomono *et al.*, 2019).

The AAV infection cycle initiates with the attachment to the primary receptors at the cell surface – glycans (see Figure 1.3). As previously described, different serotypes have different primary receptors, which are commonly followed by secondary co-receptors for optimal attachment and internalization (Nonnenmacher and Weber, 2012). The endocytosis process takes place with the invagination of the plasma membrane containing virus-receptor complexes either by clathrin-mediated endocytosis, caveolae-mediated endocytosis, or via clathrin-independent carriers / GPI-anchored-protein-enriched endosome compartment (CLIG/GEEC) (Nonnenmacher and Weber, 2012). Inside the endosome, the virion undergoes a series of pH-dependent structural changes to become able to traffic in the cytoplasm via the cytoskeletal network (Wang *et al.*, 2019). After the vesicle scission and release into the cytoplasm, the capsid undergoes profound conformational changes that are mandatory for efficient transduction and can either transit from late and/or recycling endosomes (Nonnenmacher and Weber, 2012).

To determine the importance of the conformational changes, Sonntag and collaborators microinjected intact viruses in the nucleus and cytoplasm of HeLa cells, which resulted in very low transduction (Sonntag *et al.*, 2006). They demonstrated for example that AAV2 capsid must withstand conformational changes in the endocytic vesicle for an effective transduction (Sonntag *et al.*, 2006). In this case, it is required a passage of the virions through the cytoplasm and exposure of the N-terminal domains of VP1 and VP2 outside of the capsid by endosomal processing. The N-terminal domains are essential to escape the endosomal vesicle and as nuclear localization signals for the viral capsid (Nonnenmacher and Weber, 2012).

AAV1 and AAV5 for instance undergo retrograde transport, which means they traffic from the early endosomal compartment, to the trans-Golgi network and then are released in the cytoplasm (Nonnenmacher and Weber, 2012).

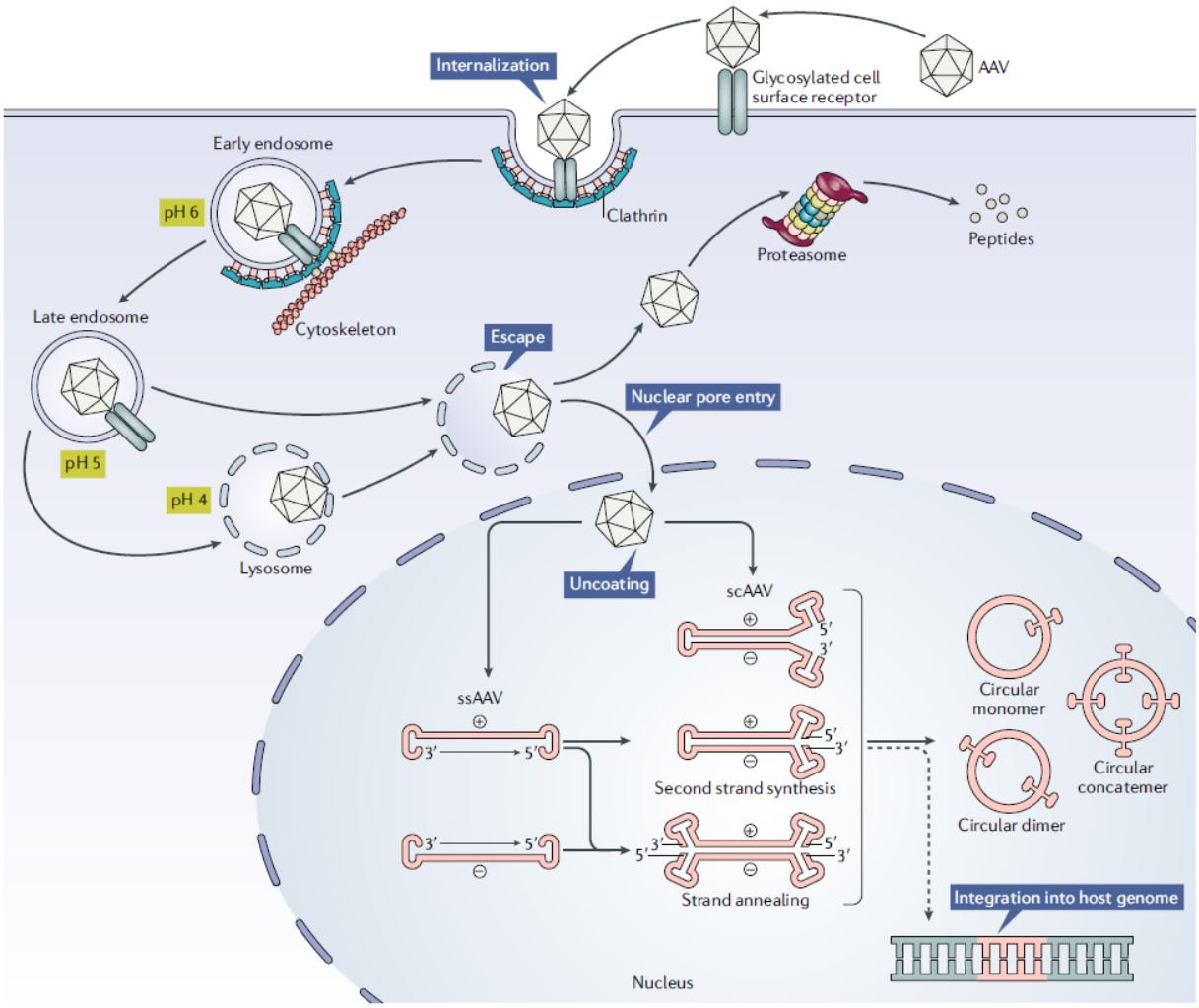

Figure 1. 3 Diagram of rAAV replication cycle. The viral particle recognizes the glycosylated surface receptor of the host cell. The virus internalization process begins via clathrin-mediated endocytosis. The virus undergoes a decrease on pH that consequently lead to a VP1 and VP2 conformation change. After endosomal escape, the virus can either be transported to the nucleus, where and the uncoating process begins, or undergo proteolysis by the proteosome. The viral particle can either transport single stranded DNA (ssDNA) or self-complementary DNA (scDNA). ssDNA, minus or positive strand depending on the packed molecule undergo second strand synthesis by the cellular machinery or by strand annealing of plus and negative strands (if they coexist in the nucleus). If the viral particle is packed with scDNA the transcription process starts immediately. Due to the viral inverted terminal repeats (ITRs) natural inter-molecular or intra-molecular recombination, the molecule forms circular episomal genomes that endure in the nucleus. When the viral particle has a wild-type genome, it is not capable of replicate itself without the co-infection with an helper virus. In this scenario the genome integrates into the host cell genome. Adapted from the Wang *et al*, 2019.

How the AAV leaves the endosome and the mechanism to enter the nucleus is still unknown (Maes *et al.*, 2019; Naso *et al.*, 2017) and is estimated that only 20% of the AAV2 infecting a cell reach the nucleus (Maes *et al.*, 2019). Once inside the nucleus, it dislocates to the nucleolus and finally egress into the nucleoplasm (Nonnenmacher and Weber, 2012). It is known that the virion enters the nucleus through the nucleus pore complex (Wang *et al.*, 2019) followed by uncoating process with subsequent release of the single stranded genome (Saraiva *et al.*, 2016). The cellular replication machinery synthesizes the complementary strand at the self-primed ITR at the 3´-end of the viral genome forming a double strand molecule (Wang *et al.*, 2019). To form the new particle, the double strand is separated, and both plus and minus ends are able to be packed into separate virions (Wang *et al.*, 2019).

The AAV life cycle is a biphasic (Ling *et al.*, 2018), because it depends on whether there is a co-infection of the host cell or not. The lytic cycle occurs when the host cell is coinfected with a helper virus, such as Adenovirus or HSV. In this scenario, the AAV engages a rapid replication process, where the viral Rep and Cap proteins are being synthesized, enter the nucleus and start assembling around the viral genome ssDNA forming the AAV viral particle. The newly formed AAV particles are then released to the extracellular milieu by helper virus cell lysis induction (Balakrishnan and Jayandharan, 2014; Saraiva *et al.*, 2016).

In the absence of an helper virus, the AAV enters in the non-lytic cycle. AAV genome persists in the host cell as an episomal state or integrates into the host cell genome in the AAVS1 site, as explained above (Balakrishnan and Jayandharan, 2014; Saraiva *et al.*, 2016). The process of integration is dependent of Rep68 or Rep78, which bind to Rep binding elements (RBE) present in the viral genome ITRs and at the AAVS1 site, originating a complex licensing the integration process (Saraiva *et al.*, 2016).

An AAV infection is not always linear; in most cases, AAV does not reach the nucleus and often is marked for degradation. After infection, epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) phosphorylate the surface-exposed tyrosine residues present in the AAV2 capsid protein, leading to ubiquitination culminating in proteasomal degradation of the vectors in the cytoplasm (Zhong *et al.*, 2007).

1.3.3 Recombinant AAVs

Nineteen years after the AAV discovery, Hermonat and Muzyczka successfully engineered recombinant AAV serotype 2 (rAAV2) as a gene delivery vehicle (Hermonat and Muzyczka, 1984). In 1996, Flotte and his collaborates used an AAV vector in humans for the first time, to deliver the correct cystic fibrosis transmembrane conductance receptor (CFTR) cDNA gene, in an attempt to treat cystic fibrosis disease (Flotte *et al.*, 1996).

The standard cassette to construct the rAAV is packed between the two viral ITRs (Deverman *et al.*, 2018) and is commonly composed by a mammalian promoter, a gene of interest and a terminator (Naso *et al.*, 2017). The choice of promoter is an essential step because it influences the pattern and longevity of transgene expression in the nervous system cells (McCown, 2005). The cytomegalovirus (CMV) promoter/enhancer or simian virus 40 (SV40), among others, are strong and constitutively active promoters with high levels of the transgene expression in most cell types, which makes them good candidates as promoters (Naso *et al.*, 2017).

rAAVs are not integrative vectors, instead form a circular concatemers that persist episomal in the nucleus of transduced cells, maintaining a long-term and stable expression that has showed to be effective neuronal transducers, once inside the CNS (McCown, 2005). Studies using rAAV2 as a gene delivery vehicle via stereotactic surgery in patients with Parkinson Disease demonstrated a long-term expression of the transgene and safe rAAVs administration (Marks *et al.*, 2016). This extrachromosomal expression does not guaranty however high transgene expression levels when transducing dividing cells. In this case it will be eventually diluted over cellular replications process, with the rate of transgene loss dependent on the turnover of the target cell (Naso *et al.*, 2017).

Like natural AAVs, rAAV genome replication is also dependent of the host cell replication machinery for the complementary strand synthesis. To overcome this limitation and improve gene expression, self-complementary AAVs (scAAV) were developed, in which a single stand packed genome complements itself to achieve a double stranded configuration (Naso *et al.*, 2017). Nevertheless scAAVs have their packing capacity reduced to only 2.2 kb, half of the ssAAV capacity to transport the transgene of interest and the type-specific promoter (Maes *et al.*, 2019). A study to determine the maximum packing capacity of the scAAV vectors have demonstrated that genomes with approximately 3.3 kb can be successfully encapsidated and purified and are capable

of high efficiency transduction (Wu *et al.*, 2007). Also, scAAV vectors have successfully transduced motor neurons upon intravenous injection in adult animal models (Duque *et al.*, 2009).

1.3.3.1 rAAV Production

The crescent use of rAAV in clinical trials has exposed the need for a production technology that fits the current good manufacturing practice (cGMP), with good and scalable manufacturing and quality control assays (Clement and Grieger, 2016). For the rAAV manufacturing, two fundamental technologies exist: transfection system and helper virus complementation system. Both relay in three fundamental commonalities: i) the host cell successfully receives and amplify the necessary genetic material, ii) finetuning of Rep-Cap expression levels and iii) adjustment of the cellular medium to provide a better AAV environment (Aponte-Ubillus *et al.*, 2018).

Helper virus complementation systems, very succinctly, insert in the packing cells two plasmids, one with the transgene of interest flanked by ITRs, and another with wild-type AAV Rep and Cap genes, along with replication competent (rc) helper virus, i.e. rc adenovirus in HeLa cells, rc herpes virus in human embryonic kidney 293 (HEK-293) or rc baculovirus in Sf9 insect cell line (Keeler and Flotte, 2019). The main obstacle is that complementation systems add inherent complexity to the production process, due to the AAV dependent interaction with the host genetic elements. The AAV production yield is easily altered with subtle changes on the biological and chemical inputs and may contain replication competent AAV and helper virus due to process-related impurities (Aponte-Ubillus *et al.*, 2018).

To overcome this hurdle, it was tested an alternative method - the transfection method - that discard the use of helper viruses and uses a plasmid just containing the proteins from the helper virus which are essential for the AAV replication. The transient triple transfection of HEK0293 cells is nowadays the most used method for rAAV production (Naso *et al.*, 2017; Saraiva *et al.*, 2016). As the name suggests, the cells are transfected with three plasmids, a transfer vector, a helper plasmid, and the pAAV-RC or packing construct (see Figure 1.4). The transfer vector contains the ITRs, the flanked expression cassette that comprises a promoter and the transgene, and a polyadenylation (PolyA) signal or a string of five thymidines to terminate the transcription. The ITRs regulate the replication and the packing of the vector genome. The transgene, with a limited size of 4.5kb in the case of ssAAVs, encodes for the gene of interest (Saraiva *et al.*, 2016). To

replace the cellular coinfection necessary for AAV replication with the helper virus, a helper plasmid with the adenoviral 5 helper regions E2a, E4 and virus associated non-coding RNAs (VA RNAs) is added to a cell line constitutively expressing the E1a and E1b adenoviral genes (Keeler and Flotte, 2019). Lastly, the pAAV-RC or packing construct contains the rep genes that are needed for the genome replication and cap genes essential for the capsid assembly (Saraiva *et al.*, 2016).

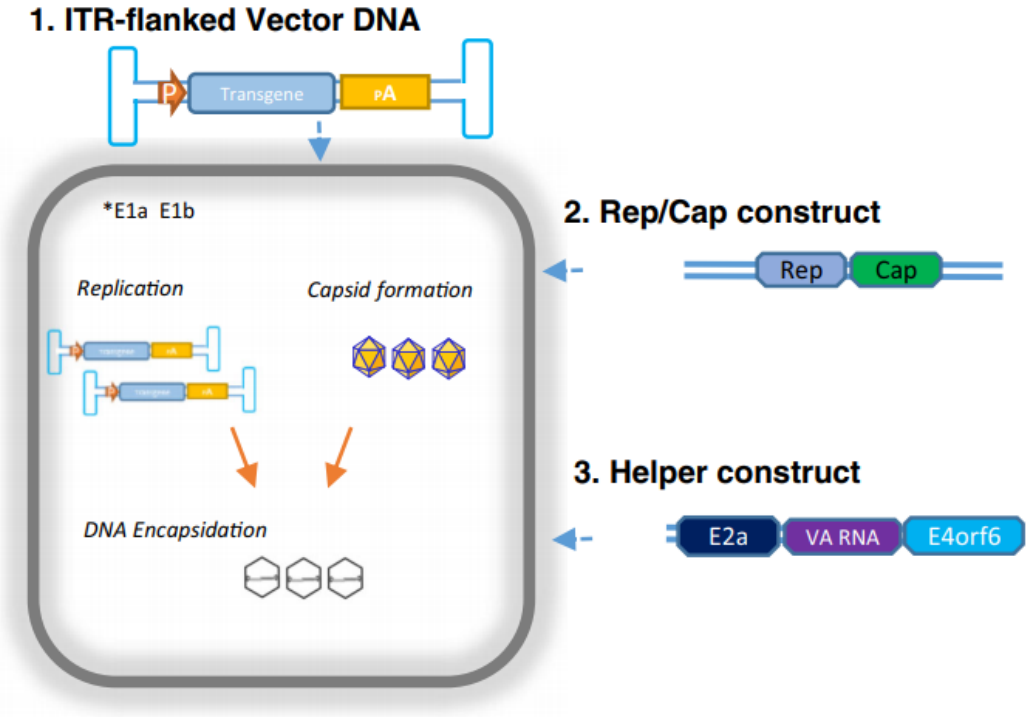

Figure 1. 4 Triple transfection scheme. **1.** "Vector transgene plasmid", were the wild-type Rep and Cap genes are replaced by the transgene of interest, a promoter (p) and a polyadenylation (pA) sequence. **2.** pAAV-RC plasmid or packing construct, contains genes encoding for the replication (Rep) process and capsid proteins (Cap) production. **3.** Helper construct plasmid, encodes for the helper genes from adenovirus. The packing cell is transfected with the three plasmids. Replication process begins with support of the helper construct plasmid, which provides helper genes from adenovirus. Cap proteins are synthesized in the cytoplasm and shuttled to the nucleus where the assembly process begins with the help of the AAP protein. Adapted from Ubillus *et al*. 2018.

Despite the improvement of the triple transfection protocol, this remains inefficient since not all cells take in optimal ratios all the plasmids needed for efficient packing and to produce fully packed capsid vectors (Wang *et al.*, 2019).

A good purification method must separate rAAV particles from cellular and particle waste, separate empty from full rAAV capsids and maintain the stability and potency of the purified

particles. The purification process begins with the host cell chemical or physical lysis induction followed by enzymatic digestion for nucleic acid removal (Clement and Grieger, 2016).

The purification method commonly used are density ultracentrifugation and affinity or ion exchange chromatography (IEC) (Naso *et al.*, 2017). The density gradient ultracentrifugation method separates the rAAVs from cellular and viral impurities and empty capsid particles, using a gradient separations process of either iodixanol or cesium chloride. However this is a very slow process that often brings out risk of AAV contamination during the processes, it is a low scalable method and requires the removal and a test trace for iodixanol or cesium chloride (Clement and Grieger, 2016) .The affinity chromatography relays rAAV attachment to cell membrane-associated carbohydrates or AAV-specific binding proteins/resins while IEC separates the AAV capsids based on electrostatic interactions between the capsid and the column matrix (Naso *et al.*, 2017). The elution process occurs by increasing ionic strength and subsequent AAV particles removal. This is an efficient, and versatile and scalable method with already GMP compliant commercial resins (Clement and Grieger, 2016).

1.3.3.2 rAAV Immunology

The AAV immunogenic profile demonstrates to be low compared with other viruses due to its biomolecular composition, based on proteins and nuclei acids, and lacking lipids and chemical compounds (Naso *et al.*, 2017). Also, the inefficient transduction of antigen-presenting cells (APCs), such as macrophages and dendritic cells (DC), may answer why AAVs are less immunogenic (Hareendran *et al.*, 2013). In theory, the reason why the innate immune response is triggered is due to AAV infection of immature APCs (Hareendran *et al.*, 2013). The innate immune system is deeply involved in the adaptive immunity response, leading to the recruitment of macrophages and neutrophils and synthesis of proinflammatory cytokines which activate T-cell and B-cell responses. CD8+ T-cells activation leads to the generation of neutralizing antibodies (NAbs) and to transgene and AAV capsid negative response (Hareendran *et al.*, 2013; Keeler *et al.*, 2019). NAbs have a substantial influence in the transduction efficiency and distribution of the AAV particles, because it works by attaching to the virion capsid and influencing the cellular attachment and subsequent steps until transgene deliver (McCown, 2005; Smith and Agbandje-McKenna, 2018).

The immune reactions can be triggered by various factors, such as dosage of viral genomes administrated and the route of administration (Hinderer *et al.*, 2018).

Molecular factors, such as the intracellular trafficking and the type of genome carried, i.e. ssDNA or dsDNA, also influence the triggering of an immune response. For instance, Haeendrann and collaborators observed that scAAV triggered off a more prominent immune activation than ssAAV (Hareendran *et al.*, 2013; Keeler *et al.*, 2019).

The adaptive immunity can recognize the AAV capsid and react against it, on the other hand the transgene can trigger both adaptive and innate immunity initiating an immunological reaction against the AAV (Naso *et al.*, 2017).

It is estimated that 40% to 70% of the general population have been exposed to natural serotypes of the AAV (Smith and Agbandje-McKenna, 2018); depending on the globe region, the percentage of exposure to different serotypes diverge (Kruzik *et al.*, 2019). Preexisting immunity can strongly neutralize AAVs and can have a great impact in the clinical efficiency of the AAV, representing a great challenge to use in a systemic deliver and so being an exclusion criteria on clinical trials (Koerber *et al.*, 2008; Naso *et al.*, 2017; Smith and Agbandje-McKenna, 2018). As an example, a study performed in rats concluded that high titters of neutralizing antibodies (Nabs) reduces significantly the transduction efficiency of rAAV vectors when intracranially delivered in the striatum, (Sanftner *et al.*, 2004). This emphasizes the importance of pre-screening patients before the administration of rAAVs for the presence of NAbs and AAV-specific cellular response (Hareendran *et al.*, 2013).

Immunosuppression and decoys, as for example empty capsids, have been also used to subvert the immune system and enable efficient gene transfer (Smith and Agbandje-McKenna, 2018).

1.3.3.3 AAV1

Adeno-associated virus serotype 1 (AAV1) wild type has a 4 718 nucleotides length genome which 143 nucleotides comprising each terminal repeat, sharing the same integration region as AAV2 in the AAVS1 region (Xiao *et al.*, 1999). It has a natural tropism to the muscle (Xiao *et al.*, 1999), mainly conferred by the amino acids 350 and 423, which codify to the C-terminal part of the VP1 protein (Huang *et al.*, 2016). It cannot cross the BBB and has a low

transduction efficiency *in vivo* (Srivastava, 2016b). AAV1 binds to α2-3 and 2-6 N-linked sialic acid (Srivastava, 2016b), X-ray crystallography determined the AAV1 sialic acid complex was 3.0Å (Huang *et al.*, 2016). Six site directed mutations were tested on the AAV1 sialic acid region and all resulted in abolished sialic acid binding except for S472R that increased binding. Other AAV1 parameters were evaluated, for instance, transduction efficiency was abolished or decreased in all six mutations and three mutations escaped recognition by the anti-AAV1 capsid antibody ADK1a (Huang *et al.*, 2016). AAV6 is a hybrid of AAV1 and AAV2, in which only 6 out of 736 amino acids from AAV6 VP1 capsid protein differ from AAV1, therefore sharing an identity of 99.2% (Huang *et al.*, 2016; Rutledge *et al.*, 1998)

Investigating the AAV1 capsid is important, because it has lower preexisting humoral immunity in the human population compared to AAV2, AAV5 or AAV8; therefore it is probably a good gene therapy delivery vehicle (Kruzik *et al.*, 2019; Xiao *et al.*, 1999). For instance, rAAV1 became the vector of choice for muscle directed in gene therapy (Hauck and Xiao, 2003). Recently, a group of researchers altered the AAV1 properties by adding a 7-mer PHP.P peptide to the AAV1 capsid conferring it the ability to efficiently cross the BBB mice and improve transduction efficiency, in this way creating a non-invasive route of administration (Lau *et al.*, 2019).

One of the first successful Phase I/II clinical trials using AAV1 vector as a potential gene therapy treatment for lipoprotein lipase (LDL) deficiency resulted in a scientific milestone, the approval Glybera®, the first gene therapy medicine (Domenger and Grimm, 2019)

1.3.3.4 AAV2

In 1965, adeno-associated virus serotype 2 (AAV2) was the first AAV discovered (Atchison *et al.*, 1965), and is the most studied AAV serotype, due to well characterized and favorable biology of its ITRs (Burger *et al.*, 2004; Srivastava, 2016a). However, approximately 90% of the population test positive for antibodies against wild type AAV2 (Srivastava, 2016a).

AAV2 has a 4 681 nucleotides genome (Xiao *et al.*, 1999) and internalizes target cells by recognition of the heparin sulfate proteoglycan (HSPG), its primary cell surface receptor, discovered in 1998 (Summerford and Samulski, 1998).Since almost all cells express this receptor explains why AAV infects almost all cell types across the species (Srivastava, 2016a). Nonetheless binding to this receptor is insufficient for the AAV2 cell entry, since it needs the co-receptor

binding, such as FGFR1 (human fibroblast growth factor receptor 1) integrins αVβ5 and α5β1, HGFR and LamR to complete internalization process (Srivastava, 2016b). Some researchers dispute whether HSPG is the primary AAV2 cell surface receptor in human tissue or, its presence is simply explained with a tissue-culture adaptation that selected it positively (Nonnenmacher and Weber, 2012).

The AAV2 uptake into the cell is by default the clathrin-mediated endocytosis (Nonnenmacher and Weber, 2012). Later, another experiment with a constitutively active protein involved in the macropinocytosis process, it was observed that Rac1 GTPase augmented drastically the transduction of AAV2. This indicated AAV2 enters the cell through at least both pathways (Nonnenmacher and Weber, 2012). Another factor that increases the transfection efficiency is the cellular coinfection with adenovirus (Nonnenmacher and Weber, 2012).

Several human diseases, such as Leber´s, congenital amaurosis, aromatic L-amino acid decarboxylase deficiency and chloride have shown clinical efficiency of rAAV2-based gene therapies (Srivastava, 2016a).The first phase I and II human trials using rAAVs were performed by Flotte and colleagues, in 1996, using AAV2 vectors as a potential treatment for cystic fibrosis (Flotte *et al.*, 1996). Unfortunately, this strategy did not show clinical efficiency because the HSPG are predominantly expressed in the baso-lateral cell surface rather than the apical surface where the virus could not access (Srivastava, 2016a). In the CNS, rAAV2 demonstrated to efficiently deliver genes into the brain, retina, and spinal cord. It provides long term expression in neurons, although with relatively restricted biodistribution (Deverman *et al.*, 2018)

1.3.6 Routes of rAAV administration to the CNS

In the design of an experiment using AAV vectors as delivery vehicles, one of the fundamental questions is how to best administer AAVs (Haery *et al.*, 2019). To formulate the best administration route is imperative to understand the disease and the location of the affected areas (Naso *et al.*, 2017). The principal delivery administrations are, i) systemic delivery, ii) intramuscular delivery and iii) intraparenchymal delivery.

A systemic delivery involves the intravenous administration (IV) on the patient and is mainly used for monogenic diseases affecting, for example, the liver such as haemophilia A and B (Naso *et al.*, 2017). This method is non-invasive enabling of widespread gene delivery (Deverman *et al.*, 2018); however it requires high virus titters to achieve efficiency (~1×10^{14} vg/Kg) (Haery *et*

al., 2019), measurement of the NAb titters before administration and re-administration is admonished (Naso *et al.*, 2017)

In the case of gene delivery to the brain, IV administration requires the use serotypes that can cross the BBB, for example, the AAV9, which has most pronounced results when injected in the neonate stage of life (Haery *et al.*, 2019). However, limited neuronal transduction was observed in later developmental stages. With the objective of working around this limitation, a new AAV capsids were developed, AAV-PHP.B and AAV-PHP.eB that increased transduction around 50% (Haery *et al.*, 2019; Liguore *et al.*, 2019).

Intramuscular (IM) delivery can be a useful approach for antibody therapeutics targeting the CNS. An IM injection could serve as a storage for transgene expression, with a continuous release of an antibody of interest, reducing the need for repetitive IV administration as it occurs with passive immunization protocols (Deverman *et al.*, 2018).

Intraparenchymal delivery is the injection directly in the target tissue/organ. In this case, different AAV serotypes can obviously spread differently from the injection site and exhibit transduction preference among the different organs cell types (Haery *et al.*, 2019). Thereby, it is crucial to understand more profoundly the biodistribution and assess the transduction levels of each serotype in the CNS. This is the most used route of administration to reach the CNS. However, at the same time, the most difficult since require general anaesthesia, craniotomy and multiple injections at different sites are requires when multiple areas are affected (Deverman *et al.*, 2018).

CSF delivery is achieved in three ways, by: intracerebroventricular (ICV) injection, subpial injection and intrathecal (IC) injection. ICV injection deliver genes into the ventricular space and can provide widespread expression in neonatal stages, while in adult stages it provides a non-uniform expression with preference to the superficial structures, for example, ependymal cells. The subpial injection occurs below the meninges and transduction efficiencies are very similar to ICV injection. The intrathecal injection in the intrathecal space along the spinal cord delivers genes to the spinal cord motor neurons and dorsal root ganglions (Haery *et al.*, 2019). Studies performed from rodent models to non-human primates demonstrate it is an effective route of administration (Gray *et al.*, 2013).

Other very versatile technique is being developed targeting single cells (Schubert *et al.*, 2019). This innovative approach uses magnetically guided virus stamping, in which a virus-bound nanoparticle is magnetically guided to the selected cells to ease the rate of physical contact. In the

monolayer cell culture, the success rates reached 80% with reliable single cell specificity. Likewise, this approach is now being tested in organoids and brains of living animals (Schubert *et al.*, 2019). The limitations of this technic undergo the need for customized magnets and electrostatic forces (Schubert *et al.*, 2019).

1.3.7 rAAV Applications and Gene Therapy Strategies

Over the last few decades AAV gene therapy has impressively progressed (Keeler *et al.*, 2019), (Weinmann and Grimm, 2017). It is often pictured as the perfect tool for clinical application, i.e. to deliver genes and cure diseases, but it is also useful for basic research for example for optogenetics.

Its utility for basic science is poorly explored, but rAVV is an ideal vector to express short genetic sequences that help understand neural networks, thereby studying more about development, behavior and learning (Chen *et al.*, 2019). A great way to identify neural progressions and network connectivity is by taking advantage of enhanced trans-synaptic anterograde ability of natural serotypes (AAV1, AAV2, AAV6 and AAV9) or newly-design vectors (e.g. AAV2-Retro and AAV MNM008) (Chen *et al.*, 2019; Haery *et al.*, 2019) to determine gene expression, location and connectivity of specific neural cells and also of other non-neural cells in the nervous system (Haery *et al.*, 2019).

In pre-clinic studies, AAV vectors have been designed to carry genetic material with a wide variety of purposes, such as: gene replacement, gene silencing, gene addition or gene editing/ engineering (Wang *et al.*, 2019). For each purpose different strategies are used (Table 1.6).

Gene replacement is often used in the presence of loss-of-function mutations, where the treatment is based on adding the correct form of the gene (transgene / gene of interest) (Wang *et al.*, 2019). Because it is impossible to predict how many rAAVs will transfect a single cell, it is important to administer the right dose to avoid potential toxic effects owing to expression (Deverman *et al.*, 2018).

Gene silencing is quite the opposite. In the presence of the gene that is causing the disease, it is crucial to shut it off by inhibition of its expression with natural biological processes using interference RNAs, such as: microRNAs (miRNA), small interference RNAs (siRNA), or short hairpin RNA(shRNA) (Wang *et al.*, 2019).

Gene addiction is used to modulate complex genetic or acquired diseases by delivering supplies, such as antibodies to neutralize deadly infections (Wang *et al.*, 2019).

Genome editing/ engineering is often used to directly repair mutations in a two-step system, first the target gene is identified and cleaved and second repair of the DNA genome leading to the desired DNA alteration (Wang *et al.*, 2019). Such tools are meganucleases, Zinc-fingers nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases based on clustered regulatory interspaced short palindromic repeat (CRISPR) – associated CAS protein system (Kotterman and Schaffer, 2014). The big concern of this technic is the off target editing that can transform the cell into a malignant (Deverman *et al.*, 2018).

Table 1. 6 Summary of the gene therapy approaches.

Strategy	**Type of Disease:**	**Tools:**	**Example:**
Gene Replacement	Loss-of-function mutations	Transgene	LPL deficiency
Gene Silencing	Gain-of-function mutations	iRNA, miRNA, siRNA, shRNA,	Parkinson, SCA1, SCA2
Gene Addiction	Complex Genetic or Acquired Diseases	Transgene (ex. antibodies)	Cancer
Genome Editing	Directly Repair mutations	Meganucleases, ZFNs, TALENs, CRISPR-Cas	Lysosomal Storage Disorders

For the success of the above-mentioned therapies it is required efficient gene therapy vectors.

1.3.8 Next generation AAV vectors

Next generations AAV vectors arise from the need to amplify the rAAV abilities such as its efficiency, safety and targeting and downgrade its limitations. To improve the rAAV abilities, next generation AAVs were designed from the following strategies: i) “directed evolution method”, ii) “DNA shuffling method”, iii) rational design, iv) insertional mutagenesis and v) “mosaic method” (see Table 1.7)

The "directed evolution method" is used for capsid protein engineering to boost pre-existing functions or to develop new functions in proteins that lack that ability (Koerber *et al.*, 2008). This approach relies on genetic diversity and selection process to rapidly accumulate beneficial mutations that will improve the AAV function (Kotterman and Schaffer, 2014). Sundry methods are used in directed evolution, such as error-prone PCR or Cre recombination-based AAV targeted evolution (CREATE) (Wang *et al.*, 2019).

The "DNA shuffling method" is based on the natural homologous recombination process (Stemmer, 1994). Different wild-type AAV serotypes cap genes are fragmented by DNAse and randomly reassembled by polymerase chain reaction (PCR) leading to a diverse library of complex recombinants (Koerber *et al.*, 2008; Soong *et al.*, 2000). The next step is the selection of efficient viral particles and analyse the *in vitro* and *in vivo* tropism of the new viruses (Koerber *et al.*, 2008).

Rational design is the engineering of the vector capsid with modification of peptide sequences to improve tropism, disrupt cellular degradation of capsids or avoid Nabs; however it is important to make sure the design does not affect negatively the vector stability and efficiency (Wang *et al.*, 2019). To apply this method it is important to have basic knowledge about the AAV binding, internalization, trafficking, uncoating and gene expression (Wang *et al.*, 2019). Three different approaches have been used to develop rational designed vector capsids: genetic mutation of AAV capsid, insertion of non-viral parts into the AAV capsid and chemical biology for capsid modification.

By inserting point mutations in the AAV capsid, it is possible to increase the efficiency and specificity of AAV delivery (Lee *et al.*, 2018). In this way, a group of researchers created a hybrid capsid of AAV2, inserting one amino acid and replacing four amino acids from AAV1 capsid, termed as AAV2.5. This vector exhibited better transduction levels than the AAV1 and AAV2 alone (Bowles *et al.*, 2010).

In order to elicit desired functions, it is also possible to incorporate non-viral motifs into the viral capsid. For example, tetra-aspartic acid residues flanked by protease cleavage sequences have been inserted in the AAV2 capsid near HSPG binding domains. In this case, AAV2 binds to its primary receptor after extracellular proteases sequence cleavage such as metalloproteases (MMPs), preventing the transfection of non-target cells (Judd *et al.*, 2014). In another study, to trace the AAV9 movement in real-time, tetracycline motif was added to the vector capsid generating a new vector named AAV9-138(Chandran *et al.*, 2017). Upon injection of maleimide

dye, the maleimide will attach to the tetracycline and is possible to trace AAV9-138 using intravital microscopy. The interactome of AAV9-138 compared with the wild-type AAV9 serotype is still to be tested; however the ability to pass the BBB is maintained as well as the infectivity and distribution (Chandran *et al.*, 2017).

Table 1. 7 Examples of strategies to improve rAAV.

Directed evolution	Is capsid protein engineering to boost pre-existing functions or to develop new functions in proteins that lack that ability (Koerber *et al.*, 2008).	**Error prone PCR**	Uses the PCR technique with altered conditions leading to site-specific mutagenesis as for example cap genes, altering the capsid tropism (Wang *et al.*, 2019).
		Cre recombination-based evolution (CREATE);	Insertion of aleatory peptide fragments into the wild-type AAAV genome based on the CRE recombination(Wang *et al.*, 2019).
		DNA shuffeling	Is based on the natural homologous recombination process (Stemmer, 1994). Different wild-type AAV serotypes cap genes are fragmented by DNAse and randomly reassembled by polymerase chain reaction (PCR) leading to a diverse library of complex recombinants (Koerber *et al.*, 2008; Soong *et al.*, 2000).
Rational Design	Is the engineering of the vector capsid with modification of peptide sequences to improve tropism, disrupt cellular degradation of capsids or avoid Nabs, based on known features of the virus (Wang *et al.*, 2019).	**Site-directed Mutagenesis**	By inserting point mutations in the AAV capsid, it is possible to increase the efficiency and specificity of AAV delivery (Lee *et al.*, 2018).
		Insertion of non-viral parts	Incorporate non-viral motifs into the viral capsid. For example, insertion of tetra-aspartic acid residues to prevent the transfection of non-target cells (Judd et al., 2014).

To solve one of major limitations of AAV, i.e. the packing capacity, researchers designed the dual vector system (Kotterman and Schaffer, 2014). If the transgene is bigger than 7.4Kb, it is splitted in two and each strand is packed into two individual AAV vectors. This approach depends on the heterodimerization of the two AAV vectors followed by the formation of concatemers of either integrated or episomal templates (Sun *et al.*, 2000). After the transduction of the target cell with each of the virus, a full-length transgene is achieved by viral ITR-mediated recombination (dual vector *trans*-splicing vectors), homologous recombination (dual AAV overlapping vectors) or both (dual AAV hybrid vectors) (Kotterman and Schaffer, 2014). This strategy has shown successful results in preclinical studies, but its efficiency varies between species (Kotterman and Schaffer, 2014). Another way to use a dual vector is by packing the transgene in the first vector and in the second vector encoding for multiple enhancer sequences or specific promoters. This second alternative has also shown effective augmentation of the transgene expression (Duan *et al.*, 2000), however, in this case, the same cell needs to be transfected with two AAV vectors carrying different DNA sequences.

As described above many approaches have been developed to generate new AAV vectors with specific characteristic. Given the amount of known AAV serotypes and their affinity to specific receptors, it is possible to manipulate them to generate a vector combining the desired characteristics. The "Mosaic method" generates mosaic capsid shells by combining different serotypes (Rabinowitz *et al.*, 2004; Wu *et al.*, 2006). By mixing different AAV rep/cap serotype plasmids in the right proportion, during the viral production, it is possible to generate "cross-dressed" AAV virions, that have novel transduction profiles (Wu *et al.*, 2006). This method allows the vector tropism alteration, but also can help investigators understanding basic mechanisms such as capsid assembly, receptor-mediated binding, and virus trafficking, attributes essential for vector development (Rabinowitz *et al.*, 2004). Any AAV serotype can be used for the generation of mosaic vectors with the desired ratio of capsid protein serotype ratios (Rabinowitz *et al.*, 2004), that theoretically reflect the input ratio of the different plasmids (Wu *et al.*, 2006). Mosaic vectors have features of parent serotypes and surprisingly also have new properties different from either parental virus. An example is the mosaic "AAV1/AAV2" produced with the transfection ratio of 1:19, respectively, of AAV1 and AAV2 helper constructs that demonstrated to have better transduction of C2C12 cells compared with the individual parental serotypes (Wu *et al.*, 2006).

1.3.8.2 ATMPs / Commercially Available Therapy

Advanced Therapy Medical Products (ATMPs) are "medical treatments that are based on genes or cells and are intended as long-term or permanent therapeutic solutions to acute or chronic human diseases at genetic, cellular or tissue level" as defined by Yu (Yu *et al.*, 2018). Currently, three ATMPs medicines have been approved for commercialization by the United States Food and Drug Administration (FDA), Glybera® in (2012), Luxturna® (2017) and Zolgensma® (2019). European Medicines Agency (EMA) only approved Glybera® and Luxturna® (Table 1.8).

The first AAV-based therapy approved by EMA for commercialization was Alipogene Tiparvovec, marked under the trade name of Glybera® in October of 2012 (Yu *et al.*, 2018). However, considering it costs 1.11 million euros for an average adult and no cure guarantee, it is currently withdrawn from the marked due to cost limitations (Keeler and Flotte, 2019). Developed by UniQure and sponsored by the Spark therapeutics (Naso *et al.*, 2017), Glybera® was conceived to treat homozygous, heterozygous or compound lipoprotein lipase deficiency (LPLD), an autosomal recessive lipid disorder caused by dysfunction in the lipoprotein lipase (LPL) gene which is involved in the catabolism of triglyceride-rich lipoproteins (Kassner *et al.*, 2018). LPLD is an ultra-orphan rare disorder. Patients with LPLD suffer from severe hypertriglyceridemia and chylomicronemia that builds up in the blood manifesting as a milky looking plasma, therefore it is recommended ultra-low fat diets for these individuals (Kassner *et al.*, 2018). Glybera® delivered the "LPL S447X gene construct" with a constitutive active promoter carried by rAAV serotype 1 vector by sixty-four intra-muscular of either 3×10^{11} or 1×10^{12} genome copies (gc)/kg in 1 mL injections in the major skeletal muscle groups (Wierzbicki and Viljoen, 2013). Given the fact that all patients were in their adulthood and it was a systemic injection, immunosuppressants were administrated prior treatment. So far Glybera® is the best candidate for LPLD, achieving a transient 40% reduction in the triglycerides and amelioration in postprandial chylomicron triglyceride content, yet some reluctance is shown to claim Glybera® as a curative treatment (Wierzbicki and Viljoen, 2013).

Voretigene Neparvovec, marked under the trade name Luxturna®, developed by the Novartis and Spark Therapeutics program was approved in December 2017 by the FDA and later, on 23rd of November 2018, by EMA (Keeler and Flotte, 2019) as a treatment for a rare form of inherited blindness called Biallelic retinal pigment epithelium-specific 65 kilodalton (RPE65) mutation-associated retinal dystrophy (Darrow, 2019). The absence or dysfunction of the RPE65

enzyme disturb the visual cycle involved in the conversion of photon of light into an electrical signal in the eye retina (Darrow, 2019). The treatment of biallelic RPE65 consists in a single injection of 1.5×10^{11} vector genomes (vg) in 0.3mL per eye. Luxturna® is composed by a rAAV2 carrying the cDNA of the RPE65 gene. This treatment costs 850 000 dollars (450 000 dollars per eye) (Keeler and Flotte, 2019; Smith and Agbandje-McKenna, 2018), preventing the progression of the disease and improving vision function.

Onasemnogene Abeparvovec, marked under the trade name Zolgensma®, was developed in the Novartis company by AveXis and globally approved on 24th of May 2019 by the FDA to treat spinal muscular atrophy (SMA) (Hoy, 2019). Zolgensma ® consists of a scAAV serotype 9 (scAAV9) containing a transgene encoding for the full-length human *survival motor neuron* SMN gene under the action of the cytomegalovirus enhancer/chiken-β-actin hybrid promoter (Hoy, 2019). SMA is a severe autosomal recessive disorder, classified in different phenotypes, caused by the deletion or mutation of the *survival motor neuron 1* (SMN1), leading to degeneration of α-motor neurons causing skeleton muscle weakness and atrophy (Czibere *et al.*, 2020). The recommended dose is 1.1×10^{14} vector genomes (vg)/Kg in a single intravenous infusion (Hoy, 2019). Disease onset is early, at only three months old patients undergo substantial loss of motor neurons progressing throughout life (Domenger and Grimm, 2019; Hoy, 2019) with a life expectancy inferior to 2 years, therefore early interventions will improve the outcome of the therapy (Czibere *et al.*, 2020). The price tag to access the Zolgensma treatment is 2.1 million dollars being presently the world´s most expensive drug (Keeler and Flotte, 2019). Given the disease early onset, patients do not need to have immune screening.

Table 1. 8 Summary of ATMPs approved for commercialization and its features.

	ALIPOGENE TIPARVOVEC	**VORETIGENE NEPARVOVEC**	**ONASEMNOGENE ABEPARVOVEC**
Commercial Name:	Glybera®	Luxturna®	Zolgensma®
Serotype:	AAV1	AAV2	scAAV9
Disease:	Lipoprotein Lipase Deficiency	Biallelic RPE65 (inherited blindness)	Spinal Muscular Atrophy
Therapeutic Approach:	Gene Replacement	Gene Replacement	Gene Replacement
Commercial price:	1.11 million €	850 000 $	2.1 million $

1.3.9 rAAV Advantages and Drawbacks

Table 1. 9 Summary of advantages and drawbacks of AAV clinical use.

ADVANTAGES:	**DRAWBACKS:**
Safety	Limited packing capacity
Episomal expression (non-integrative)	Biodistribution
Simple to manipulate	Pre-existing antibodies to AAV
Non-pathogenic	Cost
Dependovirus	Deficiencies in scalability with the triple transfection platform
Non lytic	Inability of the currently available rAAV vectors to selectively target a given cell type, tissue or organ following systemic administration.
Transduction of Quiescent and dividing cells	Lack of standardization of vector titers and potency
Low immune response	

Chapter 2 – Project Aim and Objectives

Gene therapy has great potential to one day treat neurological diseases; however, current tools have not achieved the best therapeutic efficiency (O'Connor and Boulis, 2015). It is still necessary to combine the best existing strategies in genome engineering and more efficient vectors for the neurological disorder, with the patient personal characteristics of gene therapy (Goswami *et al.*, 2019).

Over the past half-century, AAV-mediated gene delivery vehicle endorses public investments in molecular genetics (Keeler and Flotte, 2019). The AAV vectors are simple, stable and efficient delivery systems that exhibit long term expression, broad tropism, and low carcinogenicity making it straightforward vehicles for gene therapy. Research using AAV vectors have demonstrated to be powerful tool in gene therapy and disease models of neurological disorders, due to high protein expression after long periods of time (Albert *et al.*, 2017).

This study aimed to test the biodistribution and cell tropism of AAV1/AAV2 mosaic vectors in the mouse brain, and compare with their parental serotypes (AAV1, AAV2) upon intraparenchymal injection at two different sites (see Figure 2.1). All vectors encode for the green fluorescent protein (GFP) gene, controlled by constitutive CMV promoter flanked by AAV2 ITRs.

For that, adult mice will be bilaterally intraparenchymal injected in the striatum with 3×10^9 vg of AAV1/AAV2 mosaic vectors or parental serotypes. Sacrifice will occur four weeks after the injection and the brain will be separated by the two hemispheres, one hemisphere is collected to histological analysis and a striatum punch is collected from the other hemisphere. Histological analysis will demonstrate the AAV vectors location, spreading and tropism. The molecular analysis will evaluate the copy number of AAVs per ng of DNA. The obtained results are then compared between different AAVs.

In the second part of this work, AAV1, AAV2 or mosaic capsid AAV1/AAV2 vectors will be bilaterally injected in the Deep Cerebellar Nuclei (DCN) (5×10^9 vg/DCN). Four weeks following injection the mice brain will be collected and one hemisphere will be histologically analysis and the other will be for molecular analysis.

The present study intends to provide evidence that mosaic capsid AAV1/AAV2 vector has better biodistribution and transduction than individual parental capsids AAV1 and AAV2 vector, while enabling purification based on heparin affinity chromatography and maintaining or augmenting neuronal tropism.

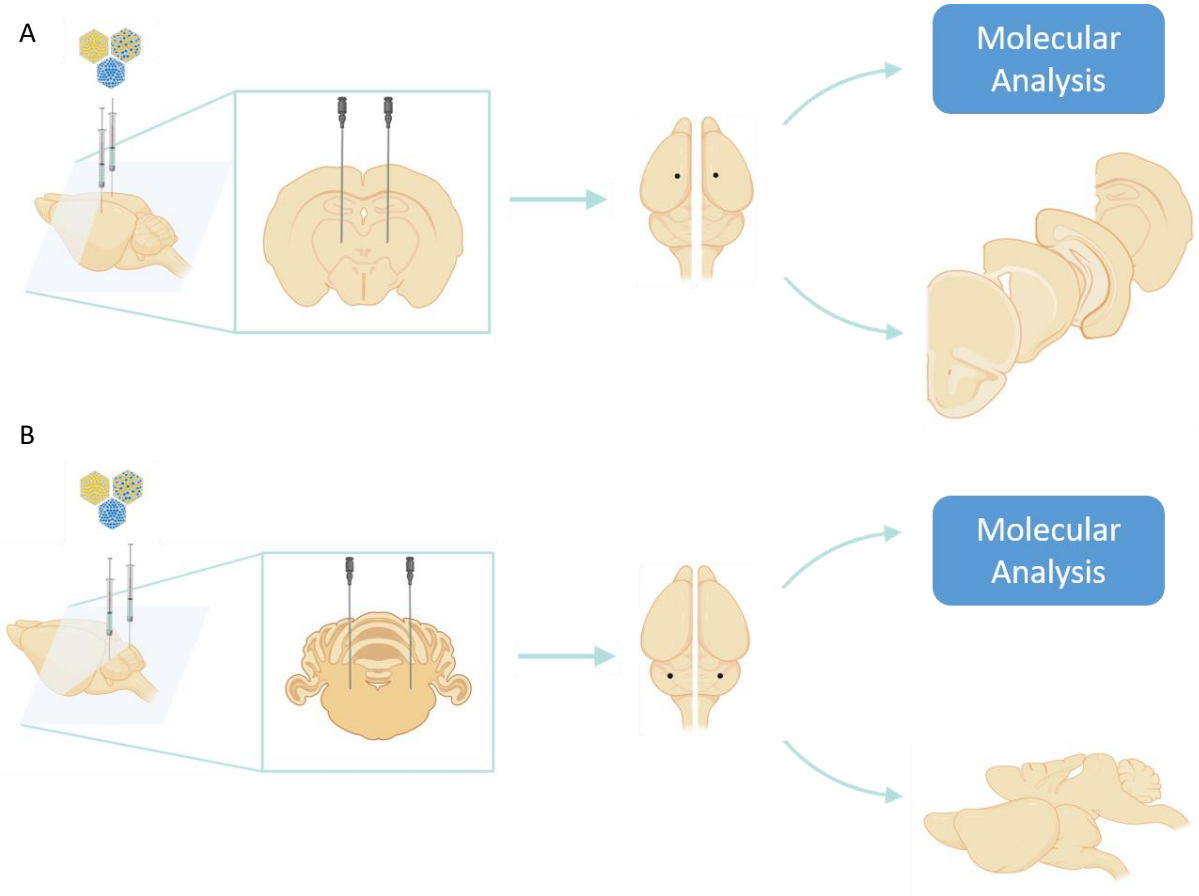

Figure 2. 1 Experimental design. **A** First part of the experiment: adult mice undergo a stereotaxic surgery for either mosaic vector AAV1/AAV2 or its parental serotypes injection in the striatum. Four weeks post injection the animals will be sacrificed, and the hemispheres will be separated. One hemisphere will be used for histological analyses and the other used for molecular analysis. **B** The same protocol will be followed in the second part of the study; but, in this case, the deep cerebellar nuclei will be the local of injection.

Chapter 3 – Materials and Methods

3.1 Adeno-associated Virus (AAV) Vector Production and Purification

HEK293 cells were grown in complete medium containing Dulbecco's Modified Eagle Medium (DMEM)/Nutrient Mixture F-12 medium with GlutaMax (Life technologies), 10 % heat inactivated HyClone FBS (Thermo Scientific), and 1 % 1 M HEPES (Life Technologies) at 37 °C in a humidified incubator with 5 % CO_2/95 % air. Twenty-four hours before transfection, 10 x 10^{16} HEK293 cells were plated onto a 15-cm dish. A triple transfection method was performed in HEK293T cells using the following plasmids: ssAAV plasmid coding for the green fluorescent protein (GFP) under the control of the cytomegalovirus enhancer, a pDF6 helper plasmid containing adenovirus helper genes and a plasmid carrying the Rep-Cap genes to generate the desired rAAV particles. In the last case, a plasmid from serotype 1 to generate rAAV1, a plasmid from serotype 2 to generate rAAV2 or both plasmids from serotype 1 and 2 to generate mosaic AAV1/AAV2 were added to originate the respective rAAV serotype. The purification of the AAV1 and AAV2 serotype was performed using iodixanol gradient centrifugation and heparin affinity chromatography was used for mosaic AAV1/AAV2 purification. The iodixanol gradient centrifugation separates the full capsid viral particles from the empty capsid viral particles by a discontinuous iodixanol gradient. The heparin affinity chromatography relays on the electrostatic interaction between the rAAV capsid and the HSPG present in the column matrix (Clement and Grieger, 2016). Viral titers were obtained via qPCR. All vectors were produced and purified by ViraVector in Coimbra https://www.uc.pt/ucbusiness/pts/viravector.

3.2 Mice Procedures

All mice used in the present work were adult wild-type C57BL6 mice (16-22 weeks old male and female, 25-30 g), obtained from the animal facility of the CBMR/UAlg, Portugal) and maintained in a 12 h light–dark cycle in a temperature/humidity controlled room and had free access to water and food. All care and procedures were in accordance with the European Union Directive 86/609/EEC. Researchers received a certified course from the Federation of Laboratory Animal Science Association (FELASA) and all procedures received prior approval from the Portuguese authorities (*Direção Geral de Alimentação e Veterinária*). The animals were housed in groups of no more than five per cage, in a licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006).

3.3Stereotactic surgery and AAV Vector Administration

3.3.1. Injection in the striatum

In the first cohort, 16-17-week-old wild-type mice were firstly anaesthetized with Nimatek (100mg/kg, ketamin and clorocresol 1000:1; IP) and Domitor (1 mg/kg, Metedionin Clorohidrate; intraparenchymal (IP) and then, received a single injection per hemisphere of AAV1, AAV2 or mosaic AAV1/AAV2 vectors in the striatum, in a final volume of 2 µL. The striatum coordinates were +/- 1.8 mm lateral to the middle line, 0.6 mm posterior to the bregma and -3.3 mm ventral to the brain surface (Marcelo *et al.*, 2019). Using a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA), administrated with an automatic injector (Stoelting Co.,Wood Dale,IL,USA), a total of 3×10^9 vg of each AAV vector serotype was IP injected at a speed of 0.5µL/min per striatum.). To minimize the backflow, the needle was kept motionless for 5 min after the vector administration.

3.3.2. Injection in the cerebellum

In the second cohort, 17-22-week-old wild-type mice were anaesthetized with Nimatek (100mg/kg, ketamin and clorocresol 1000:1; IP) and Domitor (1 mg/kg, Metedionin Clorohidrate; IP) and received a bilateral injection of AAV1, AAV2 or mosaic AAV1/AAV2 per DCN, in a final volume of 2 µL,. The DCN was defined by the coordinates +/-2 mm lateral to the middle line, -6 mm posterior to the bregma and -3.3 mm ventral to the brain surface (Sakamoto and Endo, 2013). Using a 34-gauge blunt-tip needle linked to a Hamilton syringe (Hamilton, Reno, NV, USA), administrated with an automatic injector (Stoelting Co.,Wood Dale,IL,USA), a dose of 5×10^9 vg was injected per DCN at a speed of 0.5µL/min). To minimize the backflow, the needle was kept motionless for 5 min after the vector administration.

3.4 Tissue Processing

Animals where anesthetized with lethal injection of Nimatek and Domitor IP, and cardiacaly perfused with autoclaved phosphate-buffer solution (PBS) 0.1M, 4 weeks post stereotactic injection. Brain were removed and divided in two hemispheres.

For the IP injection in the striatum, one hemisphere as used to collect the striatum punch using a Harris Uni-Core pen with 2.5 mm diameter (TedPellaInc., Redding, California, USA) and stored at -80 ºC. The other hemisphere was post-fixed over three days on 4% paraformaldehyde-phosphate buffer (PFA) (Fluka, Sigma, SigmaAldrich, St.Louis, Missouri, USA), followed by exchange of 4% PFA over three days and soaked for 48h in 20% sucrose solution before freezing.

The IP injection in the DCNs, one hemisphere was post-fixed for histological analysis as described above and the other hemisphere was stored in separate tubes, collecting the cerebellum apart from the cerebrum.

The histologic samples were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura FineTechnical Co., Ltd., Tokyo, Japan), frozen and sectioned on a cryostat (CryoStar NX50, Thermo SCIENTIFIC, Germany) and stored in a 48-well trays, free floating in 0.1 M PBS supplemented with 0.05 µM sodium azide, at 4 ºC until further usage. Striatum of injected mice were sectioned in a coronal plane with a 20-µm thickness at -20 ºC, and a total of five 48-well trays were recovered. The DCN injected mice were sectioned in a sagittal plane with a 30-µm thickness at -20 ºC, and a total of three to four 48-well trays were recovered.

3.4.1 DNA and RNA Extraction

The DNA and RNA were extracted from the punches recovered from the striatum region in mice IP injected. The extraction was performed using the AllPrep® DNA/RNA/Protein Mini kit (Qiagen, USA) and following the manufacturer instructions.

3.4.2 Quantitative Real-time Polymerase Chain-Reaction for and Viral Titter

The AAV real-time PCR titration kit (TaKaRa, Shira, Japan) was used to quantity AAV titer as well as copy number of AAV per DNA in transduced cells/tissues following the manufacturer instructions.

3.5 Immunohistochemistry with DAB Revelation

For the immunostaining process, sections where washed in PBS 0.1M and incubated with phenyl hydrazine hydrochloride solution for 30 min at 37 ºC. The coronal sections were washed

three times in PBS 0.1M for 5, 10, and 10 min under moderate agitation, then blocked in blocking solution (0.1% Triton, 10% NGS in PBS 0.1M) for 1 hour. Primary antibodies 1GFP63 (1:1000; Biolegend, CA, USA, CAT:668205) and anti-DARP-32 (1:1000; Millipore Corp., 290 concordRD, Billerica, MA, USA; AB10518) were incubated overnight in blocking solution at 4 ºC under gentle agitation. Sections were then washed three times as described above and incubated with the secondary antibody in blocking solution with gentle agitation for two hours. Secondary antibody, Goat Anti-mouse IgG antibody (H+L), biotinylated (1:200,Vector Laboratories, Inc, CA, USA, BA-9200) was used for detection of GFP and Goat Anti-mouse IgG antibody (H+L), biotinylated (1:200; Vector Laboratories, Inc, CA, USA, BA-1000) was used for DARP-32 detection. The sections were washed three times as described above and incubated in VECTASTAIN ABC kit (Vector Laboratories Inc, CA, USA, PK-6100) for 30 to 40 min under moderate agitation. Sections were washed three times and reveled under DAB revelation (Vector Laboratories, Inc, CA, USA, SK-4100). Sections were then washed three times and inserted on gelatinous covered glass slides followed by overnight drying at room temperature. Finally, they were mounted with EuKitt ® (O.Kindler GmbH&CO, Freiburg, Germany, BCBT5431) and cover slipped.

Light microscopy was performed using a Axio Imager Z2 microscope (Zeiss, Jena, Germany) with a 5x objective (EC Plan-Neofluar 5x/1.5).

3.6 Fluorescent Immunohistochemistry

For the immunostaining process, sections were washed three times in PBS 0.1M for 5, 10, and 10 min under moderate agitation, then blocked in “blocking solution” (0.1% Triton, 10% NGS in PBS 0.1M) for 1 hour. Primary antibodies, anti-FOX3 (NeuN) Antibody (1:1000, Biolegend, CA, USA, CAT: 834501), anti-Glial Fibrillary Acidic Protein (GFAP) (1:1000; Biolegend, CA, USA, CAT:644702) and anti-Iba1 (1:1000; Millipore Corp., 290 concordRD, Billerica, MA, USA; MABN92) were incubated overnight in blocking solution at 4 ºC under gentle agitation. Sections were washed three times, as described above, and incubated with secondary antibody in blocking solution with gentle agitation for two hours. Secondary antibodies, goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 (1:200; Invitrogen by ThermoFisher Scientific, A11005) was used for NeuN, GFAP and Iba-1 detection. Sections were then washed three times and inserted on gelatinous covered glass slides followed by overnight drying at room

temperature. Finally, they were mounted with Flouromount-G ™ Mounting Medium with DAPI (Invitrogen by ThermoFisher Scientific, Ref.4959-52) and cover slipped.

3.7 Quantification of GFP expression volume

Fluorescent images of the entire striatum and cerebellum were acquired using an Axio Imager Z2 microscope (Zeiss, Jena, Germany) and a 5x objective (EC Plan-Neofluar 5x/0.15). For each section, the GFP area was measured using Zen 3.1 lite software (Blue edition, Zeiss). The final volume of GFP transduction in the mice injected in the striatum was determined with the following formula: **volume = a*160**, where "a" is the total area of transduction per animal and "160" corresponds to the distance between analyzed sections. The volume of GFP transduction in the animals injected in the DCN was calculated following the formula: **volume = a*240**, where "a" corresponds to the total area of transduction and "240" is the distance between analyzed sections.

As additional information, mice injected in the striatum had a total of 24-30 coronal sections and mice injected in the deep cerebellar nuclei had 18-22 sagittal sections/animal.

3.8 Determination of the transduced brain regions

The determination of the transduced brain regions was performed by direct visualization on fluorescent microscopy (Zeiss, Jena, Germany) while following the Allen Brain Atlas online (Sciences., 2020). To determine the transduced areas in the striatum of injected mice, the corpus callosum, the ventricle size and hippocampal region were used as references. In the case of DCN-injected mice the regions used as references were the cerebellum variation, the fiber tracts, and the ventricles.

3.9 Determination of the vector cell tropism

Confocal imaging was performed at the Light Microscopy Unit of CBMR-Ualg, using a LCM 710, Axio Observer, and an objective EC Plan-Neuofluoar 40x/1.30 Oil Dic M27. A z-stack in a selected zone of the section was acquired. Zen 3.1 (blue edition) software was used for image processing.

3.10 Statistical Analysis

The GraphPad software (La Jolla, USA) was used for statistical analysis using the ANOVA for multiple comparisons. Statistically significant values were considered for $P < 0.05$ and $P < 0.001$.

Chapter 4 – Results

4.1 Results from Intraparenchymal (IP) injection of Adeno-associated Viruses (AAVs) in the Striatum

4.1.1. Global striatal transduction: comparison between mosaic AAV1/AAV2 and parental capsids

The mosaic AAV1/AAV2 was developed by the "triple transfection method", i.e. insertion in a packaging cell of the helper construct, the vector transgene and the Rep/Cap construct, that in this case were two plasmids, one coding for the AAV1 serotype capsid genes and the second coding for the AAV2 capsid serotype. This new strategy allows the creation of an AAV1/AAV2 mosaic vector that, apart from enabling purification based on heparin affinity chromatography, may increase the capacity for greater distribution and tropism than individual parental capsids. To evaluate this, a ssAAV encoding for the green fluorescent protein (GFP) reporter protein driven by a Cytomegalovirus (CMV) promoter in between of two AAV2 inverted terminal repeats (ITRs) were packed into AAV1, AAV2 or mosaic AAV1/AAV2 vectors (see Figure 4.1). The CMV promoter is a small size, immediate early promoter able to act simultaneously as an enhancer (Fitzsimons *et al.*, 2002; Schmidt *et al.*, 1990). It is a constitutive promoter that has demonstrated to drive AAV transgene long-term expression in the brain (Fitzsimons *et al.*, 2002; Kaplitt *et al.*, 1994).

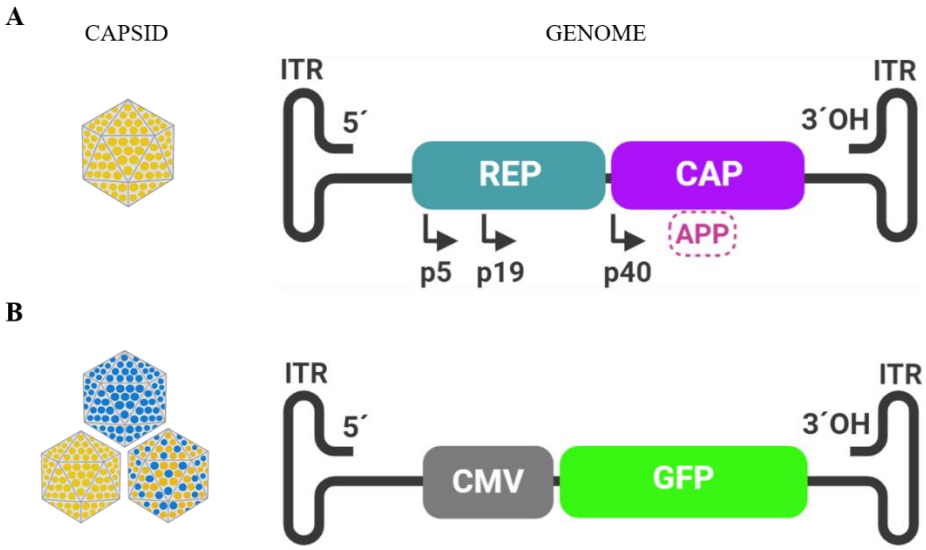

Figure 4. 1 Wild-type AAV and recombinant AAV Vectors used in the present study. **A** Wild type AAV capsid with representative genome, codifying for REP, CAP and APP proteins controlled by p5, p19 and p40, promoters. **B** Recombinant AAV Vectors used in study encoding for GFP protein under the control of CMV promoter. Blue viral capsid represents the AAV1 serotype. Yellow viral capsid represents the AAV2 serotype. And the blue and yellow capsid represents the mosaic AAV1/AAV2. AAV- Adeno-associated Virus; REP - – replication genes; CAP – Capsid genes; APP – Assembly gene; ITR – Inverted Terminal Repeats; CMV – Cytomegalovirus Promoter; GFP – Green Fluorescent Protein.

The vectors were IP injected in the striatum and animals sacrificed one-month post injection. Sections from the striatum region were collected and analyzed in fluorescent microscope (see Figure 4.2 A and B). Light microscopy was also performed to identify regions expressing GFP (see Annex 1), however fluorescent microscopy provided a better GFP visualization. The measurement of individual GFP transduced areas was calculated using the Zen 3.1 software, which determined the transduction volume per animal (see Figure 4.2 C). Mosaic AAV1/AAV2 and its parental serotype AAV1 had a similar global level of transduction, with an average value of 7.3mm^3 and 5.7mm^3 per animal, respectively. AAV2 had a much lower global volume of transduction, approximately 2.6 mm^3 per animal.

Total DNA was also isolated from the striatum punch and copy number of AAVs analyzed by qPCR (see Figure 4.2 D). The number of vg per ug of DNA correlates with the GFP global transduction. The mosaic AAV1/AAV2 and the AAV1 parental serotype had similar number of vg/ng (2925 vg/ng and 2105 vg/ng, respectively), while AAV2 had 438.7 vg/ng.

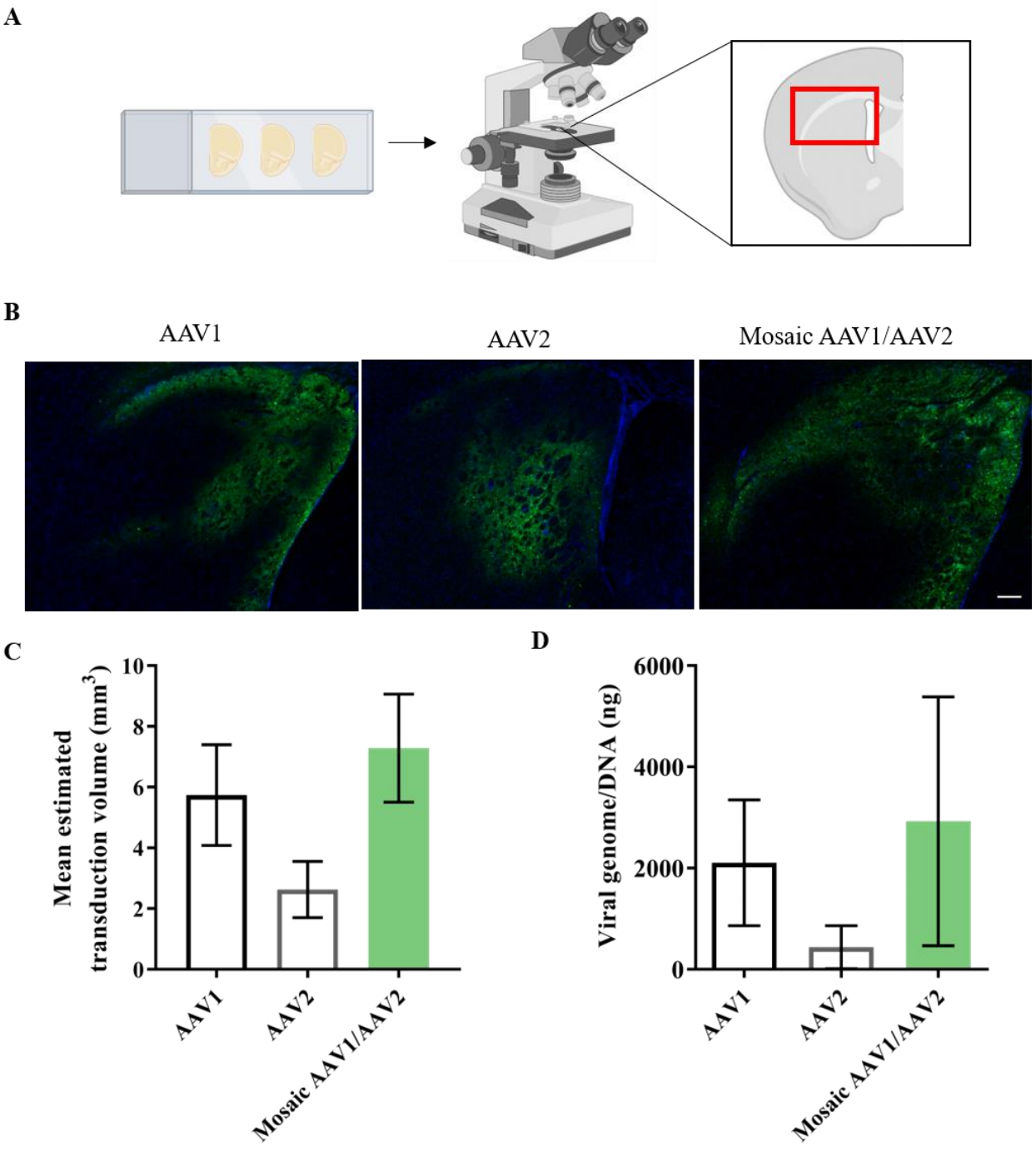

Figure 4. 2 Global striatal transduction between mosaic AAV1/AAV2 and parental capsids, following IP injection in the striatum. **A** Schematic representation of the mounted sections and microscopic field of vision. **B** Representative images from fluorescent microscope Imager.z2. Coronal sections displaying GFP transduced regions (green). Blue – nucleus staining. **C** Estimated transduction volume of mosaic AAV1/AAV2 and parental serotypes in the striatum. **D** Mean viral genomes (vg) per group of animals transduced with each AAV vector. Scale bar 200µm. Values are expressed ± SEM. Number of animals per group (AAV1, n= 4; AAV2, n= 3; AAV1/AAV2, n= 3).

4.1.2. AAV spreading in the striatum: comparison between mosaic AAV1/AAV2 and parental capsids

From the previous results, it is clear that mosaic AAV1/AAV2 and AAV1 vectors spread was larger than AAV2 vectors. To determine the vectors biodistribution from the injection site, the transduced region was divided in smaller fractions and the volume was calculated for each vector. The injection site is located between the 1.42 to 0.85 mm relative to bregma (see Figure 4.3 A). The results are concomitant with the global spreading, indicating that mosaic vector AAV1/AAV2 and AAV1 have a higher level of transduction throughout the striatum than AAV2 serotype (see Figure 4.3 C). However, it is interesting to note that AAV vectors do not spread uniformly from the injection site, instead they have a more anterioposterior spreading. The vectors have a higher tendency to have a dorsal flow from the injection site, transducing regions dorsal to the striatum.

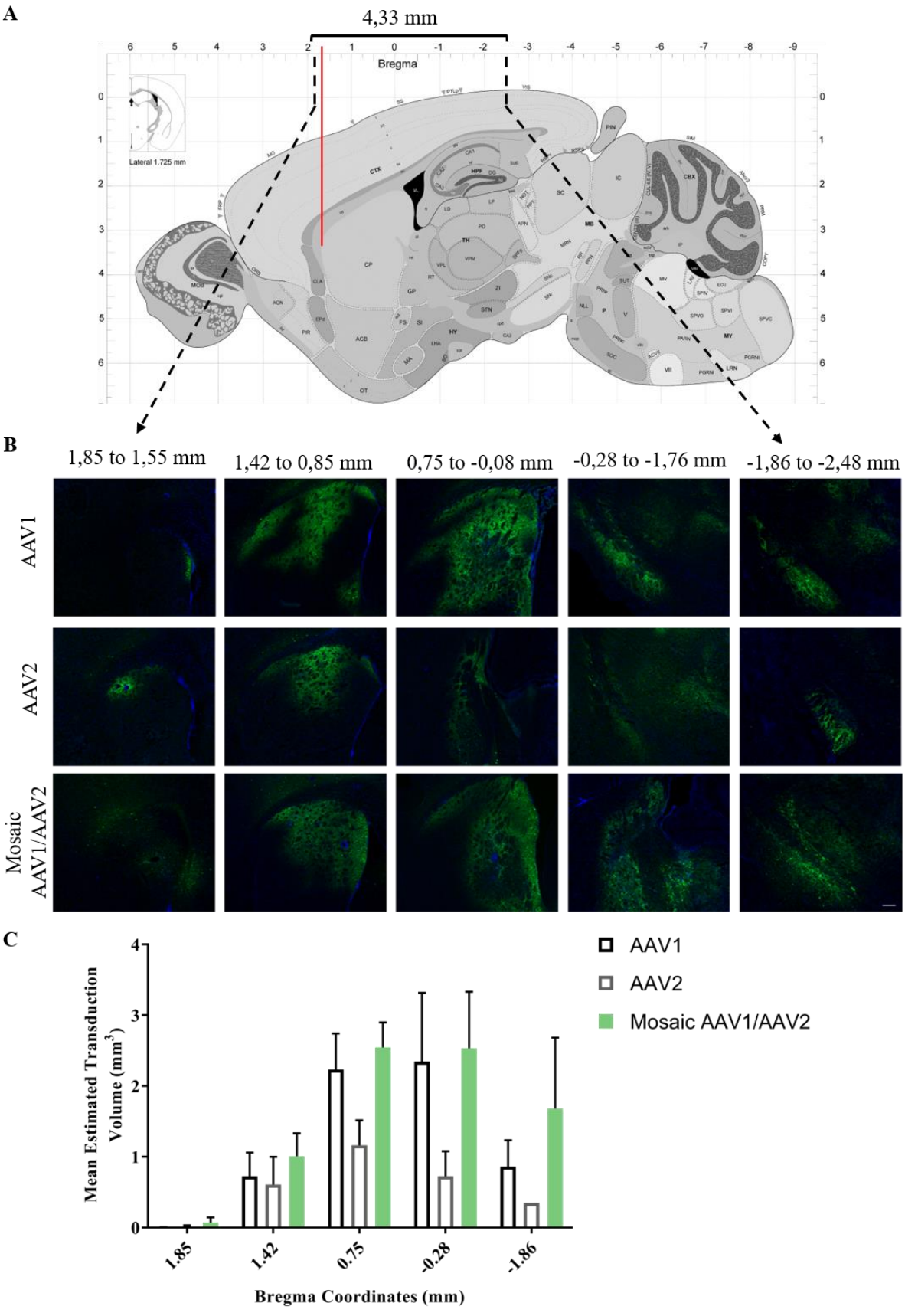
A
4,33 mm
Bregma
Lateral 1.725 mm
B
1,85 to 1,55 mm
1,42 to 0,85 mm
0,75 to -0,08 mm
-0,28 to -1,76 mm
-1,86 to -2,48 mm
AAV1
AAV2
Mosaic AAV1/AAV2
C
Mean Estimated Transduction Volume (mm3)
AAV1
AAV2
Mosaic AAV1/AAV2
1.85
1.42
0.75
-0.28
-1.86
Bregma Coordinates (mm)

Figure 4. 3 Spreading of the three serotypes following IP injection in the striatum. **A** Schematic sagittal representation of the 4.33mm region expressing GFP. Adaptation from an Allen brain atlas image. Injection site – Red line. **B** Representative images of coronal sections expressing GFP (green) from the begging of the striatum region forward, as despised in the schematic sagittal plane. Reference to bregma is given on top image. Blue – nucleus staining. Scale bar 200µm. **C** Mean estimated transduction volume per brain striatal region. Values are expressed ± SEM. Number of animals per group (AAV1, n= 4; AAV2, n= 3; AAV1/AAV2, n= 3).

4.1.3. Tissue tropism of vector AAV1, AAV2 and AAV1/AAV2

Next, to identify the specific brain regions transduced by the mosaic AAV1/AAV2 and parental serotypes, all sections were analyzed following the Allen brains atlas online (Sciences., 2020). The results are displayed in Table 4.1 Mosaic AAV1/AAV2 is expressed in all regions. To distinguish how the transduction varies between the viral vectors, a qualitative scale which indicate the level of GFP expression was defined. Starting with the mosaic AAV1/AAV2 levels of expression, the highest levels occurred in the caudadoputamen in the striatum and the globus pallidus. High levels of expression were detected in the caudate nucleus and subtalamic nucleus. Moderate levels of expression were also found at the primary motor and somatosensory cortex are and nucleus accumbens, whereas low levels of expression were observed at the corpus callosum, and cerebral cortex.

The parental serotype, AAV1, had a very similar pattern of transduction compared with the mosaic AAV1/AAV2 vector, except for 1) failing to transduce the posterior parietal association areas of the cerebral cortex and, 2) had low levels of transduction of the caudate nucleus region. The AAV2 serotype, had distinct levels of transduction compared with the two other vectors, with no areas where the transduction was superior to the mosaic AAV1/AAV2 or AAV1 vectors. It did not transduce the nucleus accumbens, caudate nucleus, corpus callosum, and the cerebral cortex regions. High levels of transduction were detected at the caudadeputamen and moderate levels of transduction at the globus pallidus. Low levels of transduction occurred at the primary cortex regions and subthalamic nucleus. Illustrative images of different brain regions transduced by the mosaic AAV1/AAV2 vector and parental serotypes are depicted in Figure 4.4.

Table 4. 1 Brain regions transduced by mosaic and parental serotypes upon striatal injection.

Brain region	**AAV1**	**AAV2**	**Mosaic AAV1/AAV2**
Cortex			
Primary Motor Area	++	+	++
Primary Somatosensory Area	++	+	++
Striatum			
Nucleus Accumbens	++	-	++
Caudadoputamen	++++	+++	++++
Caudate Nucleus	+	-	+++
Corpus Callosum	+	-	+
Hypothalamus			
Subthalamic Nucleus	+++	+	+++
Pallidum			
Globus Pallidus (External Segment)	++++	++	++++
Cerebral Cortex			
Claustrum	+	-	+
Posterior Parietal Associations Areas	-	-	+

-, No expression levels of expression; + relatively low level of expression; ++, moderate level of expression; +++, high level of expression; ++++, highest level of expression.

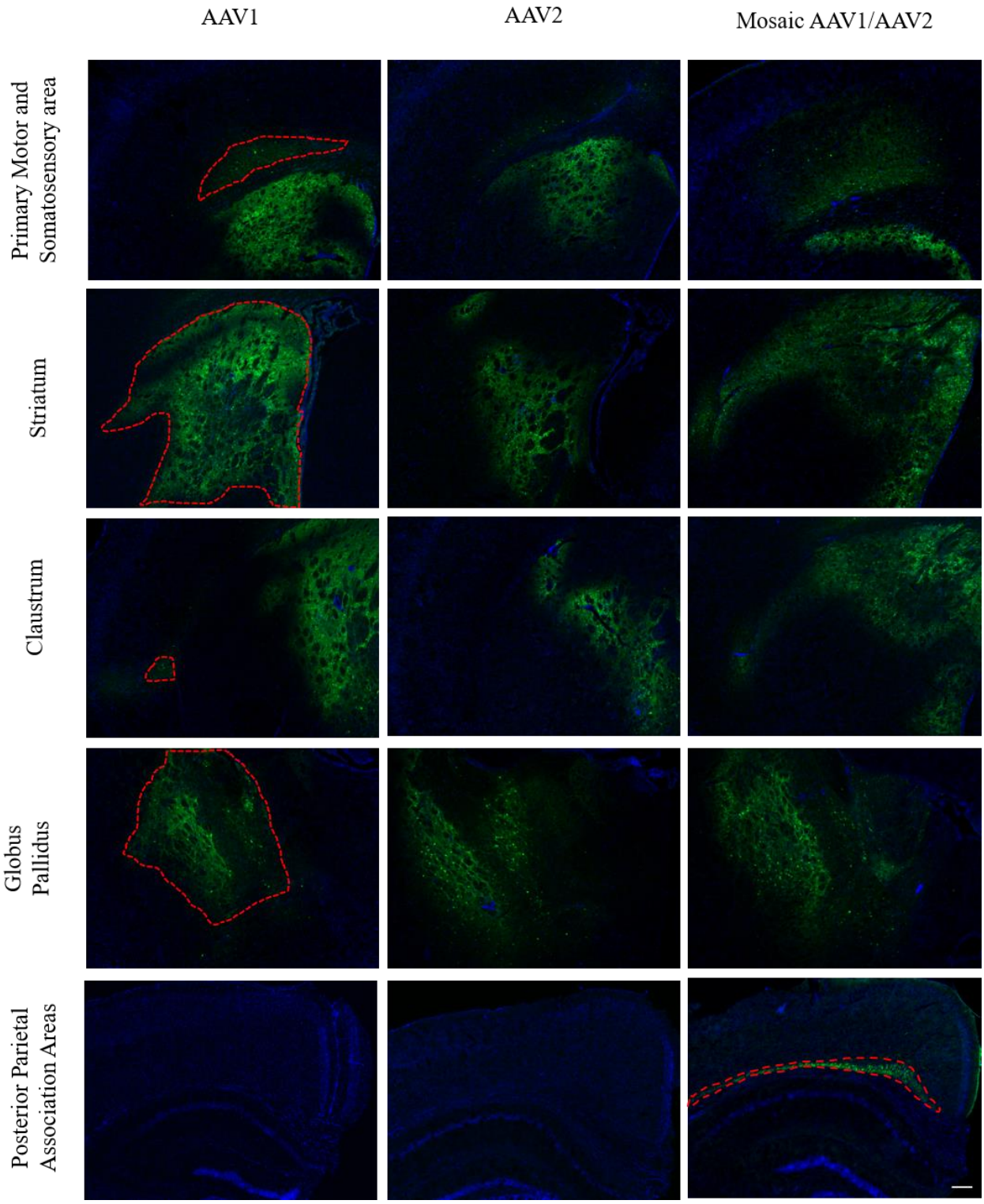

Figure 4. 4 Brain regions transduced by mosaic AAV1/AAV2 vector and parental serotypes, following IP injection at the striatum. Coronal cuts of brain regions expressing GFP (green), for mosaic AAV1/AAV2 and parental serotypes. Each dotted line delimitates the respective brain region. Blue – DAPI staining. Scale bar 200 µm.

4.1.4. Cell Tropism of vector AAV1 AAV2 and AAV1/AAV2

After investigating the global spreading and brain regions transduced by the mosaic AAV1/AAV2 vector and parental serotypes, a more detailed study was performed to determine which brain cells does the mosaic AAV1/AAV2 targeted, as well as parental serotypes. Previous reports have demonstrated that AAV vectors have a neuronal tropism when injected in the CNS (Kaplitt *et al.*, 1994). Confocal microscopy was performed to determine the colocalization of GFP and CNS cell markers. The antibodies used were NeuN, GFAP and Iba-1 for neurons, astrocytes, and microglia cells staining, respectively. Strikingly, results suggest no vector colocalization with neural marker NeuN (see Figure 4.5).

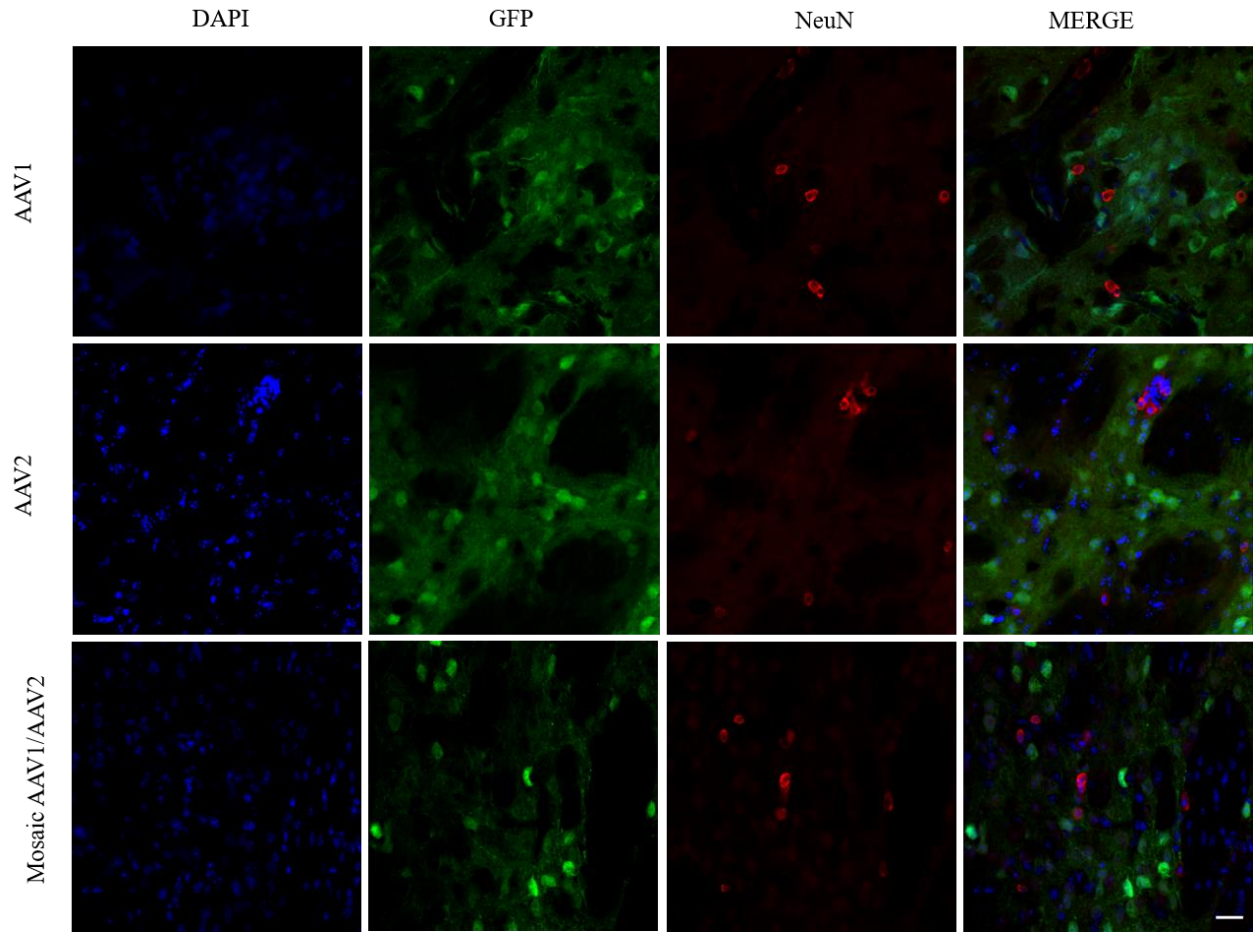

Figure 4. 5 Mosaic AAV1/AAV2 and parental serotypes do not colocalize with NeuN-positive cells in the striatum of injected animals. Confocal microscopy was performed for colocalization of GFP (green) and NeuN (red), a neuronal antibody. Blue – DAPI staining; Scale bar 20µm.

As expected, no colocalization between the vectors and the astrocytes or microglia cells was observed in confocal microscopy (see Figure 4.6).

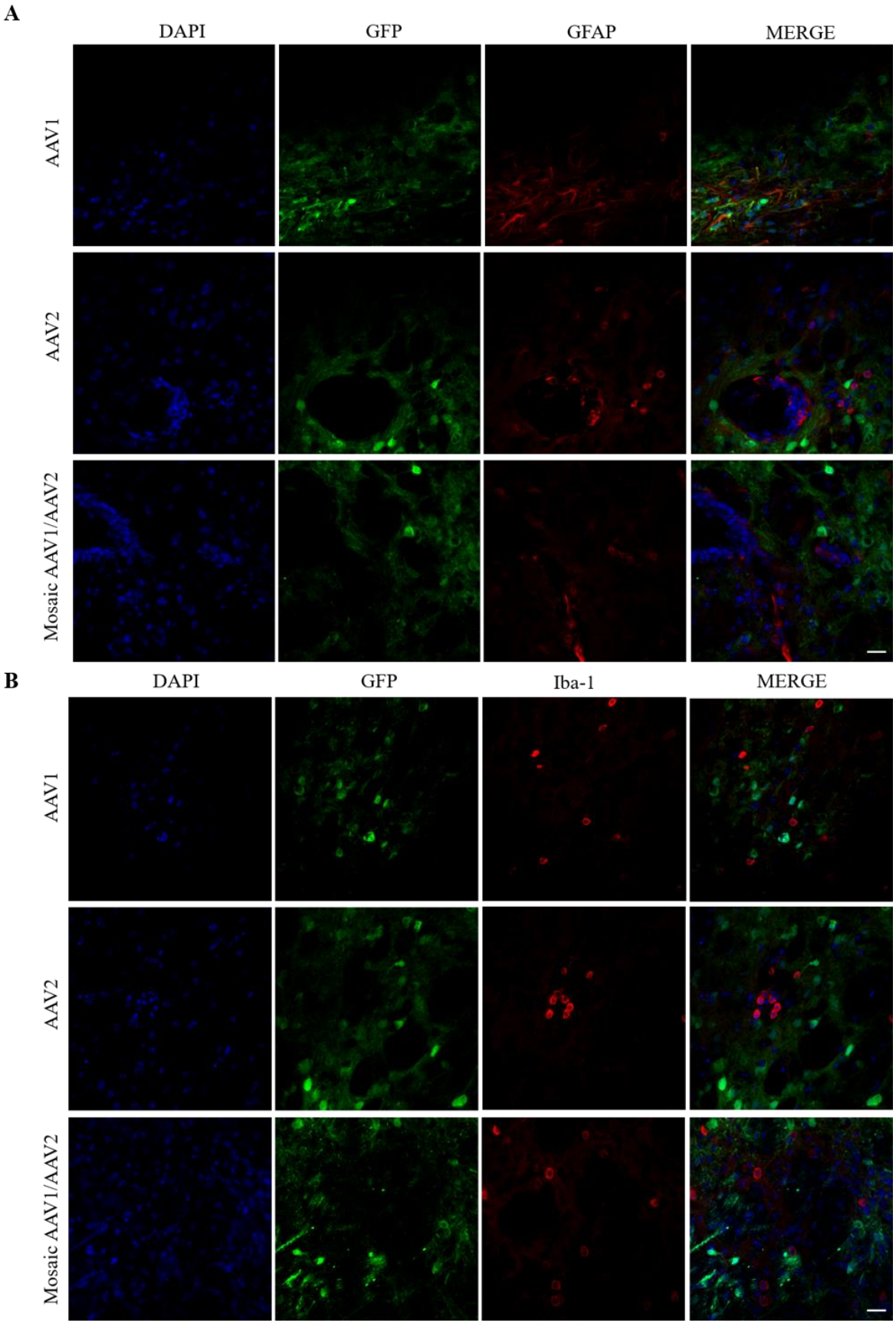
A
DAPI
GFP
GFAP
MERGE
AAV1
AAV2
Mosaic AAV1/AAV2
B
DAPI
GFP
Iba-1
MERGE
AAV1
AAV2
Mosaic AAV1/AAV2

Figure 4. 6 Mosaic AAV1/AAV2 do not have tropism to astrocytes and microglia cells, in striatum injected animals. **A** Confocal microscopy to demonstrate the colocalization of transduced cells (green) and astrocytes (Red). **B** Confocal microscopy to demonstrate the colocalization of transduced cells (green) and microglia (Red). Blue – DAPI; Scale bar 20µm.

4.1.5. Toxicity profile of vectors AAV1, AAV2 and mosaic AAV1/AAV2

One important feature of delivery systems to gene therapy is to be safe, i.e. able to deliver the nucleic acid without damaging the target tissue. To assess the safety of mosaic AAV1/AAV2 and parental serotype vectors, neuronal staining was performed for all animals (see Figure 4.7). The DARP-32 is a cytosolic protein prominent in spiny neurons of the neostriatum (Nishi *et al.*, 1997). When the DARP32 protein is impaired is an indicator of downregulation of efficient dopaminergic neurotransmission (Fienberg *et al.*, 1998). The results suggest no loss of neuronal marker DARP-32 antibody in the striatum following the injection of the mosaic vector and parental serotypes.

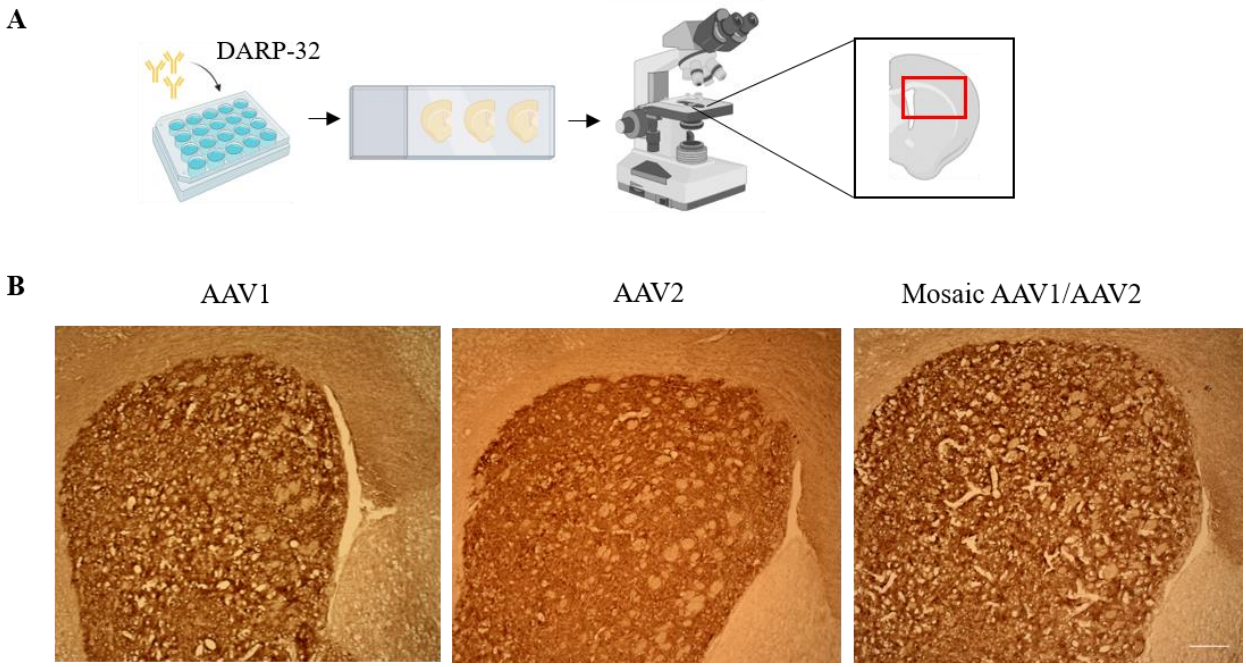

Figure 4. 7 Mosaic AAV1/AAV2 and parental serotypes do not induce DARP-32 depletion upon striatal injection. **A** Visible immunohistochemistry of DARP-32 staining. **B** Representative image of DARP-32 staining in the striatum transduced with different AAVs serotypes at the same quantity ($3x10^9$ viral genomes) per striatum, demonstrating no neuronal loss. Scale bar 200 µm.

4.2 Results from the IP injection of AAVs at the Cerebellum

4.2.1. Global cerebellar transduction comparison between mosaic AAV1/AAV2 and parental capsids

To determine how the vectors behaves upon IP injection in a different brain region, the mosaic vector and the parental serotypes were also injected at the DCN region in the cerebellum. As described above, one-month post injection the mice were sacrificed, and brain was collected. Then, fluorescence microscopy analysis was performed to measure the GFP area per section aiming to calculate the total transduction volume per animal (see Figure 4.8). In agreement with the results obtained in the striatum injection, the mosaic AAV1/AAV2 and parental serotype AAV1 has similar results. However, AAV1 vector had a greater mean transduction value of 54.3 mm^3, while mosaic AAV1/AAV2 had a mean transduction volume of 31.6 mm^3. AAV2 serotype had the lowest mean transduction value of 17.1 mm^3.

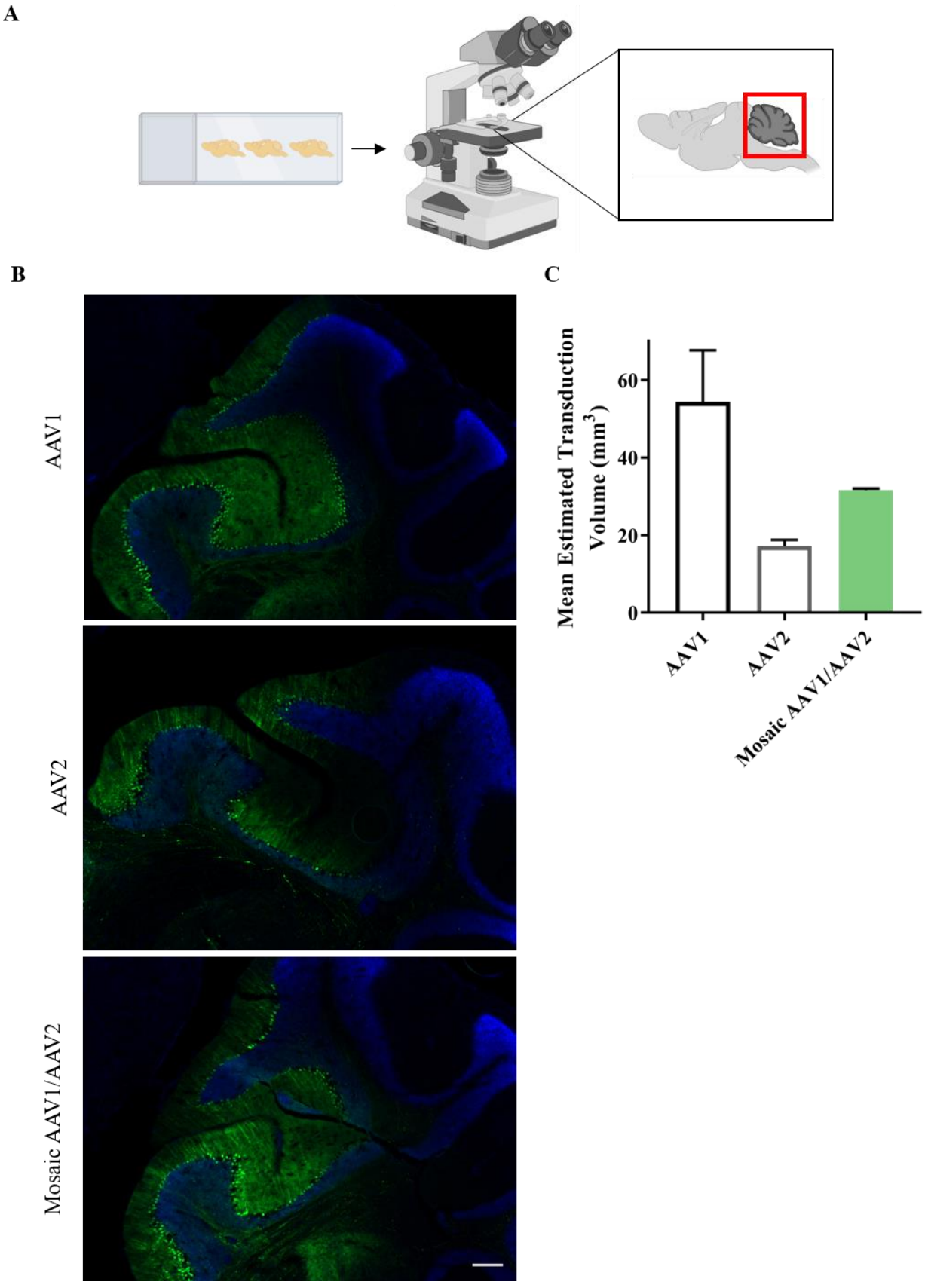
A
B
C
AAV1
AAV2
Mosaic AAV1/AAV2
Mean Estimated Transduction Volume (mm3)
60
40
20
0
AAV1
AAV2
Mosaic AAV1/AAV2

Figure 4. 8 Mosaic AAV1/AAV2 and parental serotypes spreading and transduction in DCN injected mice. **A** Schematic representation of the mounted sections and microscopic field of vision. **B** Representative images from fluorescent microscope Imager.z2 of the sagittal sections displaying the transduced regions (green). Blue – DAPI staining. **C** Estimated transduction volume of mosaic AAV1/AAV2 and parental serotypes. **D** Value of viral genomes (vg) per DNA. Scale bar 200µm. Values are expressed ± SEM. *P<0.05 by one-way ANOVA. Number of animals per group (AAV1, n= 3; AAV2, n= 3; AAV1/AAV2, n= 3).

4.2.2. Spreading of mosaic AAV1/AAV2 upon DCN injection

After the global analysis, it was important to determine how does the mosaic vector spread from the DCN injection site in comparison with the parental serotypes. Thus, the transduced region was separated per brain regions according with the sections collected (see Figure 4.9 A). The mean volume per brain region in DCN injected mice revealed higher volumes of transfection closer to the medio part the brain than in the lateral part of the brain. In the 2.35 to 1.53 region significant p-values between AAV1 and AAV2 and between the AAV1/AAV1 and AAV1/AAV2 were found.

The set of imagens from the 2.38 to 1.53 mm brain region of the animals injected with the mosaic AAV1/AAV2 and parental serotypes (see Figure 4.9 B) indicates the transduction of purkinje cells (green stained).

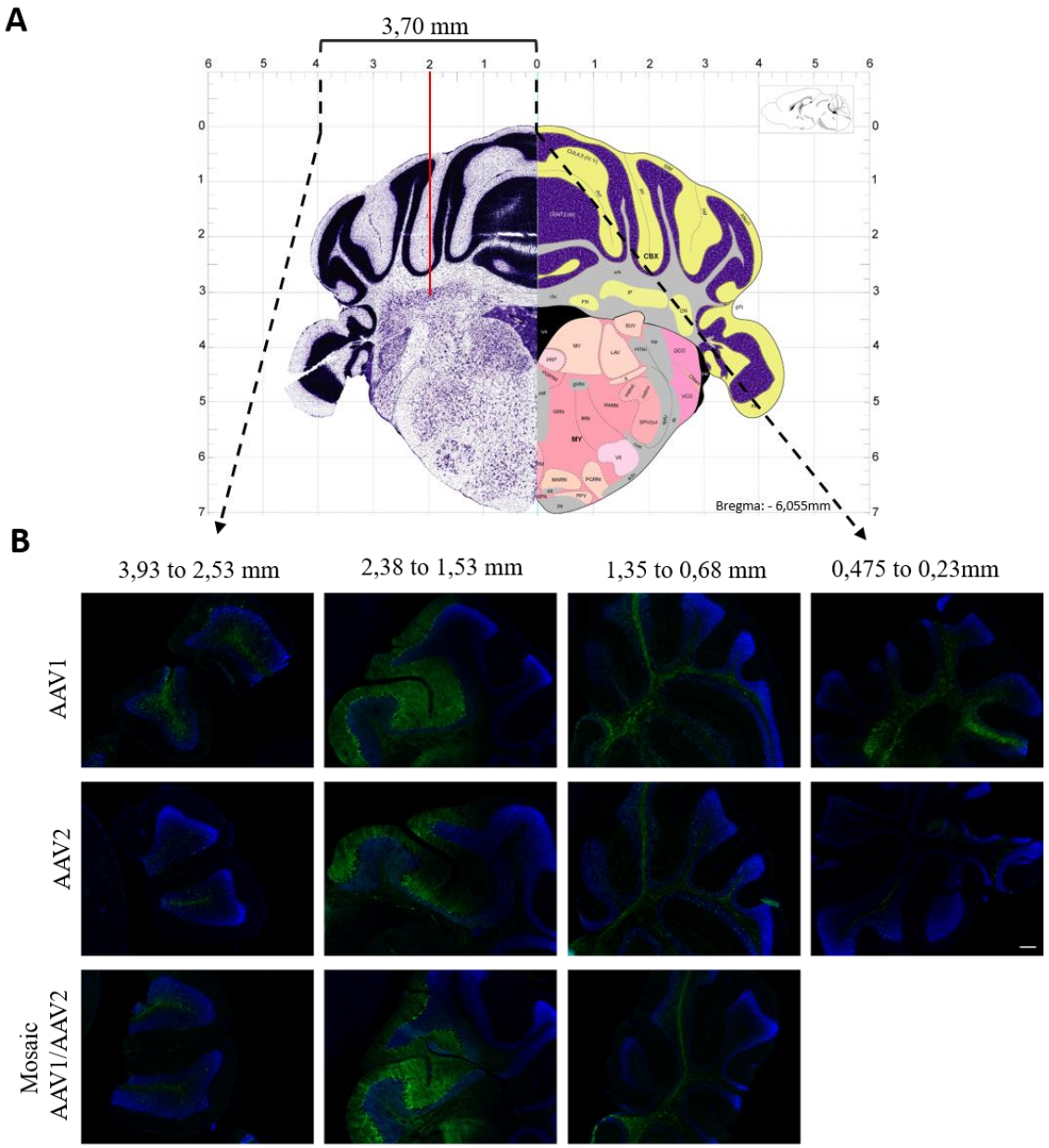
A
3,70 mm
Bregma: - 6,055mm
B
3,93 to 2,53 mm
2,38 to 1,53 mm
1,35 to 0,68 mm
0,475 to 0,23mm
AAV1
AAV2
Mosaic AAV1/AAV2
C
Mean Estimated Transduction Volume (mm3)
Medio-lateral (mm)
3.93
3.38
1.35
0.45
AAV1
AAV2
Mosaic AAV1/AAV2

Figure 4. 9 Spreading of the three serotypes following IP injection at the DCN. **A** Schematic coronal representation of the 3.70 mm region expressing GFP. Adaptation from an Allen brain atlas image. Injection site – Red line. **B** Representative images of sagittal sections expressing GFP (green) from the beginning of the cerebellum region forward, as depicted in the schematic coronal plane. Blue – nucleus staining. Scale bar 200µm. **C** Mean estimated transduction volume per region. Values are expressed ± SEM. *P<0.05; ***P < 0.001 by one-way ANOVA. Number of animals per group (AAV1, n= 3; AAV2, n= 3; AAV1/AAV2, n= 3).

4.2.3. Tissue Tropism of vectors AAV1, AAV2 and AAV1/AAV2

Brain regions transduced by mosaic AAV1/AAV2 and parental vectors upon DCN injection are depicted in Table 4.2 .The mosaic AAV1/AAV2 vector has the highest level of expression at the simple lobule; high level of expression was found at the paraflocculus, fiber tracts, medulla and pons; moderate levels of expression at the ansiform lobule, granular layer, flocculus and mid brain; and low levels of expression found at the Cul 4 and 5, thalamus and central amygdala nucleus.

The AAV1 serotype has similar transduction levels of expression to the mosaic vector. It has the highest level of expression at the simple lobule and cul 4 and 5; high levels of expression at the ansiform lobule, paraflocculus, granular layer, flocculus, fiber tracts, medulla and pons; moderate levels of expression at the thalamus and mid brain; and low levels of expression at the central amygdala nucleus.

Like in the striatum, AAV2 serotype vectors had lowest levels of transduction compared with the mosaic AAV1/AAV2 and AAV1 vectors. The regions with moderate levels of transduction are the granular layer, simple lobule and cul 4 and 5; relative low levels of transduction at the ansiform lobule, paraflocculus, flocculus, fiber tracts, midbrain, central amygdala nucleus, medulla and pons; and no levels of expression at the thalamus region. Representative images of the mosaic AAV1/AAV2 and parental serotypes transduced areas are shown in Figure 4.10.

Table 4. 2 Brain regions transduced by mosaic AAV1/AAV2 and parental vectors upon DCN injection

Brain Region	**AAV1**	**AAV2**	**Mosaic AAV1/AAV2**
Cerebellum			
Ansiform Lobule	+++	+	++
Paraflocculus (Granular Layer)	+++	+	+++
Granular Layer	+++	++	++
Flocculus	+++	+	++
Fiber Tracts	+++	+	+++
Simple Lobule, Molecular Layer	++++	++	++++
Culmen - Cul4 and 5	++++	++	+
Thalamus	++	-	+
Mid Brain	++	+	++
Central Amygdala Nucleus	+	+	+
Medulla	+++	+	+++
Pons	+++	+	+++

-, No levels of expression; + relatively low level of expression; ++, moderate level of expression; +++, high level of expression; ++++, highest level of expression.

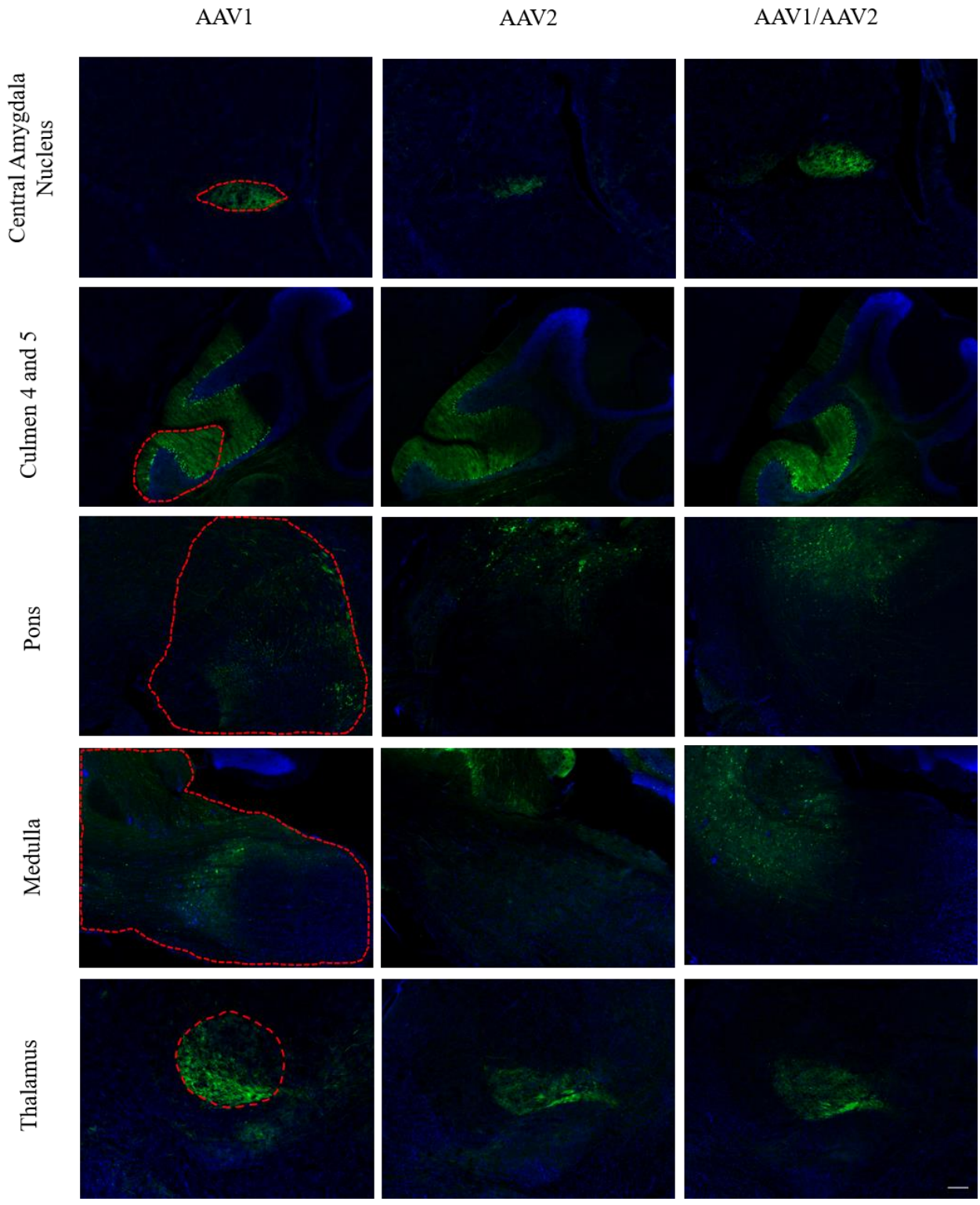

Figure 4. 10 Representative images of the mosaic AAV1/AAV2 and parental transduced brain region, in DCN injected mice. Sagittal cuts of brain regions (inside the dashed line) expressing GFP (green), for mosaic AAV1/AAV2 and parental serotypes. Blue – DAPI staining. Scale bar 200µm.

4.2.4. Cell Tropism of vector AAV1, AAV2 and AAV1/AAV2

The cerebellum is structurally divided in two main structural components: the laminated cerebellar cortex and the deep cerebellar nuclei (DCN), which is a very important region responsible for the motor control. The neuronal cell types that compose the cerebellum are specific for its role in motor control, thus not being able to be stained by the same neuronal marker. Hereupon, it is important to detect what cell types are being transduced by the mosaic AAV1/AAV2 vector when injected at the DCN. To uncover its tropism, fluorescent immunohistochemistry was performed using the same antibody as in the striatum injected mice. As expected, no neuronal tropism was evident AAV vectors (see Figure 4.11). Despite the NeuN antibody being a neuronal specific marker, cerebellar cells are not immunohistochemically stained with this antibody (V. V. Gusel'nikova, 2015).

Amplified confocal imagens also demonstrate the vectors ability to transduce the purkinje cells (see Figure 4.11 and 3.12 A).

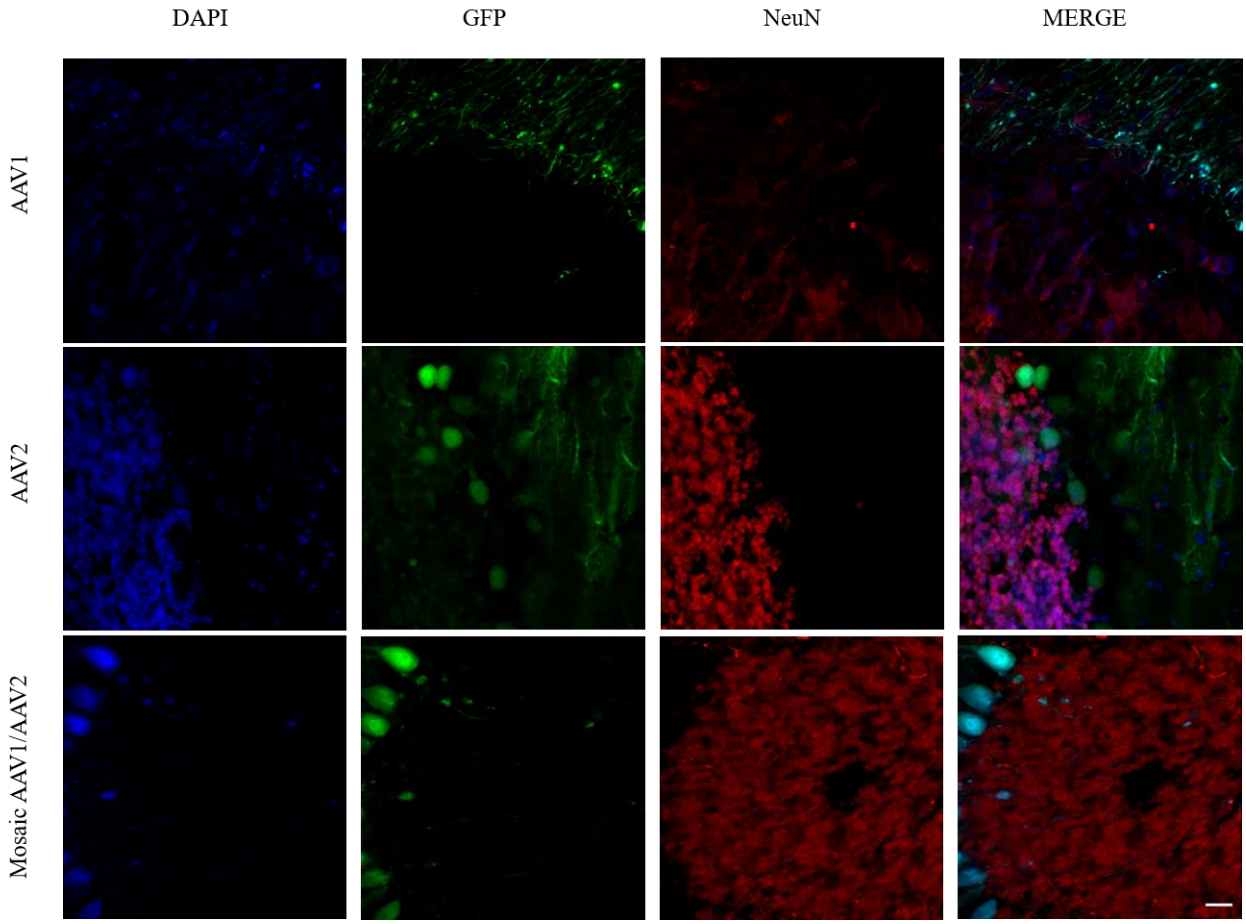

Figure 4. 11 Mosaic AAV1/AAV2 and parental serotypes do not have tropism for neuronal cells. Confocal microscopy was performed to demonstrate the colocalization of transduced cells (green) and neurons (Red). Blue – DAPI; Scale bar 20µm.

In accordance with the results obtained in the striatum, nor the mosaic AAV1/AAV2 or the parental serotypes have a tropism with astrocytes or glial cells (see Figure 4.12).

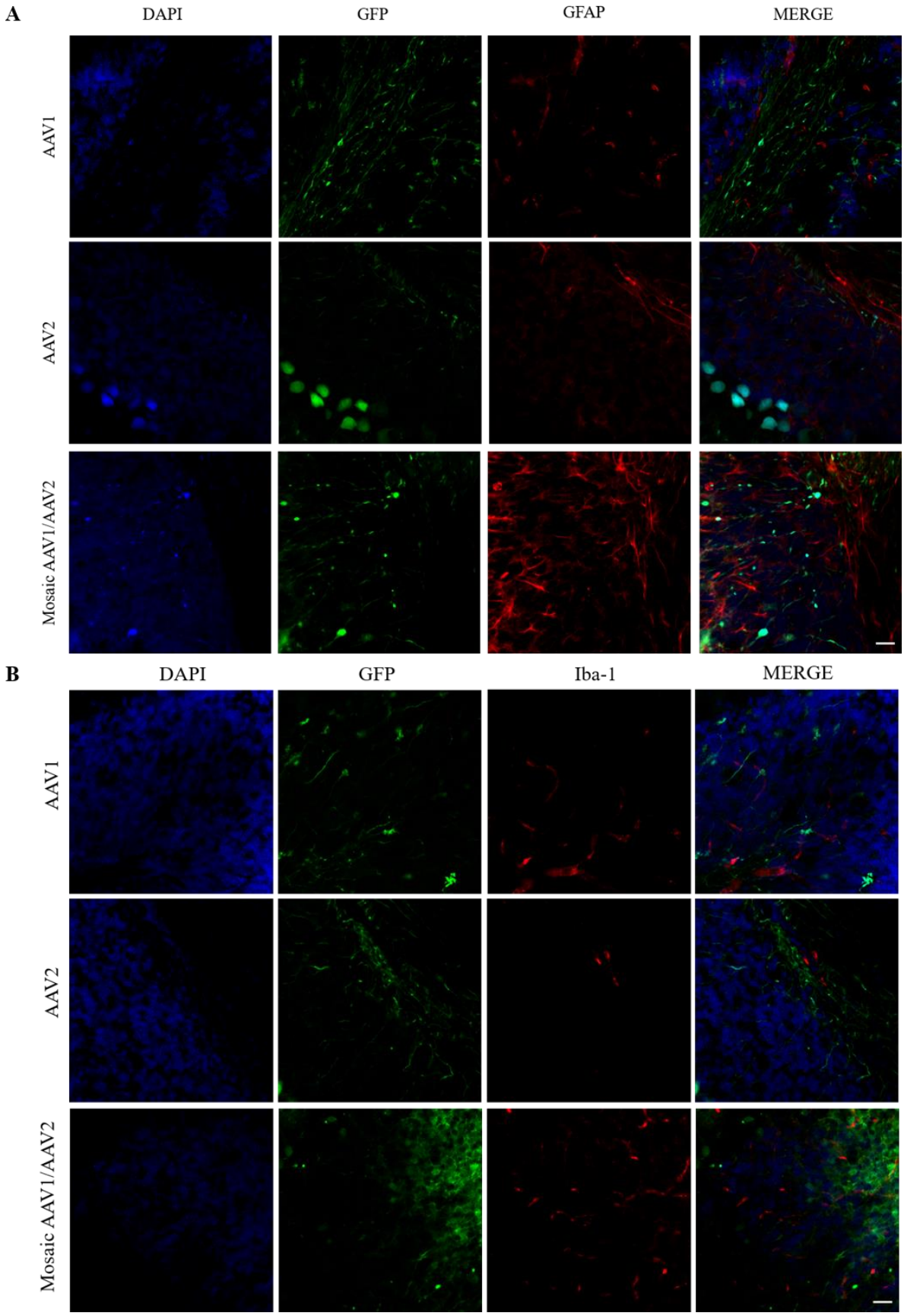
A
DAPI
GFP
GFAP
MERGE
AAV1
AAV2
Mosaic AAV1/AAV2
B
DAPI
GFP
Iba-1
MERGE
AAV1
AAV2
Mosaic AAV1/AAV2

Figure 4. 12 Mosaic AAV1/AAV2 do not have tropism to astrocytes and microglia cells, in DCN injected animals. **A** Confocal microscopy was performed to demonstrate the colocalization of transduced cells (green) and astrocytes (Red). **B** Confocal microscopy was performed to demonstrate the colocalization of transduced cells (green) and microglia (Red). Blue – DAPI; Scale bar 20µm.

Chapter 5 – Discussion

5.1 Animals injected with Adeno-associated Viruses (AAVs) in the striatum

The experiments aforementioned aimed to demonstrate that the mosaic AAV1/AAV2 sustain the features of parental serotypes to become a good delivery vehicle, while preserving the AAV1 neuronal tropism and the AAV2 ability to bind heparin and allow affinity chromatography purification.

From the work here presented, the injection in the striatum of the AAV1/AAV2 mosaic vector showed a greater ability of spreading and transduction when compared with parental serotypes. Although there is a visible difference between the groups, no statistical significance difference was obtained when using the one-way ANOVA test due to the reduced number of animals per group. These results suggest that the mosaic AAV1/AAV2 vector can preserve the AAV1 serotype properties, without interference of the AAV2 serotype. Another interesting feature of the mosaic vector is the ability to transduce the posterior parietal association area (see Figure 4.4), a region that none of the parental serotypes was able to transduce, suggesting the mixture of capsids augmented the mosaic vector transduction ability.

The mosaic AAV1/AAV2 vector spread from the injected site was consistent with the parental serotypes. This pattern followed the striatum structure in the mice brain (see Figure 5.1) and was able to spread throughout other areas such as the cortex, corpus callosum, hypothalamus, pallidum (Figure 4.4 and Table 4.1). Also, it is important to refer that the transduction of the primary motor and somatosensory area can be originated from the needle track when the injection syringe is pulled out, and not spreading from the local of injection.

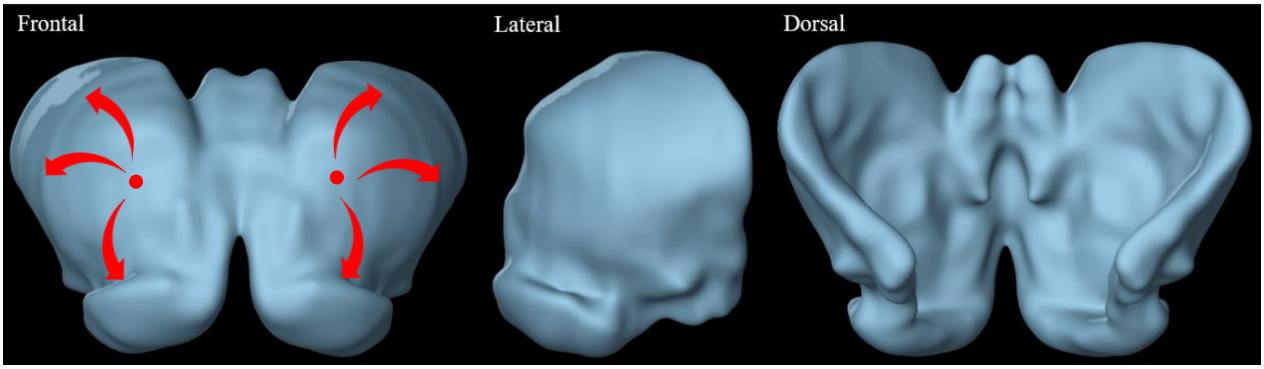

Figure 5. 1 Striatum frontal, lateral and dorsal view. The vectors in the present study spreading (red arrows) following the injection at the injection site (red dot). Adapted from the Brain explorer 2.

Previous research have demonstrated colocalization of the NeuN-positive cells and some AAV vectors upon intraparenchymal (IP) injection in mice brain (Tadokoro *et al.*, 2017). Therefore, it was unexpected to have no colocalization between the green fluorescent protein (GFP) signal and the NeuN neuronal marker in the present study. A more extensive neuronal marker screening should be performed to further understand the mosaic AAV1/AAV2 tropism. The astrocytes and microglia cells are not natural targets of the parental serotypes AAV1 and AAV2, thence, the results are in conformity with the literature.

As previously mentioned, the safety is a very important feature when choosing a delivery vehicle for gene therapy. Previous studies have demonstrate low toxicity when injecting AAV vectors carrying therapeutic treatment (Dufour *et al.*, 2020; Stoica *et al.*, 2013; Wang *et al.*, 2014) None of the vectors in this study indicated loss of neuronal marker DARP-32 when injected in the striatum. This suggests that the mosaic AAV1/AAV2 vector could potentially be part of the *in vivo* delivery system armament.

5.2 Animals injected in the DCN

The results from the DCN injected animals also indicate that the mosaic AAV1/AAV2 and parental AAV1 serotype have a better spreading than the AAV2 vector. However, in this specific location, the AAV1 serotype displayed higher spreading. This could be explained by the brain region where the vectors were injected. Since striatum and DCN have very distinct shape and cellular composition, the AAV1 serotype vector had easiness to spread in the DCN than the mosaic AAV1/AAV2, thence better global spread values. Taking this into consideration, the AAV2 serotype appears to negatively affect the mosaic AAV1/AAV2 spreading in the DCN. Previous studies concluded that the delivery route influences the pattern of CNS transduction and also has an impact in the cellular tropism of the injected vector (Han *et al.*, 2020; Hori *et al.*, 2019; Naidoo *et al.*, 2018; Samaranch *et al.*, 2012). Likewise, other studies conclude that the choice of AAV serotype influences the vector spreading and transduction activity to other organs (Rocha *et al.*, 2011; Zincarelli *et al.*, 2008). The studies above mentioned, help understanding that the spreading and transduction both depend on vector of choice and route of administration of the viral vector. This can help to explain different spreading of the mosaic AAV1/AAV2 and parental serotypes in the present study in the striatum and DCN upon IP injection.

The AAV vector spread per regions follows the cerebellum area per cut (see Figure 4.9 C). To analyze these results, sagittal cuts of the mice brain from the medio-lateral orientation were performed, starting at the most lateral region thus having smaller cerebellum areas than when closer to the medial region (see Figure 5.2). Thus, it is logical that the lateral part has minor area expressing GFP than the medial region. Also, another factor that could have contributed for GFP volume increase toward the medial region. Such areas as the medulla and thalamus regions, are more internal regions in the brain. These regions are only visible in sections closer to the medial point; thus, the transduced areas increase because it is where the medulla and thalamus regions emerge.

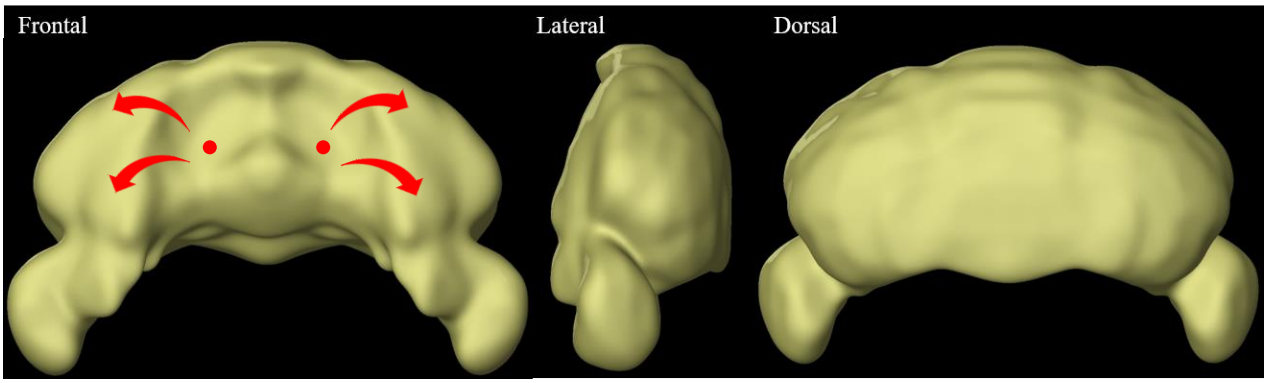

Figure 5. 2 DCN frontal, lateral and dorsal view. The injection site is represented by a red dot and the arrows indicate AAV vectors spreading. Adapted from the Brain explorer 2.

Contrary to the striatum, the cerebellum is a separate region from the rest of the brain, only communicating with other regions through the pons. Structurally, the cerebellum is divided between the laminated cerebellar cortex and the DCN (D. Purves, 2004), the injection location. Despite not being directly connected to the cerebrum, the vectors were able to spread from the cerebellum to close regions such as the pons and medulla, but also to distant regions such as the thalamus and the central amygdala nucleus. This results from the cerebellum connectivity. It receives projections from the cortex, vestibular system, spinal cord and brainstem, and projects to the thalamus (Hintzen *et al.*, 2018); (D. Purves, 2004). The anterior and central amygdala nucleus is part of the amygdala region, which together communicate with the hypothalamus and brainstem that might explain how that area was transduced.

Owing to cerebellum very specialized function, specialized type of cells make part of its composition, namely Purkinje cells. This type of neuron is not stained by the NeuN Ab, whence

no colocalization with the GFP was an expected result. However, it is remarkable how clearly the purking cells are green stained, indicating the vector transduced the purkinje cells. This means mosaic AAV1/AAV2 could prospective viral vector for diseases that impair the function of purkinje cells, such as spinocerebellar ataxia type 3 (SCA3).

As mentioned above, the AAV1 and AAV2 serotypes do not have tropism to astrocytes or microglia cells, therefore they also did not colocalize with GFP in DCN-injected mice. A study investigated which cells are transduced when different serotypes are injected into the cortex, striatum, hippocampus and thalamus of adult mouse brains (Cearley *et al.*, 2008). They concluded that AAV serotypes transduced neurons, however some vectors appeared to also transduce astrocytes or oligodendrocytes, supporting the hypothesis that AAV1/AAV2 has a neuronal tropism.

Chapter 6 – Conclusions

In general, these are the conclusions taken from our study and for the mosaic AAV1/AAV2 vectors.

- The mosaic AAV1/AAV2 vector has a similar transduction than AAV1 and is able to transduce other brain regions when injected in the striatum.
- The mosaic AAV1/AAV2 vector is a safe delivery vehicle for CNS.
- In the DCN, the mosaic AAV1/AAV2 has better results than AAV2, however AAV1 achieved better results than AAV1/AAV2.
- The AAV2 serotype seems to negatively influences the mosaic AAV1/AAV2 vector when injected in the DCN, considering the lower spreading values compared with AAV1 serotype.

Overall, this study demonstrated that the mosaic AAV1/AAV2 sustain the features of parental serotypes to become a good delivery vehicle. Indubitably, the mosaic AAV1/AAV2 vector sustains the AAV1 known ability to transduce CNS cells, while simultaneously enabling the AAV2 purification method based on the heparin affinity chromatography.

Chapter 7 - Future perspectives

In the future it would be important to repeat the experiment with a larger number of animals in each group to better understand if there is a beneficial effect on using the mosaic vector AAV1/AAV2 in striatum injections instead of parental serotype AAV1.

Moreover, it would be of extreme importance to study the mosaic AAV1/AAV2 vector cellular tropism. Therefore, fluorescent immunohistochemistry with other markers such as oligo2, an oligodendrocyte marker or neuronal markers, MAP2, β-III tubulin, DARP-32 (striatum) or calbindin (cerebellum) should be performed.

It would be of interest to study if the mosaic AAV1/AAV2 and parental serotypes vectors, AAV1 and AAV2, have a non-toxic profile upon injection in the DCN to corroborate and support the obtained results in the striatum.

Following neuronal tropism determination, it would be of interest to test the AAV1/AAV2 packing the therapeutic transgene for a neurological disease. Impaired striatum function is associated with Alzheimer's Disease (Hanseeuw *et al.*, 2019), Parkinson´s disease (Tanimura *et al.*, 2018), psychotic bipolar disorder and schizophrenia (Karcher *et al.*, 2019), obsessive compulsive disorder and autism spectrum disorder (Naaijen *et al.*, 2017). Inherited neurological disorders, such as polyglutamine diseases can lead to cerebellum degeneration(Carlos Adriano Matos, 2018). Perhaps the mosaic AAV1/AAV2 vector will have a role in the treatment or cure for these neurological diseases.

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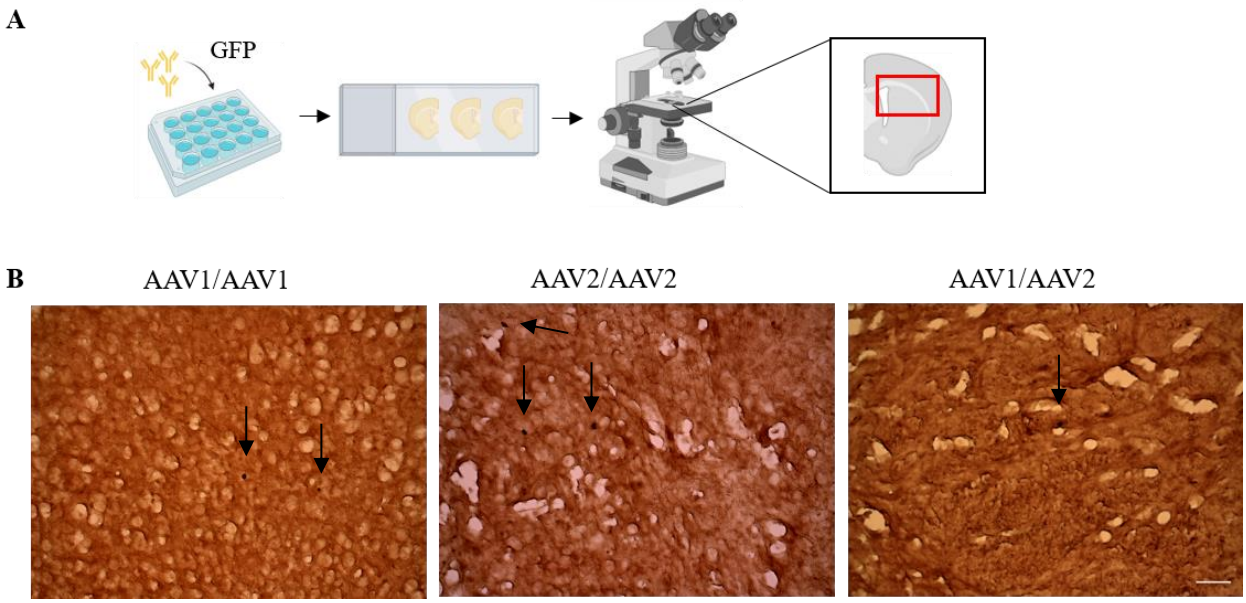
Annex



Annex 1: GFP staining in mice injected with mosaic AAV1/AAV2 or parental serotypes, in the striatum. **A** Schematic representation of immunochemistry using GFP Ab and the area of image acquisition during microscopy. **B** Light microscopy was performed to identity GFP (black arrows) in mice injected with 3×10^9 vg per striatum of mosaic AAV1/AAV2 or parental serotypes. Scale bar 50µm.