SCUOLA DI DOTTORATO

UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

PhD program Translational and

Molecular Medicine, DIMET

Cycle XXX

Gene therapy approaches for neurological disorders using newly generated AAV strains

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ACADEMIC YEAR
2016/2017

"*We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs*"

Molecular biology, nucleic acids, and the future of medicine

Edward L. Tatum, 1966

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Chapter 1. General Introduction

Gene therapy is considered to be one of the most promising strategies to develop effective treatments for a wide range of serious acquired and inherited diseases. Several clinical trials have been started in the recent years, providing successful data of safety and efficacy for many disorders; in some cases, these results leaded to the approval by European or American regulatory authorities.

Gene therapy is defined as the introduction of nucleic acids into cells to treat a disease and represents a simple method depending on either restore or add a specific gene function. Unfortunately, in practice Gene Therapy finds large limitations due to the complexity to reach the target area in the body. For this purpose, in the years many methods have been established for gene delivery. These strategies, in some cases, have been successfully used for the treatment of patients with specific disorders. Although the adopted procedures can be divergent each other, they but gather a series of tools that can be employed to treat several

disorders. Basically, genetic material is delivered by a vector, after administration, to a specific cell population or tissue where the transfer occurs providing a therapeutic effect. Once injected, however genetic material can be rapidly degraded; thus, the choice of the vector is crucial to achieve successful gene transfer. Essentially there are two main possible strategies to convoy therapeutic DNA to the target of interest: while the first one is based on chemical protection of the genetic material, the other takes advantage of modified viruses, commonly named viral vectors, which have been naturally selected for gene transfer.

However, the gene therapy in the brain remains a challenging topic due to the complexity of this organ and to date, all the possible approaches required invasive injections often with poor outcomes.

In the next paragraph will be discussed the historical perspective and ethics in gene therapy. In following sections, the basic methods used and most common vectors will be explored. In the following section, a

detailed analysis of Adeno-associated viruses (AAVs) will be presented.

Finally, an important focus will be pointed on gene therapy in the nervous system, in particular for Parkinson Disease, and on promising non-invasive application based on a newly generated AAV vectors.

1.1 Historical and Ethical Perspectives

The gene therapy is a recent field, that was born in 1970s[1]. In the past decade, after the initial evidences that phages could transfer genetic material from one bacterium to another, Howard Temin, in 1961, discovered that in a similar fashion specific genetic mutation could be inherited as a result of virus infection. Based on his experimental observations he concluded that chicken cells infected with the Rous sarcoma virus (RSV) stably inherited viral specific gene mutations that contained the information for the generation of RSV progenies[2]. The first example of use

of genetic material as rescue strategy is due to Waclaw Szybalski. He demonstrated that a genetic deficiency of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) can be recovered by a transfer of functional DNA from an external source. In his work, Szybalski isolated DNA from human bone marrow cell line D98S, which he used to transform $HGPRT^{-}$ recipient cells. These cells, as result, did not die in presence in conditional HAT medium, but they survived and proliferate[3]. This is the first documented heritable gene transfer in mammalian cells. In addition, he was the first scientist to use the term "gene therapy", although he knew that performing and documenting in vivo gene repair for clinical applications would be extremely difficult[4].

In that period the awareness of use genetic material as medicine increased, and in 1966, Edward Tatum published a paper evoking the effectiveness of viruses to be used in somatic cells and possibly in genetic therapy[5]. Two years later, Rogers et al. demonstrated an initial proof-of-concept of virus mediated gene transfer[6]. Noteworthy, in 1972 Theodore Friedmann

and Richard Roblin reasoning on the recent evidences, layered the foundation for Gene Therapy and established the first criteria and ethical aspects for the field[7]. However, in the 1970s, the first direct human gene therapy failed to rescue an enzymatic deficiency in urea cycle[8].

Remarkably, the turning point was in 1980s, driven by the progress of recombinant DNA technologies and virology. This new wave of works in the field were focused on blood disorders since the possibility to bring the cells to the vectors (ex vivo transduction), followed by cell engraftment results in a controlled and efficient delivery. Early studies used transduced and then transplanted T cells or hematopoietic stem cells (HSCs), to treat adenosine with deaminase deficiency causing severe combined immunodeficiency (ADA-SCID)[9–11]. The patients, however, continued to require ongoing enzyme replacement therapy (ERT), most likely due to insufficient engraftment of corrected stem cells. With the use of new optimized protocols for gene delivery to HSCs, in the late 1990s two clinical trials, for X-linked severe combined immunodeficiency (X-

SCID) and ADA-SCID, demonstrated long-term successful reconstitution of the immune system in the absence of ERT or protective living environment[12–14]. However, the first trials for direct in vivo gene therapy, using adenoviral vectors, had less success than the ex-vivo approach. In particular, in 1999, in a trial for treatment ornithine transcarbamylase deficiency, a young patient died following systemic inflammation and multiorgan failure[15]. This tragic result, led to reconsider the use of viral vectors for safety beyond the moral doubts raised by the gene therapy field.

Another major concern regards the control of the expression of therapeutic DNA[16], which could lead to uncontrolled genetic changes, including modification in germline and offspring. This important aspect, however, is often disregarded for therapies that use approved drugs that can cause genetic alterations as well as radiation therapy. In general, interventions in germline are legally forbidden by legislation in many countries while others have advisory guidelines or are very restrictive making very difficult to perform germline modifications[17].

The possibility of insert corrected genes or in general to supply genetic deficiencies in human, gave rise to debate. A series of ethical issues, beyond technical aspects (i.e. biochemical characterization of the disease and mutation) were identified, including safety and risk assessment, fairness in the selection of the candidates in trials, and also privacy and confidentiality[7,18]. More important issues, that require regimentation, raised with the clinical trial experience[19], including ethics of informed consent for patients which cannot provide consent to treatment (i.e. children and dementia-affected patients).

In addition, these important considerations, however, were established before the effectively exploitation of human genetic modification, essentially with the CRISPR/Cas9 technology[20]. Several clinical trials, based on gene correction strategies, are currently in recruitment phase (www.clinicaltrial.gov). The principles governing human gene editing can be considered as part of gene therapy ones. Moreover, these fields share the mission of curing severe genetic disorders. The National Academy of Sciences and the

National Academy of Medicine, launched an initiative to inform decision-making related to recent advances in human gene-editing research. This committee clearly established the overarching principle for research and clinical application for human gene editing[21].

Another important aspect to consider are the treatment costs. Since these therapies are developed to treat especially rare genetic diseases, these therapies present a particularly acute pricing challenge[22]. The first gene therapy approved in Europe, uniQuire's Glybera™, failed to win national reimbursement in any European country. To date, since it has been used on a unique patient, uniQure recently announced that it would not seek to renew Glybera™'s European marketing authorization. This experience demand an urgent debate about how much will be invested by health systems for the gene therapies in late stage trials[22,23].

1.2 The common strategies of Gene Therapy

Basically, the approaches used in gene therapy are various and can be classified, depending on the type of DNA intervention and the target area[23].

The gene therapy can be employed in germ line or in somatic cells. In the first case, a functional gene, as previously mentioned, can be delivered to reproductive cells, producing an inheritable modification in patients. In the second case, the therapeutic gene is transferred in patient's somatic cells, and the modifications are restricted to the individual. Moreover, to reach the site of interest, two main ways can be pursued: cells can be treated outside the body and subsequently transplanted or infused (ex vivo strategy) or the specific population can be corrected directly into a patient[26].

Furthermore, gene therapy can be divided in four subclasses based on therapeutic strategy to adopt[25]:

a) **gene addition** is defined as the delivery of a corrected copy of a defective gene (without removal of the endogenous mutated one) and is well suited for disorders due to loss-of-function mutations
b) **gene reprogramming**: modifications of messenger RNA (mRNA) levels by inhibiting expression of the mutated gene
c) **gene supply**: addition of a gene which does not correspond to the mutated gene, but its expression in the diseased cells or tissues can have a therapeutic function (preventing or arresting the disease progression
d) **gene repair**: correction of a mutated sequence in a specific gene

All these procedures are based on delivering therapeutic DNA to the patients, and the success of gene therapy essentially depends on ensuring that the therapeutic genes enter the targeted cells efficiently without any form of biodegradation[27]. Therefore, DNA not only must be protected from degradation by

nucleases, but must be sure that it transcribes inside the cell[28].

The ideal transfer system, named vector, should satisfy several criteria[29,30]:

- Safety profile, it must not trigger a strong immune response, genotoxicity and citoxocity
- Limited or specific integration, it must either remain in episomal position or integrate into a specific region of the genome.
- Specificity for the target area, the vector must deliver the gene to only certain types of cells, especially when they are scattered throughout the body, or when it they are part of a heterogeneous population, especially after systemic administration
- Stable expression of therapeutic product, it must be expressed for a defined length of time

Moreover, it must be at commercially available high concentration. No single vector system is likely to be optimal for all the potential gene therapy applications[30,31]. The best solution is selected based on

vector properties and the benefit for experimental rationale. Although many progress have been reached in gene therapy, the continuous development and improvement of vectors will increase the number of potential applications, and will contribute to find new promising alternatives for the treatment of many disorders.

Mainly, the vectors used for gene delivery are classified in two types[24]: non-viral and viral vectors. Both classes have their advantages and drawbacks. The legitimate concern about vectors safety, is probably the most important brake for gene therapy in humans. Although the majority of them are viral-based, the gene therapy vectors are continuously modified to improve the effectiveness and safety.

Non-viral vectors

Non-viral vectors are relatively safe and cause a lower immune response compared to viral ones. Moreover, they can be produced easily and in large quantities.

However, their low transfection efficiency limits their use on a large scale[24,32].

Non-viral vector can be delivered in different ways based on physical or chemical properties[27]. In case of physical methods, no carrier for DNA is required and the physical forces are used to weaken the membrane cell to make it more permeable to the transgene[27]. Physical methods include electroporation, gene-gun, ultrasound and hydrodynamic injection[24]. The chemical vectors are proposed as alternatives to viral ones to overcome the drawbacks of the latter. These vectors have three properties that improve gene transfer into the cell nucleus: (i) They mask DNA-negative charges, (ii) compress the DNA molecule to make it smaller, and (iii) protect it from degradation by intracellular nuclease[33]. To overcome the degradation issue, nucleic acids can be modified to be resistant to nucleases, such as Locked Nucleic Acids (LNA)[34]. Other strategies take advantage of liposomes, to entrap and deliver Nucleic Acids (lipoplexes), or as alternative of cationic polymers (natural or synthetic)

mixed with DNA to form the, so-called, polyplexes[27,33].

One of the challenges to systemic delivery of DNA therapeutics is the potential degradation of the therapeutic gene by endonucleases in physiological fluids and the extracellular space. For this reason, entrapment of the DNA in a nanoparticulate carrier is desirable both to provide protection from endonuclease degradation and to improve circulation time[24]. Nevertheless, few of these vectors have so far been developed clinically owing to their low delivery efficiency[27,35].

1.3 Viral vectors

Viruses are biological entities positive selected for their ability to perform gene transfer[30]. The viral particles, also known as virions, consist of two or three elements[36]: (i) the viral genome made of either DNA or RNA, that carry genetic information;

(ii) a protein coat, called capsid, which surrounds and protects the genetic material; and in some cases (iii) an envelope of lipids that surrounds the capsid when they are outside the cells. In the last decades, they have been successfully modified into relatively safe vectors to take advantage of the efficient gene transfer[30,37].

The most important concern about the use of viral vectors is the safety. In general, the viral genome is replaced by a transgene, that is the therapeutic agent[38]. In addition, for vector generation, the trans-elements (viral genes) and the cis-acting sequence are divided, into distinct nucleic acid molecules, to prevent the reconstitution of functional and infective viruses[30]. The generated vectors can only perform dead-end infection (non-replicative), named transduction[30], that introduces the functional genetic information into the host cell. To date, viral vectors are the vectors most often used to transfer genes. Unfortunately, as previous outlined, they have drawbacks to be discussed[24,30]: they can cause an acute immune response; the production in large quantities is difficult and expensive

and can package limited sized transgenes. Moreover, viral vectors can elicit integration of the transgene, which potentially can result in insertional mutagenesis and oncogenesis[39]. In order to avoid these problems, targeted integration to predetermined genomic sites has been one of the most primary topics in current viral vectors development[40].

However, each viral vector has its specific properties, depending on the origin virus, that can allow to reach a stable level of transduction. Among all the possible vectors generated, the most exploited ones are divided in four classes[38], depending on the origin viruses: Retroviruses and Lentiviruses, Adenoviruses, and Adeno-associated viruses.

Retroviral vectors

Retroviral vectors derived from *Retroviridae*, which are lipid enveloped particles comprising a single-stranded RNA genome of 7 to 11 kilobases[29,30]. The viral RNA is retrotranscribed into linear double stranded DNA and integrated into the host genome.

These viruses, are characterized by their use of viral reverse transcriptase and integrase for stable insertion of viral genomic information into the host genome. All retroviral genomes have two long terminal repeat (LTR) sequences at their ends. LTR sequences act in cis during viral gene expression, and packaging, retro-transcription and integration of the genome. The LTR sequences frame the tandem *gag*, *pol* and *env* genes encoding the structural proteins, nucleic-acid polymerases/integrases and surface glycoprotein, respectively[30,38]. Disruption of the nuclear membrane is required for the preintegration complex to gain access to the chromatin[41] and productive transduction by retroviral vectors is strictly dependent on target cell mitosis shortly after entry[42].

Use of retroviral vectors leaded to achieve the first encouraging clinical results of gene therapy. They were used to vehicle the suicide gene HSV-TK donor lymphocytes to control graft-versus-host disease developing in an allogeneic graft-versus-leukemia response[43]. Another result was the transduction of the

common cytokine receptor γ-chain was transduced into the bone marrow stem cells of two children affected by severe combined immunodeficiency (SCID)-X1[12]. After infusion, these transduced cells were able to reconstitute immune function.

The main limitation of retroviral vectors has been their inability to infect non-dividing cells, meaning that post mitotic cells are not prone to be targeted[29,30]. Successfully, In 2016, the European Commission granted market approval for a gammaretroviral vector, Strimvelis™ (GlaxoSmithCline), this is the first ex vivo hematopoietic stem cell (HSC) gene therapy for the treatment of ADA-SCID[14].

Lentiviral vectors

As Retrovirues, Lentiviruses (LVs) belong to family of *Retroviridae*[29]. However, unlike other retroviruses, LVs can infect non-dividing cells, owing to their specific nuclear targeting strategy[44]. LV vectors can cause slowly progressive diseases, including immunodeficiency, anaemia, pneumonitis and

encephalitis, in their specific hosts (human, monkey, cat, horse, cow, goat and sheep)[39]. Although LV vectors are considered promising for gene therapy, safety represent a serious concern since most lentiviral vectors originated from HIV[39]. Compared to Retroviruses, LVs have a more complex genome: in addition to the *gag*, *pol*, and *env* genes, they encode two regulatory genes, *tat* and *rev*, essential for expression of the genome, and a variable set of accessory genes[39]. In the third generation vectors, *tat* is eliminated and replaced by addition of a chimeric 5' LTR fused to a heterologous promoter on the transfer plasmid, making the vector safer than previous generated[45].

The first generated LV vector, derived directly from Human Immunodeficiency Virus (HIV-1)[39]. Since HIV-1 Env recognizes human CD4 as a primary receptor, the probably unique application of this vector, is the transduction of $CD4^+$ T cells[39]. To confer a broader tropism to LV vectors, the HIV-env was

substituted with VSV-G envelope. This vector efficiently transduces the neurons and glial cells of the central nervous system (CNS) of rodents and non-human primates[46,47]. As for retroviral vectors, they are object of concerns about integration, that could lead to oncogenesis[40]. A possible strategy to prevent this problem, is the use of non-integrating LV vectors. These vectors typically have mutation in the *IN* gene, reducing vector integrations[48].

Adenoviral vectors

Adenoviruses (Ads) are DNA containing viruses, with no lipid envelope[49]. Ad vectors have become a very popular tool for gene transfer into mammalian cells[49] due to their numerous advantages: they can infect a wide variety of dividing or non-dividing cells and they are easily purified to high titres[50]. The genome encodes approximately 35 proteins that are expressed in two general phases[51]: "early" phase, which occurs prior to the initiation of viral DNA replication, and "late"

which occurs following the initiation of DNA replication. The early proteins have regulatory functions that allow the virus to take control of the cell and to carry out viral DNA replication

The strains commonly used to construct recombinant viruses (Ad2, Ad5) are well characterized; they can accommodate up to 37 kb foreign genetic material, but is believed that the viral proteins contribute to genome stabilization. As result, Ad vectors lacking of viral protein have reduced packaging capacity[52]. Their genome rarely integrates into the host chromosome, which is suitable for applications requiring transient gene expression. They can be employed as replicant vector (oncolytic), for cancer gene therapy[51].

Since they present these properties are considered suitable for temporary transgene expression and several gene therapy trials have been or are being conducted with Ad vectors[53]. Most of these trials are for treatment of cancer, although some are for use of Ad vectors as vaccines in which the vector expresses a foreign antigenic protein[51,53]. In the last years, many methods for manipulating the viral genome have been

developed, including helper dependent and hybrid vectors[54]. Helper dependent vectors are devoid of all viral-protein-coding DNA sequences[55]. Hybrid vectors combine the highly efficient infection capacity of adenoviruses with the long-term genomic-integrating potential of other viruses are currently being tested. Such hybrid systems showed efficacy in murine cancer models[56].

Other vectors

Many other vectors derived from common viruses, have been modified for gene delivery strategies. Among these, the vectors derived from Herpes virus type-1 (HSV-1) are object of active studies[57]. These vectors replicate specifically in actively dividing tumour cells have been used in Phase I–III human trials in patients with glioblastoma multiforme[58].

1.4 Adeno-associated viral vectors

Adeno-associated virus (AAV) is one of the most studied for gene therapy purposes. AAV is a

nonenveloped, single-stranded DNA-containing virus, firstly found as a contaminant of adenovirus preparations[59]. This virus belongs to the *Parvoviridae* family, to the genus *Dependovirus*, since it needs the presence of a helper virus to replicate and complete its life cycle[60].

In general, recombinant AAV vectors (hereafter rAAVs) are considered safe and well tolerated, thus they are valid candidates for gene therapy approaches. Remarkably, in 2012, Glybera™, the first gene therapy approved by European regulatory authorities, is based on a rAAV[26,61]. In addition, Glybera™ as medication for lipoprotein lipase deficiency that is an orphan disease, indicates that gene therapy of rare diseases could be interesting from a medical point of view. Furthermore, this year a US Food and Drug Administration advisory committee unanimously voted to approve another AAV-based experimental gene therapy (Voretigene Neparvovec) for patients with Retinal dystrophy due to mutation of RPE65[62]. Importantly, other AAV-based gene therapies phase II clinical trials are ongoing or already concluded[62].

These progresses emphasize the importance of AAVs for gene therapy, demonstrating the large possibilities of gene therapy intervention with these vectors.

AAV genome consists in two open reading frames (ORFs), named *rep* and *cap*, flanked by inverted terminal repeats (ITRs) on the 5′ and 3′ end[60] (fig.1). The ITR sequences form dsDNA hairpin structures at each end. The *rep* ORF encodes four proteins. These Rep proteins, named based on their molecular weight, are needed for AAV replication, transcription, integration and encapsidation. On the other hand, the *cap* ORF encodes for three structural proteins (VP1, VP2 and VP3), which assemble in a ratio of 1:1:10 to produce an icosahedral capsid of approximately 25 nm in diameter[63,64].

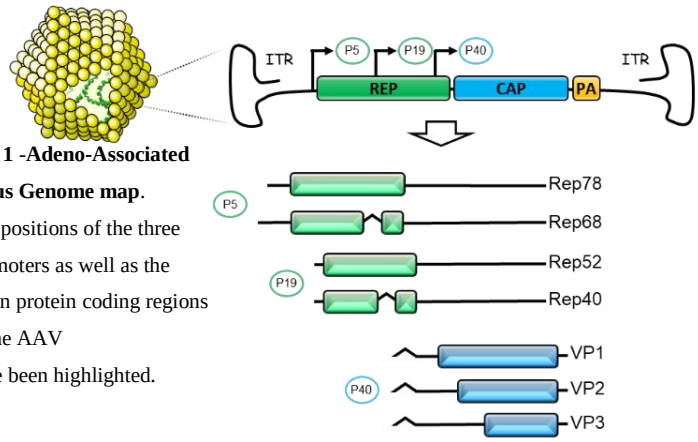

Fig. 1 -Adeno-Associated Virus Genome map. The positions of the three Promoters as well as the seven protein coding regions of the AAV have been highlighted.

The production of rAAV is relatively simple. The viral genome is removed and replaced with the transgene of interest (followed by a Polyadenylation site), inserted between the ITRs, while *rep* and *cap* genes can be supplied separately in co-transfection of HEK293 cells[65]. The ITR sequences and *rep* ORF of AAV2 are the most commonly used, coupled with *cap* sequences from other AAVs. Thus, the transfer plasmid can be combined with specific rep and cap genes, generating different types of rAAVs. In addition, for the production an additional plasmid, which contains adenoviral helper genes, is required[65,66](fig.2).

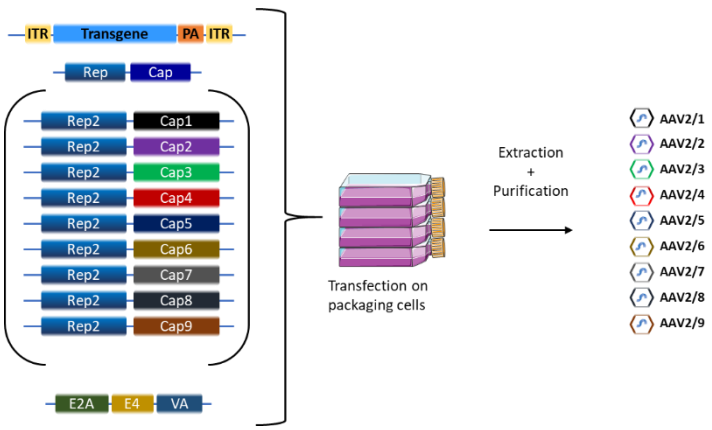

Fig. 2 - Generation of recombinant vectors based on different AAV serotypes. Transfection in the packaging cells of the plasmids containing: the transgene

(between the viral inverted terminal repeats, ITRs) and the packaging sequences (the rep open reading frames belong to serotype 2 to allow the most efficient packaging of a viral genome with AAV2 ITRs) results in production of recombinant virions. The hybrid virions containing the genome of one serotype (i.e. AAV2) and a different capsid (AAV2/1–9) have tropism dependent on the capsid sequence.

Since rAAVs do not carry any viral coding sequence, transduced cells will not produce any viral product. AAVs can infect both dividing and non-dividing cells, resulting in long lasting episomal transgene expression that can be persistent for at least 10 years[67]. Despite AAV is considered to keep an episomal presence in the cells, it is well known that the viral genome can integrate in a site-specific manner in a region on the long arm of chromosome 19 (19q13-qter), termed the AAVS1[68].

Over the last years, twelve natural serotypes have been detected and isolated from humans and other primates[69,70]. Different serotypes are defined by capsid protein motifs that are identified by distinct neutralizing antibodies. One of the major breakthrough, in AAV field, was the testing of these different serotypes. In 1998, Rutledge et al.,

characterized AAV6, which differs from AAV1 by only 14 amino acids but had different in vivo properties[71]. Moreover, Gao et al. showed that transduction with AAV8 in murine liver was 10- to 100-fold higher than with AAV2[72]. A comprehensive evaluation of their tropism is certainly likely to play a key role in all future studies, both basic science as well as clinical.

To date, the analysis of the tissue tropisms and biodistribution of AAV1-9 serotypes in mouse models have been published[67,73]. The possibility of use different AAV serotypes for specific purposes, including clinical trials, raised the interest on AAV capsids and their properties[31,74]. The differences in AAV capsids contribute to selectivity in cell transduction. The different capsids show specific properties in sugar-binding preferences, influencing the transduction of the various AAV variants. AAV has evolved to enter cells through initial interactions with carbohydrates present on the surface of target cells[73]. These carbohydrates represent the primary cell surface receptor and is followed by interaction with a

secondary receptor that is capable of facilitating viral entry. AAV serotypes 2, 3, and 6 interact with heparan sulphate proteoglycans, while AAV9 interact is reported to bind to N-terminal galactose[75,76]. Secondary receptors include fibroblast growth factor receptor (FGFR) and integrin for AAV2; hepatocyte growth factor receptor (c-Met) for AAV2 and 3[75]. Recently, Pillay et al., using a genome wide haploid genetic screen identified a factor, named AAVR, necessary for transduction by multiple AAV serotypes including AAV2 and 9; importantly AAVR-KO mice showed resistance to infection in vivo[77]. Acting as universal receptor, AAVR raised new questions on the mechanism of infection[78]. AAVs are described to use multiple independent pathways as result of specific to specific cell types and environmental conditions[79,80]. AAVR encodes for a type I transmembrane protein containing a MANSC domain, five polycystic kidney disease (PKD) domains, and a C6 region near the N terminus[81]. A subsequent study showed that AAV2 interacts predominantly with the second PKD repeat domain of AAVR. In contrast, AAV5 interacts

primarily through the first, most membrane-distal, PKD domain to promote transduction. Furthermore, other AAV serotypes, including AAV1 and -8, require a combination of PKD1 and PKD2 for optimal transduction[82]. These results suggest that despite their shared dependence on AAVR as a critical entry receptor, different AAV serotypes have evolved distinctive interactions with the same receptor. Moreover, splicing variants for AAVR are predicted[78]; this heterogeneity can contribute to explain the divergent selectivity of AAVs for organs and tissues.

Despite all the properties that justify the use of rAAVs for gene therapy, they have limitations that have to be addressed. It is known that AAVs can trigger innate and adaptive immune response[67]. As already mentioned, for AAVs are present in human population neutralizing antibodies (Nabs) for specific capsids, which can limit the gene delivery for many serotypes[83]. Furthermore, following cellular transduction, AAV capsid epitopes can be presented on major histocompatibility complex (MHC) class I molecules, which leads to the elimination of transduced cells by

capsid-specific cytotoxic T lymphocytes and the corresponding loss of gene expression[84,85]. Large human populations have been exposed to natural AAV serotypes, and the presence of specific neutralizing antibodies (Nabs) in patients is an exclusion criterion for clinical studies with AAV vectors[86,87]. Curiously, Nabs for the most common AAVs are also present in sera of numerous animal models, including naive mice obtained directly from commercial vendors[88]. This phenomenon can alter the outcome of the studies with misleading results. The presence of Nabs could limit the use of AVV vectors, but is possible to adopt complementary strategies to overcome this issue:

The first strategy, commonly named capsid switch[89], requires the use of an alternative AAV capsid, with close properties to the first one. Unfortunately, is possible that the second capsid has a close but different tropism. Moreover, possible crossreactivity of anti-AAV antibodies can results in neutralization of a wide range of AAV serotypes[90]. On the other hand, the Capsid Decoy strategy, takes advantage of empty AAVs to buffer the Nabs in a dose dependent manner,

furthermore these decoy capsids can be modified (see below) to be unable to enter in the cells[91]. Alternatively, to reduce Nabs titre, saline flushing and plasmapheresis have been successfully tested in nonhuman primates[92–94]. Finally, the immune response to AAVs can be modulated by pharmacological treatments[95,96]. The last strategy discussed is represented by Vexosomes[97] which are AAV particles associated coupled with extracellular vesicles (exosomes). By this way, is possible to protect AAVs from Nabs, and importantly they perform efficient transduction in vitro and in vivo compared to naked AAV particles[98,99].

The other important limitation of AAV gene therapy is the packaging capacity. In general, AAV vectors have shown to package genomes of up to 5 kilobases, including ITR sequences[38]. Thus, a putative cassette, made by promoter and transgene, for rAAVs, has to be no longer than 4.6/4.7 kb. Beyond 5 kb the packaging efficiency markedly decreases, and genomes with 5′ truncations become encapsidated[100,101], resulting in a decrease of gene transfer efficiency. Furthermore, it is

believed that the various AAVs can have different packaging capacity[102]; AAV5 capsid capacity is estimated in 8.9 kb.

Since rAAVs acts as episomal viruses, with minimal integration capability, is difficult to estimate the stability of transgene expression. Any AAV genome that reaches the nucleus will still require the synthesis, or recruitment, of a complementary strand in order to achieve stable gene expression. The ITRs, serve as replication origins during productive infection and as priming sites for host–cell DNA polymerase to begin synthesis. This step can effectively limit transgene expression but can be bypassed through the use of self-complementary AAV (scAAV) vectors[102]. The relative efficiency of single stranded (ss) AAV vectors will rely more on the multiplicity of infection than scAAV, making the expected distribution of vector within the target tissue an important consideration. Because parameters such as the size of the tissue and the route of vector administration vary widely in different applications, direct comparisons between ssAAV and scAAV transduction, as well as therapeutic efficacy,

are needed to have a comprehensive evaluation of, as well as therapeutic efficacy, are needed to have a comprehensive evaluation of advantages and disadvantages of these two kinds of AAVs. Although, the transgene capacity of scAAVs is split in a half (2.2 kb), it is still sufficient for a wide range of application. Moreover, the careful optimization of transcriptional and post-transcriptional regulatory elements, as well as codon optimization, is likely to provide significant reward in overall levels of transgene expression[102].

For gene therapy approaches rAAVs vectors able to target specific tissues and organs with high efficiency are needed. For this reason, the development of new engineered capsids represents a valid source of vectors that can be tested in pre-clinical studies. AAV capsids with novel or enhanced properties have been developed through display of targeting peptides, rational design and directed evolution[103,104].

As successfully used for other viral vectors[105], the identification of capsid variants by display of targeting peptides, allows the selection of targeted vectors; no prior knowledge of the potentially binding receptor

and ligands is needed[106]. For display on the vector surface, the targeting ligand can either be coupled to the assembled particle or genetically fused to capsid. In the first instance, coupling is usually noncovalent[107]. For this purpose, immunoglobulin G-binding domains have been displayed on the envelope proteins of or the AAV capsid[108]. However, this type of strategy is problematic for in vivo applications, including systemic injections, where blood components may dissociate the targeting ligand from the vector particle. In the genetic approach, targeting ligands, usually short peptides, are inserted at the tip of protrusions of the AAV capsid[109–111]. However, the peptides identified by display might result in conformational changes of the peptide when incorporated directly into the viral surface or might not function efficiently in vivo.

In rational design, the AAV variants are developed starting from knowledge of delivery mechanisms and structural studies[103]. An example of this approach is the mutagenesis of tyrosine residues in capsids. Tyrosine residues can be phosphorylated as signal for

ubiquitylation and proteasome degradation of viral particles[112]. Mutated tyrosine in phenylalanine residues showed up to 30-fold higher efficiency of transgene expression[112]. Moreover, AAV2 tyrosine-to-phenylalanine mutant capsid showed a reduced risk of cytotoxic T lymphocyte immune responses, that can be a limitation for clinical AAV-mediated gene therapy[113].

Directed evolution emulates the process of natural selection, in which genetic diversification and selection progressively improve a molecule's function[104]. To modify AAVs, this process has involved mutating wild-type cap genes to generate libraries of viral particles (fig.3). Then, a selective pressure is applied, such as high-affinity antibodies against the AAV capsid, the need to bind new cell surface receptors or circumvent intracellular barriers, or tissue structures that bar the virus from accessing target cells in vivo. The selective pressure promotes the emergence of variants able to overcome these barriers[103]. After the selection step, the successful variants can be recovered and used as the starting

material for the next cycle of selection to further enrich the improved variant[104].

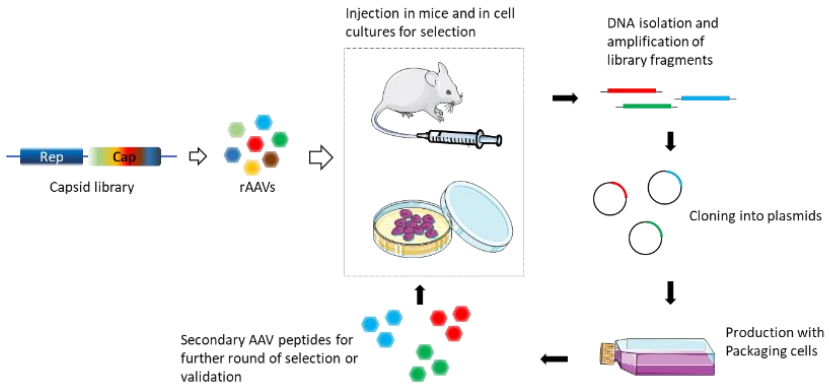

Fig. 3 - Targeted selection of a random library for identification of efficient and specific capsid targeting peptides. A random AAV peptide library is injected intravenously in mice, or used to transduce cell cultures. After library administration, total DNA is isolated from the tissue/cell of interest. The viral DNA of particles enriched in the target tissue or cells is amplified and cloned into library plasmid backbones to generate a new AAV peptide library by transfection of AAV producer cells. The newly generated rAAVs can be used for validation experiments; as alternative the new library can be used for validation a subsequent round of selection

A nice exemplification of the method is represented by AAV-DJ[114]. This rAAV was generating by a library of shuffled capsid-encoding genes: the newly generated hybrid serotypes, were then tested and selected for efficient transduction in hepatocytes and resistance to

immune neutralization[114]. Similarly, a chimeric variant composed of AAV1, 6, 7 and 8 was identified through in vivo biopanning for its efficient muscle transduction[115]. Directed evolution allows also to generate rAAVs able to transduce in vitro specific cell populations like stem cells[104]: This method was applied to isolate a capsid variant capable of efficient transduction of neural stem cells (NSCs)[116]. In this case, the selection was conducted using an error-prone AAV2 library, an AAV2 with random peptide insert library and an AAV2 pairwise shuffled library on NSCs (from the adult hippocampus). The AAV2 variant mediated 50-fold increased transduction of murine and human NSCs[116].

AAV vectors have been firstly detected essentially in human tissues[69]. All the modification methods allow the generation of new variants for specific aim. However, tropism validation experiments are performed in animal models, essentially in mice, and it can mislead the results for human applications. An attractive way to study rAAVs transduction of human tissues in vivo is represented by xenotransplantation.

Human xenografts are appropriate to evaluate viral transduction in vivo but outside human body. Using a xenograft liver model, Lisowski et al., evaluated the transduction efficiency of various serotypes of human liver in vivo[117]. Furthermore, by a shuffling capsid-encoding genes, they generated an hybrid rAAV, able to perform the first species-specific hepatocytes transduction[117].

1.5 Gene therapy in Central Nervous System

Diseases of the Central Nervous System (CNS) represent a large class of conditions affecting the brain and the spinal cord[118]. These diseases are often caused by inherited genetic mutations that can cause impairment of neuronal development and function and lead to neurodegeneration and death. Unfortunately, these pathologies are the most difficult to treat with

traditional pharmacological and surgical approaches due to the complexity of the CNS pathophysiology. Moreover, Blood brain barrier (BBB) limits possible widespread delivery of therapeutic agents within the nervous system, from systemic administration[119].

Efficient and specific delivery to the brain and spinal cord is a rate limiting step in gene-based strategies[120]. Moreover, diseases of CNS can be localized to restricted areas or due to specific cell populations, while other present an extent pathology through the whole organ. This heterogeneity made in the last years brain gene therapy extremely challenging[121]. The correct choice of methods of administration has been represented the major issue to date[121]. Peripheral administration can be performed for disease related to sensorial organs as inner ear[122] and eye[123]. The most common way to vehicle vectors to the brain is the intracranial injection. Unfortunately, the direct injection is a surgical procedure and can be considered invasive for both human and pre-clinical animal models. However, this technique is suitable to target specifically restricted areas and cell populations.

AAVs and LVs vectors are the most used vector in this context[70,124]. A direct injection can lead to diffuse delivery through the cerebrospinal fluid (CSF). Intracerebroventricular injection (i.c.v.) of AAVs in neonatal mice model showed widespread transduction[125,126]. Unfortunately, i.c.v. delivery of AAVs to the adult animals results mostly in ependymal cell transduction[127] which limits the therapeutic applicability of this strategy.

The use of AAVs allowed to use also alternative accesses to the brain. Specific viral vectors can be transported to target area through retrograde or anterograde axonal transport[128]. Since, this approach requires the administration in a peripheric organ is less invasive than the previous reported. However, the transduced population can be limited by anatomical conformations and nervous circuitry and strictly dependent on the injected viral dose[129].

The emerging strategy to target the brain is the use of AAV9 through systemic injection, allowing a less invasive strategies to target the CNS efficiently, and

provide a suitable platform for several diseases with diffuse brain pathologies, both inherited or acquired[130].

1.6 Gene therapy for neurodegenerative disorders: the case of Parkinson Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss degeneration of mid-brain dopaminergic neurons constituting the nigrostriatal pathway[131]. Degeneration of dopaminergic neurons leads to serious motor symptoms (tremor and bradykinesia) and cognitive changes[132]. Beyond the motor symptoms, the second pathological landmark of PD is the presence of eosinophilic intracytoplasmic protein aggregations in the surviving neurons known as Lewy bodies (LBs). The major component of LBs is the alpha-synuclein protein (α-Syn), and its accumulation is directly correlated with the progression of the disease[133]. The association between PD and α-synuclein protein (α-

Syn) represents one of the major milestones in our understanding of the diseases etiopathology.

While there is no consensus on how misfolded α-Syn aggregate species initially arise, the consequences of these aggregates in the neurons are much better understood. In fact, defects in protein degradation and quality control machinery directly increase intracellular/vesicular α-Syn concentration, enhancing the rate of α-Syn aggregate formation[134]. Conversion of α-syn to a toxic oligomeric form(s) might be influenced by interactions with lipids or small molecules and post-transcriptional modifications, including phosphorylation of α-syn at S129 residue[135]. Many other pathologies are linked to α-Syn aggregation and are collectively named synucleinophaties[136]. All these diseases are characterized by α-Syn misfolding and multiple aggregations forming toxic species that are able to spread in a temporal-space fashion across all the brain, and leading to the progressive neurodegeneration[136].

The current therapies for PD have been essentially focused on symptoms treatment at early stages of the

disease; however, drug's effectiveness declines with progressive pathology[137]. Since the progressive neurodegeneration is not adequately treated with pharmacological approaches, PD is an ideal target for gene therapy. Moreover, the cause of the major motor symptoms is well defined, and associated with dysfunctions of a specific neuronal population that can be effectively targeted with gene transfer methods for brain cells[138]. Importantly, the recent advances in understanding the genetic causes of PD, leaded to the development of several approaches based on delivery of genes involved essentially in neuroprotection or in neurotransmitters biosynthesis[138].

To date, five divergent GT approaches have been developed to treat the major motor symptoms of PD, all with the use of AAV or LV vectors:

AAV2 was used to express the aromatic amino acid decarboxylase (AAD) gene, which encodes for an enzyme that metabolizes L-DOPA into dopamine in the striatum[139]. The aim was to optimize the symptomatic effects due to an excess of L-DOPA that occurs in the patients treated pharmacologically.

However, ADD overexpression resulted in more aggravate dyskinesia, caused by a non-regulated dopamine production[140]. To improve this approach, a single lentivirus was used to express the 3 enzymes GTP cyclohydrolase 1 (GCH1), tyrosine hydroxylase (TH) and AAD. This treatment has been renamed for clinical and commercial use as ProSavin™[141]. After good therapeutic evidences both in rats and in non-human primate model of PD, this approach entered into a clinical trial. In the first phase, it was demonstrated to be safe and able to reduce the syntomatic "OFF" state in patients[142]. New studies are now under execution for assessing long-time therapeutic effects in more patients. The second AAV-based approach aimed to express the glutamic acid decarboxylase (GAD) enzyme into the subthalamic nucleus. The final goal was to stimulate GABAergic inhibitory transmission in this region and, thereby, reducing the overactivation of subthalamus-nigral neuronal circuit associated with PD pathology. This strategy has also been tested in a phase I clinical trial showing that this procedure was safe in patients, but

showed just a modest clinical improvement[143,144]. Finally, the last approach is known as CERE-120 and is an AAV2-based gene transfer strategy to deliver the neurotropic factor human neurturin (NRTN) in the Substantia Nigra. Neurotrophic factors are used to restore normal function and prevent death in damaged neurons. Unfortunately, phase I and II trials with this virus failed to show statistically significant clinical improvements[145].

Despite the clinical trials successfully provided safety, targeted and controlled proteins for very long periods of time in human brain, the efficacy data generated did not demonstrate sufficiently robust or consistent benefits to patients, compared to that achieved by placebo controls. To date, the presented gene therapy strategies essentially aim to restore dopamine balance and motor functions. Unfortunately, α-Syn aggregates can spread throughout the brain, in a prion-like manner[146], resulting in progressive cell death and dementia, making ineffective all the proposed interventions[147]. Thus, Parkinson disease is also ideal for testing widespread delivery methods.

Intriguingly, GBA1 is an ideal candidate for gene therapy in PD. This gene encodes for lysosomal β-Glucocerebrosidase (GCase) enzyme, which catalyses the conversion in glucosylceramide in glucose and ceramide. Homozygous loss of function mutation of GBA1, cause the Gaucher's Disease, a lysosomal storage disorder (LSD) that affects many of the body's organs and tissues[148,149]. The signs and symptoms of this condition vary widely among affected individuals. Researchers have described several types of Gaucher disease based on their characteristic features (basically divided in nonneuropathic and neuropathic)[150]. There are many links between GCase and α-Syn. Firstly GBA1 heterozygous mutations are well known a risk factor for PD; Importantly, PD patients carrying a GBA1 mutations had a more diffuse distribution of LBs in particular in the brainstem and limbic areas[151,152]. Thus, GBA1 overexpression can be considered a valid option to clear α-synuclein aggregates in the brain with a strategy of diffuse delivery.

1.7 AAV9 and Non-invasive gene therapy in the nervous system

To achieve systemic administrations of vectors for the brain, the major obstacle is represented by the Blood Brain Barrier (BBB). The BBB is characterized by a vascular structure that divides the CNS from the peripheral blood circulation. The brain blood vessels are formed by endothelial cells with continuous intercellular tight junctions (TJs), lacking fenestrations which are present in other districts[153]. As result the transport through BBB is finely regulated and limits the passage of molecules including potential therapeutic AAVs[154–156]. The possibility of use a systemic injection represents an important non-invasive method to treat complex diseases. Firstly detected in rhesus monkey brain and subsequently characterized[72,157], AAV9 has the unique property to cross BBB and transduce neuronal and glial cells after systemic administration. Foust et al.[158], showed for the first time, that intravascular (i.v.) injection of AAV9 in

mice resulted in widespread transduction of CNS. In neonates a single injection of AAV9 leads to transduction in brain areas and motor neurons and dorsal root ganglia in spinal cord. In adults however, the transduction appears selective for astroglial cells. Importantly, the tropism of AAV9 to spinal cord motor neurons is consistent across species[158,159]. The possibility of use a systemic injection represents an important non-invasive method to treat complex diseases. In 2010, Kaspar and collaborators demonstrated for the first time that systemic injection of AAV9 carrying *smn1* gene can treat Spinal Muscular Atrophy (SMA) type1 in a mouse model[160] showing the clinical potential of AAV9. Currently, A Phase 1 clinical trial (NCT02122952, www.clinicaltrial.gov) evaluated the safety, tolerability and efficacy of gene transfer in 15 infants up to six months old with SMA type 1. The treatment resulted in a longer survival and improvement of motor functions[161]. Despite the remarkable results for the treatment of SMA type1, the use of AAV9 systemic injection for brain diseases showed several issues to be

discussed. For first, to reach a good level of targeted cells resulting in therapeutic effect, high titre is required[158,162]. For second, AAV9 tropism is not limited to CNS and the vectors can target other organs (i.e. liver and heart) with possible secondary effects[159,163]. Moreover, independent reported studies did not show consistence with data[164]: although AAV9 can cross BBB and reach the brain cells the results are not completely reproducible. The age-dependent efficiency and the transduction of spinal motor neurons are highly accepted, but the data on neurons and glia transduction in adult are not in accordance, raising issues on the efficacy of treatment with AAV9 for brain diseases. AAV9 can be exploited for the treatment of CNS disorders through systemic administration, but the concerns about efficacy in brain transduction need an in-depth analysis. Since its unique properties, several AAV9 variants have been developed to improve its efficiency and the transduction in adult CNS, in particular for the brain. Using an in vivo selection Choudhury et al., characterized an AAV9 variant named AAV-B1.

Systemic injection of AAV-B1 vector in adult mice and cat resulted in widespread gene transfer throughout the CNS with transduction of multiple neuronal subpopulations, but it is still selective for other organs including skeletal muscle and β-cells[165]. The vector AAV-AS, generated by the insertion of a poly-alanine peptide in AAV9.47[163] capsid, is capable of extensive gene transfer throughout the CNS after systemic administration in adult mice[166]. AAV-AS is demonstrated to be up to 15-fold more efficient than AAV9 in brain, but there was not significant difference in transduction of liver and skeletal muscle compared with AAV9. These recent reported capsid variants showed an increase in efficiency of brain transduction but did not show an improved selectivity since the transduction in other organs was well represented.

1.8 AAV-PHP.B and PHPs vectors.

Comparing all the AAV9 variants, we assume that capsids developed by Deverman et al. are the most valid choice for brain transduction with systemic injection[167]. Using a cell type-specific capsid selection method, named CREATE (Cre recombinase-based AAV targeted evolution), the authors recently identified several capsid variants of AAV9, including AAV-PHP.B and AAV-PHP.A and more recently AAV-PHP.eB and AAV-PHP.S[167,168].

Using transgenic Cre-expressing mice, the method allows to identify enriched rAAVs with modified capsid in specific cell population, including neuronal cells[167]. The method is based on two components: The first is an AAV vector library in with variable DNA sequences, encoding a heptamer peptide, into the middle of the capsid gene; the second component is a transgenic mouse line with a cell type labeled with Cre recombinase[167]. After the infection, few days later, the

Cre-expressing cells mediate recombination of the viral genome, thus is possible to detect the sequence by PCR and obtain back the most enriched capsid in a tissue[167]. After intravenous injection in adult mice, AAV-PHP.B is able to target and transduce multiple brain areas (including cortex, hippocampus) and spinal cord[167,169]. Moreover, measuring the number of viral genome copies (vg) detected in the tissues, the authors demonstrated AAV-PHP.B provided significantly greater gene transfer than AAV9 to each of the CNS regions. In the same report, it was shown that AAV-PHP.A is highly selective for astrocyte transduction[167]. Then, the same authors in a subsequent round of selection, using other specific Cre-expressing mice, identified a new variant, named PHP.eB, which displays enhanced properties compared to its ancestor vector. Briefly, AAV-PHP.eB allows a more efficient transduction in neurons after intravenous injection; importantly to reach this result a titre 10-fold reduced is sufficient, meaning that AAV-PHP.eB has an increased selectivity for adult neurons.

1.9 Aim of the thesis

Given the property of AAV-PHP.B to transduce adult brain, the aim of the thesis is to further characterize this new vector variant for applications in the central nervous system. In Chapter 2, will be presented a report where are discussed interesting applications of AAV-PHP.B, including targeting and modulation of specific brain populations, and the rapid generation of conditional-like knock out mice. A special focus will be reserved to a Gene Therapy strategy in a mouse model of Parkinson disease, with α-synuclein aggregates. The presented report is the first example of non-invasive gene therapy for neurodegenerative disorders, therefore performed in adult brains.

In Chapter 3, will be discussed the conclusions and future perspectives, including the possible translational applications of AAV-PHP.B in human patients.

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Chapter 2

AAV-PHP.B-Mediated Global-Scale Expression in the Mouse Nervous System Enables GBA1 Gene Therapy for Wide Protection from Synucleinopathy

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Morabito, G., Giannelli, S. G., Ordazzo, G., Bido, S., Castoldi, V., Indrigo, M., ... & Sessa, A. (2017). AAV-PHP. B-Mediated Global-Scale Expression in the Mouse Nervous System Enables GBA1 Gene Therapy for Wide Protection from Synucleinopathy. *Molecular Therapy*.

Highlights

- AAV-PHP.B vascular delivery sustains CNS and PNS global transduction
- Gene expression and neuronal activity control by AAV-PHP.B gene transfer
- Alpha-synuclein pathology is fully reversed by GBA1 expressing AAV-PHP.B
- Brain-blood barrier is not affected by the AAV-PHP.B brain penetration

Summary

The lack of technology for direct global-scale targeting of the adult mouse nervous system has hindered research on brain processing and dysfunctions. Currently, gene transfer is normally achieved by intraparenchymal viral injections, but these injections target a restricted brain area. Herein, we demonstrated that intravenous delivery of AAV-PHP.B viral particles permeated and diffused throughout the neural parenchyma, targeting both the central and the peripheral nervous system in a global pattern. We then established multiple procedures of viral transduction to

control gene expression or inactivate gene function exclusively in the adult nervous system and assessed the underlying behavioral effects. Building on these results, we established an effective gene therapy strategy to counteract the widespread accumulation of α-synuclein deposits throughout the forebrain in a mouse model of synucleinopathy. Transduction of A53T-SCNA transgenic mice with AAV-PHP.B-GBA1 restored physiological levels of the enzyme, reduced α-synuclein pathology and produced significant behavioral recovery. Finally, we provided evidence that AAV-PHP.B brain penetration does not lead to evident dysfunctions in blood-brain barrier integrity or permeability. Altogether, the AAV-PHP.B viral platform enables non-invasive, widespread and long-lasting global neural expression of therapeutic genes, such as GBA1, providing an invaluable approach to treat neurodegenerative diseases with diffuse brain pathology such as synucleinopathies.

Introduction

Genetic modification of adult brain neurons is an indispensable tool to determine the detailed anatomy and function of defined neuronal circuitries. However, genetic engineering in mice is a laborious and time-consuming technology, and it has grown increasingly challenging with the elevated complexity required for the dynamic assessment of gene function in time and space. Virus-mediated gene-transfer has become a fundamental strategy for gene modification in the nervous system. In particular, recombinant adeno-associated viruses (AAVs) are commonly used as gene transfer vehicles in the brain due to their broad range of infectivity, high safety profile and relatively rapid diffusion[1-3]. However, intraparenchymal injection of AAVs supports robust but relatively localized transduction in the brain tissue[4]. Thus, while this approach is meaningful for assessing the function of small neuronal clusters, its application to wider neuronal circuitries or large neural areas up to the entire brain remains unfeasible. More widespread CNS transduction has been achieved through intravenous

(i.v.) delivery of AAVs able to cross the blood-brain barrier (BBB)[5-7]. Intravenous infusions represent an ideal non-invasive delivery route for viral agents that feature brain tropism[8]. Among all the AAV serotypes, AAV9 has shown the greatest ability to permeate the BBB after peripheral vascular administration. In fact, i.v. administration of AAV9 in neonatal mice resulted in extensive and diffuse transduction in the CNS both in neurons and in astrocytes[5-7]. However, the same approach results in a much lower transduction efficiency by the AAV9 in adult mice, with increased targeting of the glial cells with respect to the neuronal fraction[5,7,9]. Thus, recent research has focused on generating novel AAV9 variants through selected mutagenesis of the capsid proteins to obtain more efficient gene transfer in the brain after viral peripheral delivery. In particular, Deverman and colleagues (2016)[10] conceived a Cre-recombination-based AAV targeted evolution strategy (CREATE) to isolate a novel engineered AAV9 capsid, named PHP.B, with the 7-amino-acid insertion TLAVPFK in the VP1 capsid protein. AAV-PHP.B was shown to outperform

the standard AAV9 in transducing neurons after i.v. administration in adult mice. Considering these properties, we sought to use AAV-PHP.B as a common platform for building a set of molecular tools for the straightforward genetic manipulation of the neonatal and adult mouse nervous system on a global scale to interrogate gene function and modulate neuronal activity within the entire nervous system. In addition, this system offers an unprecedented opportunity for treating diseases that globally affect the nervous system. In particular, widespread accumulation of alpha-synuclein (α-syn) protein aggregates in Lewy bodies is a key neuropathological hallmark of Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), leading to complex and heterogeneous symptomatic manifestations. GBA1 encodes the enzyme lysosomal glucocerebrosidase (GCase), and heterozygous mutations of the gene are the most common genetic risk factor for PD and DLB[11,12]. Mounting evidence suggest that GCase activity impairment is affecting lysosomal activity and overall autophagic flux

negatively affecting α-syn aggregates degradation and catabolism[13-15]. Intriguingly, focal expression of exogenous GCase is sufficient to limit α-syn protein inclusions and partially prevent dopaminergic neuronal cell death[16,17]. Nonetheless, an exclusively global approach targeting large brain areas might be considered for therapeutic exploitation, given the pervasive distribution of α-syn deposits throughout the brain. Herein, we showed that AAV-PHP.B-mediated GBA1 overexpression enabled a robust and long-lasting reduction of α-syn inclusions in the whole forebrain accompanied by a significant recovery in lifespan and cognitive performance.

Results

Global-scale neural transduction and single neuronal cell labeling by AAV-PHP.B intravenous delivery in neonatal and adult mice.

An AAV2 transfer plasmid was used to clone the GFP cDNA downstream of a constitutive CBA promoter and combined with the PHP.B rep/cap and helper plasmids for productive viral infection. Viral particles were then harvested from both cells and supernatants, separately concentrated and finally mixed together in order to obtain high-titer viral preparations. A dose of 2 x 10^{12} vg of AAV-PHP.B-GFP was administered by tail vein injection into 8-week-old mice (**Figure 1A**). Transduction efficiency was evaluated between 3 and 5 weeks post-injection by assessing GFP expression in various organs. As previously reported[10], GFP signal was widely detected in all CNS regions, with diffuse and robust staining in the forebrain, midbrain, and cerebellum and along the entire spinal cord axis. (**Figures 1B-E** and **S1**). Co-labeling for regional neuronal markers and GFP expression revealed that a very significant fraction of neurons, generally higher than 65%, was targeted by AAV-PHP.B in these regions (**Figures 1F-J**). Interestingly, beyond the CNS, robust GFP expression was detected in the dorsal root (DRG) and sympathetic (SG) ganglia as well

(**Figures 1K-M**). In fact, the majority of bIII-tubulin^{+} DRG and tyrosine hydroxylase (TH)$^{+}$ thoracic SG neurons were effectively transduced by the virus (**Figures 1K-M**). Therefore, a single i.v. administration of AAV-PHP.B-GFP is sufficient for global and robust transduction of the adult mouse CNS and provides new evidence of efficient tropism for PNS structures as well. Furthermore, we assessed the AAV-PHP.B viral distribution after i.v. injection in neonatal mice. Interestingly, 3 weeks after viral administration, global targeting of the nervous system was confirmed, with a pattern similar but not identical to that obtained in adult mice (**Figure S2**). In fact, the GFP signal was particularly strong in selected glial populations in the cerebral cortex, hippocampus and striatum (arrows in **Figure S2**). Although neuronal transduction was generally very efficient, especially in the cortex, hippocampus, cerebellum and spinal cord, it was very limited in the substantia nigra, revealing notable differences with respect to the transduction pattern obtained through i.v. delivery in adult animals (**Figure S2**). Thus, at the perinatal stage, although

administered by a comparable systemic delivery route, the AAV-PHP.B had a close but distinct tropism for glial and neuronal cells, probably as a result of some phenotypic differences between neonatal and adult cells likely associated with their maturation state.

Sparse and selective labeling of distinct neuronal populations is a prerequisite for accurate tracing of nerve projections. To facilitate morphological analysis, we infused the AAV-PHP.B-Cre at different doses in adult Ai9 reporter mice[18] and evaluated the extent of transduction in the brain parenchyma (**Figure S3A**). Interestingly, a low viral dose enabled sparse to single-cell labeling in the brain tissue as detected by tdTomato immunofluorescence imaging (**Figures S3B-K**). Given the whole-brain targeting profile of this virus, single-cell labeling was simultaneously obtained in different brain areas including the cortex, hippocampus and cerebellum (**Figures S3E, H, K**). These results demonstrate that a single administration of this virus at a low titer enables single-neuron visualization in multiple brain regions simultaneously.

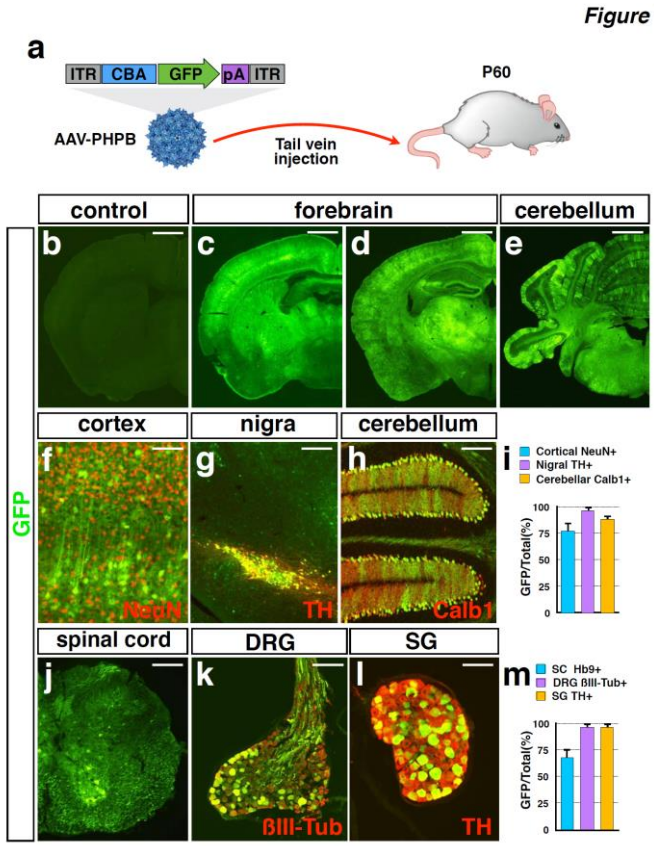


Figure 1. Global GFP expression in the central and peripheral nervous system with a single AAV-PHP.B intravenous injection. A. Schematic view depicting the transgenic cassette integrated in the AAV-PHP.B vector and injection of the viral particles into the tail vein of an adult mouse. **B.** GFP immunofluorescence in brain transduced with an empty AAV-PHP.B (negative control). **C-E.** GFP localization on coronal hemi-sections at different rostro-caudal coordinates of an AAV-PHP.B-GFP transduced brain. **F-H** High-magnification

images of cerebral cortex (**F**), substantia nigra (**G**) and cerebellum (**H**) showing the double staining for GFP and the neuronal marker NeuN, TH and Calb1, respectively. **i.** Bar graph showing the fraction of cells positive for a specific neuronal marker (NeuN, TH and Calb1) and expressing the viral GFP transgene. **J.** GFP staining on coronal section of an AAV-PHP.B-GFP transduced spinal cord. **K, L**. Section of thoracic dorsal root (DRG) or sympathetic ganglion (SG) co-stained for GFP and beta-III-Tubulin (**K**) or TH (**L**) , respectively. **M.** Bar graph showing the fraction of cells positive for a specific neuronal marker (Hb9, beta-III-Tubulin and TH) and expressing the viral GFP transgene. (n = 12 mice). Scale bars: 500 µm (B-E,J); 100 µm (G,H); 50 µm (K,L).

Facile AAV-PHP.B-based Cre-loxP conditional gene activation and control of neuronal activity in selected neuronal subtypes throughout the brain

Global targeting of the mouse nervous system by AAV-PHP.B has considerable implications, but it might represent a drawback when the aim is to study more specific neuronal targets or circuits. Thus, we conceived the idea to combine the widespread targeting of AAV-PHP.B with Cre-loxP technology in order to target specific neuronal subtypes in large brain areas (**Figure 2A**). Initially, we confirmed that systemic transduction of Cre-expressing AAV-PHP.B into Ai9 mice carrying a fluorescent tdTomato protein

downstream of a loxP-flanked STOP cassette triggered very efficient Cre excision of the cassette and subsequent expression of the reporter throughout the brain (**Figure 2B**). Next, we generated an AAV-PHP.B carrying GFP in a FLEX switch cassette whose expression is activated by Cre recombinase. Thus, AAV-PHP.B-FLEX-GFP was infused in different transgenic mouse strains expressing Cre in specific neural subtypes (**Figure 2A**). Interestingly, this configuration enabled very selective expression of the GFP transgene according to the Cre expression pattern for each transgenic line. In infused NeuroD6-Cre mice, GFP expression was specifically confined to neurons in the cortex and hippocampus (**Figures 2C, D and data not shown**). Co-staining with NeuN and GFP revealed that more than 80% of all the neurons in the aforementioned territories expressed GFP (**Figures 2E, F**). Conversely, neither $S100^+$ nor $GFAP^+$ glial cells were found to co-express GFP (data not shown). Likewise, systemic viral transduction of parvalbumin (PV)-Cre and dopamine transporter (DAT)-Cre transgenic mice led to a very specific pattern of GFP

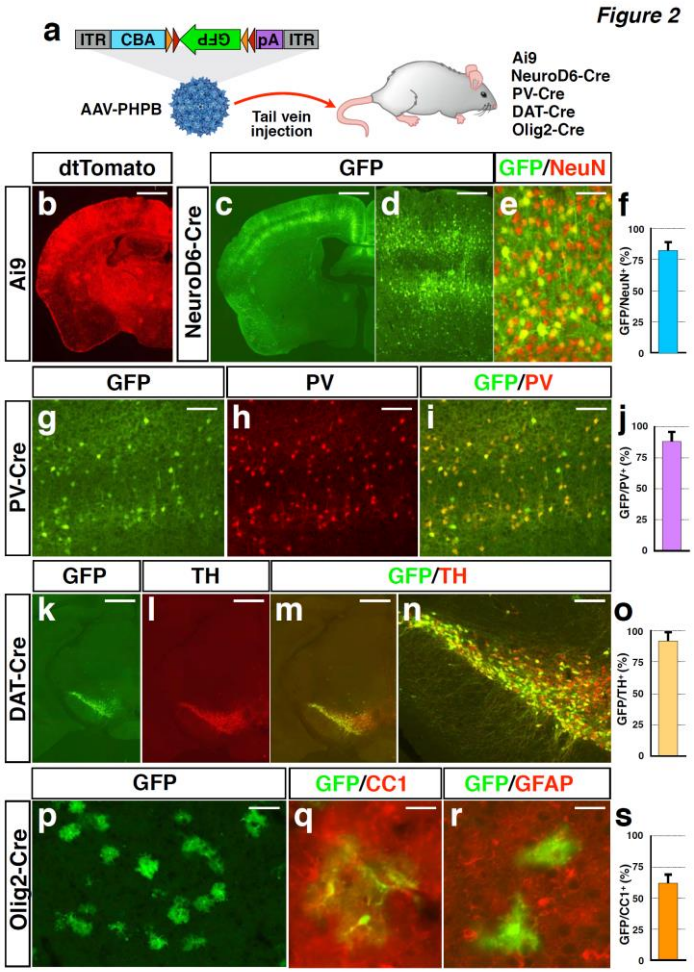
expression restricted to the forebrain GABAergic interneurons or the midbrain dopaminergic neurons, respectively (**Figures 2G-O**). In both cases, GFP expression was detected in the majority of these neuronal cell populations and not in other neuronal or glial cell types (**Figures 2J, O**). However, a similar strategy could be equally employed to selectively target glial cells. In fact, i.v. viral transduction of Olig2-Cre mice enabled specific GFP labeling of $CC1^+$ oligodendrocytes but not $GFAP^+$ astrocytes (**Figures 2P-R**). Although the overall viral targeting was not as efficient as for neurons, approximately 60% of the oligodendrocyte lineage was targeted (**Figure 2S**).

Thus, combining Cre-loxP cell lineage specificity with spatially broad AAV-PHP.B transduction enabled the targeting of a specific neural subtype in wide regions up to the whole brain. This is a favorable setting for evaluating the function of a specific neuronal cell type within the brain and its resulting effects in live animals. Current optogenetic methods evaluate the effects of altering a specific neuronal circuit, but only in a

confined brain territory limited by the intraparenchymal spreading of conventional viruses[19]. To move beyond this, we conceived the idea to combine the AAV-PHP.B/Cre-loxP system with the chemogenetic DREADD technology to obtain global modulation of the neuronal activity[20]. Moreover, the DREADD receptors are activated by the brain-penetrant small molecule clozapine N-oxide (CNO), which additionally provides fine temporal control of this system. Thus, as a proof of concept, we sought to test the effects of altering PV^+ neuronal function in the whole brain. Although PV has some expression in subcortical regions, especially in cerebellar Purkinje neurons, its major expression selectively localizes to the forebrain fast-spiking inhibitory interneurons. Therefore, we transduced adult PV-Cre transgenic mice with an AAV-PHP.B-FLEX-DREADDM4-mCherry (PHP.B-FLEX-M4C) to inhibit activity exclusively in the PV-expressing neurons (**Figure 3A**). Double immunofluorescence for the viral mCherry reporter and PV showed a common pattern of staining in the somatosensory cortex (**Figures 3B-C**).

Quantitative analysis confirmed that the mCherry reporter was restricted exclusively to the PV^+ interneurons (**Figure 3E**). Conversely, a small fraction of PV^+ cells did not express mCherry, probably because they were not transduced by the virus (**Figure 3E**). To determine whether the system was functional, we acutely sliced brains from transduced mice and the recorded the electrical activity of $mCherry^+$ neurons during patch-clamp experiments. Soon after CNO was added to the slice extracellular medium, recorded neurons silenced their activity with an abrupt loss of action potentials and membrane potential hyperpolarization (**Figure 3F**). Next, PHP.B-FLEX-M4C-transduced PV-Cre mice were implanted with epidural electrodes and EEG recordings performed before and after CNO injection. Interestingly, after CNO, the EEG showed slowed background activity and mild epileptic abnormalities (sharp waves), with no clear epileptic behavior in mice recorded for 12 hrs (n = 3) (**Figure 3G**). We then hypothesized that loss of PV forebrain interneuron activity could lead to increased seizure susceptibility. Accordingly, kainic

acid-induced seizure activity was strongly enhanced in PHP.B-FLEX-M4C, leading to animal death immediately after treatment (5/5), while the majority of control mice treated with PHP.B-GFP survived the



same treatment (4/5) (**Figure 3H**). These findings exemplify a strategy, sophisticated yet extremely easy to implement, by which to control neuronal activity in the whole brain and determine its underlying behavioral consequences in live animals.

Figure 2. AAV-PHP.B transduction associated to Cre-loxP technology enables the labeling of a specific neural subpopulation throughout the brain.

A. Schematic view depicting the GFP flex cassette integrated in the AAV-PHP.B vector and injection of the viral particles into the tail vein of Cre-expressing transgenic mouse strains. **B.** tdTomato staining on a forebrain coronal section of AAV-PHP.B-Cre transduced Ai9 tdTomato reporter strain. The highly diffuse activation of the reporter demonstrates the highly efficient Cre-mediated recombination occurred after viral transduction (positive control). **C,D.** GFP localization in the transduced NeuroD6-Cre forebrain (**C**) and cortical tissue (**D**). **E.** Co-labeling of GFP and NeuN in NeuroD6-Cre transduced cortical tissue. **F**. Bar graph showing the percentage of cortical GFP positive on total NeuN positive cells. **G-I.** Co-staining between GFP and Parvalbumin (PV) in the PV-Cre transduced cortical tissue. **J**. Bar graph depicting the fraction of GFP positive on total PV expressing neurons in the cortex. **K-O**. Double staining for GFP and TH on infected DAT-Cre ventral midbrain tissue and quantification of the percentage of GFP expressing cells within the TH cellular fraction. **P.** GFP immunofluorescence on infected Olig2-Cre cortical tissue. **Q,R**. GFP transduced cells co-express the oligoglial CC1 (**Q**), but not astrocytic GFAP (**R**) marker, identifying them as oligodendrocyte glial cells. **S.** Bar graph quantifying the percentage of transduced GFP cells within the CC1 expressing cellular fraction. (n = 3 mice for each Cre-transgenic line). Scale bars: 200 µm (A,B); 200 µm (K-M); 100 µm (D); 50 µm (G-I,N,P); 30 µm (E); 20 µm (Q,R)

Figure 3

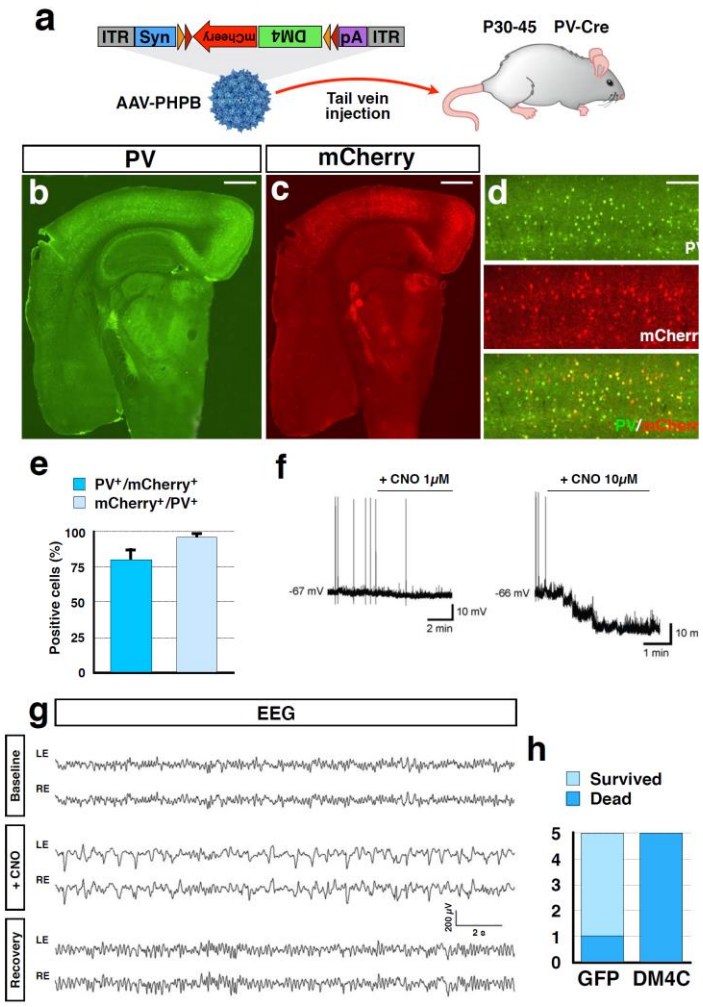


Figure 3. AAV-PHP.B-mediated targeting of the DREADD M4 chemogenetic inhibitory receptor in PV^{+} cortical interneurons sensitizes the mice to pro-epileptic insults.A. Schematic view depicting the chemogenetic DREADD M4

inhibitory receptor fused to mCherry cloned in a flex cassette and integrated in the AAV-PHP.B vector and injection of the viral particles into the tail vein of a PV-Cre adult mouse. **B, C.** Parvalbumin (PV) (**B**) and mCherry (**C**) immunofluorescence on transduced forebrain coronal sections. **D**. High-magnification images of cortical tissue co-stained for PV and mCherry. **E.** Bar graph depicting the relative fractions of PV and mCherry of GFP double-positive cells. (n = 4 PV-Cre mice). **F**. Electrophysiological recordings on transduced PV-Cre brain slices showing that CNO perfusion strongly inhibits the membrane excitability of mCherry positive neurons (n = 6). **G**. Representative EEG traces of 12hr recordings after CNO injection into transduced PV-Cre mice. (3 recordings in 4 mice). **H.** Bar graph showing the number of mice succumbed after treatment with kainic acid (KA) between the two animal groups treated either with the GFP or the DM4C expressing viruses. Scale bars: 500 µm (B,C); 100 µm (D)

Rapid analysis of Tsc1 gene function in adulthood by systemic injection of Cre-expressing AAV-PHP.B

Global nervous system targeting by AAV-PHP.B might also be convenient to regulate gene activity in transgenic mice carrying floxed gene alleles. In fact, deleting genes with Cre-loxP technology to study their effects exclusively in adulthood requires a rather extended time to obtain the mutant mice for phenotypic analysis. Thus, we generated a constitutively Cre-expressing AAV-PHP.B and systemically injected it in adult mice carrying a floxed allele for Tsc1 (**Figure**

4A). Mutations in TSC1 in humans are responsible for tuberous sclerosis complex (TSC), a disorder characterized by severe intellectual disability and intractable seizures[21,22]. Inactivation of TSC1 or its homolog TSC2, with which it forms a multimeric complex, causes hyperactivation of mTOR complex 1 (mTORC1) and hyperphosphorylation of its downstream effectors including ribosomal protein S6[23,24]. TSC patients present with focal brain lesions, known as cortical tubers and subependymal nodules, characterized by general cellular disorganization and giant cells. It is believed that these structural brain alterations are the primary cause of the chronic epileptogenic state[25]. Homozygous Tsc1 or Tsc2 mutant mice recapitulate the pathological milestones described in patients[26]. In fact, Tsc1/2 gene deletion causes overt brain pathology associated with severe epileptic crises and consequent death soon after birth[26]. Whether epilepsy is a result of the cortical tissue disorganization occurring during development or, conversely, is caused by a cell-autonomous dysfunction in the mutated neurons has remained

controversial. Recently, full-body acute Tsc1 inactivation in adulthood by classical mouse transgenic breeding was found to lead to profound epileptic seizures in the absence of neurodevelopmental brain lesions[27]. To extend this analysis, we infused AAV-PHP.B-Cre at a high dose (2×10^{12} vg) in adult $Tsc1^{f/f}$ and Ai9 reporter mice. Starting 1 week after systemic viral injection, mice developed severe epileptic seizures, with a minimum of 6 crises detected in a 12-hr continuous EEG recording (**Figures 4B, C**). About half of these animals died in the following 4 weeks (4 out of 9). A similar dose of AAV-PHP.B-Cre robustly activated tdTomato in the brains of the Ai9 mice (**Figure 4D**). To assess mTOR activation at the cellular level, we performed immunohistochemistry for phospho-S6 (pS6). As expected, pS6 staining was strongly increased in transduced floxed Tsc1 brains but not in Ai9 control brains, with most of the neurons in the cerebral cortex and hippocampus presenting a strong positive cytoplasmatic signal (**Figures 4E-H**). We then asked whether loss of Tsc1 in only a fraction of neurons

would be sufficient to cause a disease state. Thus, we injected AAV-PHP.B-Cre at a low dose (10^{11} vg) in $Tsc1^{f/f}$ mice. This dose of virus transfused in the brain of Ai9 conditional mice activated the tdTomato reporter in approximately 35% of cells in the cerebral cortex (**Figure 4K**). Nonetheless, even with this dose of virus, all the animals developed severe seizures starting 3 weeks after treatment, although the number of crises was reduced to an average of 1-2 events in 12 hrs (**Figures 4I, J)**. Remarkably, strong pS6 staining was detectable only in a mosaic fashion in the cortex and hippocampus, accounting for only a 25% of neurons, in treated Tsc1 mice but not in Ai9 control mice (**Figures 4K-O**). These results demonstrate that the Tsc1 gene has an indispensable cell-autonomous role in adult neurons and that its loss triggers severe epileptogenesis in mice, even when only a fraction of neurons carry mutant alleles for this gene.

Figure 4

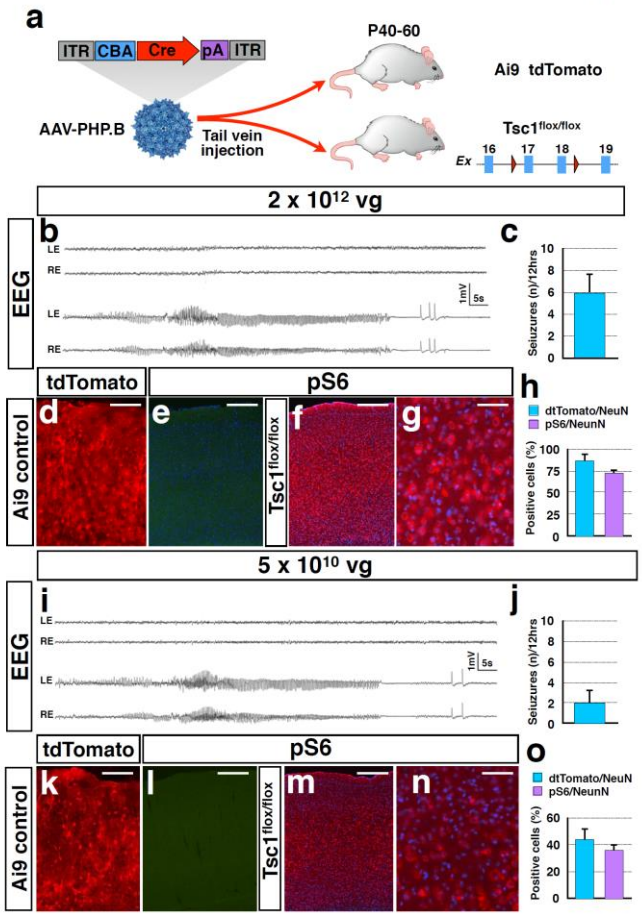

a
ITR CBA Cre pA ITR
AAV-PHP.B
Tail vein injection
P40-60
Ai9 tdTomato
Tsc1flox/flox
Ex 16 17 18 19
2 x 10¹² vg
b
EEG
LE
RE
1mV
5s
c
Seizures (n)/12hrs
tdTomato
pS6
Ai9 control
d
e
Tsc1flox/flox
f
g
h
dtTomato/NeuN
pS6/NeunN
Positive cells (%)
5 x 10¹⁰ vg
i
j
k
l
m
n
o

Figure 4. Complete or partial loss of Tsc1 in the adult brain mediated by the Cre-expressing AAV-PHP.B leads to severe epileptic seizures.

A. Experimental set up for the tail vein injection of the virus AAV-PHP.B-Cre in $Tsc1^{flox/flox}$ mice. **B.** Representative traces of EEG recordings in baseline state (above) and during seizure (bottom) in treated $Tsc1^{flox/flox}$ mice injected with a viral dose of $2x10^{12}$ vg (LE: left emisphere; RE: Right emisphere). **C.** Quantification of epileptic events in 12 hrs (3 recordings in 3 mice). **D,E.** tdTomato direct signal and pS6 immunofluorescence on cortical tissue of transduced Ai9 tdTomato reporter mice. **F,G.** Images at different magnification of transduced $TSC1^{flox/flox}$ cortical tissue stained for pS6. **H.** Quantification of tdTomato and pS6 positive cells on the NeuN neuronal fraction in transduced Ai9 or $TSC1^{flox/flox}$ mice, respectively (n = 3 mice). **I**. Representative traces of EEG recordings in baseline state (above) and during seizure (bottom) in treated $Tsc1^{flox/flox}$ mice injected with a viral dose of $5x10^{10}$ vg (LE: left emisphere; RE: Right emisphere). **J.** Quantification of epileptic events in 12 hrs (3 recordings in 2 mice). **K,L.** tdTomato direct signal and pS6 immunofluorescence on cortical tissue of transduced Ai9 tdTomato reporter mice. **M,N.** Images at different magnification of transduced $TSC1^{flox/flox}$ cortical tissue stained for pS6. **O.** Quantification of tdTomato and pS6 positive cells on the NeuN neuronal fraction in transduced Ai9 or $TSC1^{flox/flox}$ mice, respectively. (n = 3 mice). Scale bars: 100 μm (D-F,K-M); 50 μm (G,N).

Whole brain GBA1 gene transfer significantly prevents α-syn inclusion formation in A53T-SCNA transgenic mice

The brain-penetrating AAV-PHP.B is an unprecedented platform to exploit gene therapy protocols to treat neurodegenerative disorders

affecting the whole nervous system. In particular, this system can be explored to sustain diffuse expression of GBA1 in the brain to potentially counteract the gradual widespread accumulation of α-syn inclusions in the nervous system. To test this hypothesis, we employed A53T-SCNA transgenic mice that overexpress the SCNA mutation responsible for a genetic form of PD in humans. Starting from 6 months of age, these mice gradually accumulate insoluble α-syn deposits throughout the brain, with particular enrichment within the cerebral cortex, the midbrain and the pons, and at 10-12 months of age most of them die after developing a severe and rapid loss of voluntary movements and fatal paresis[28]. We focused particularly on the somatosensory (SCx) and visual cortical areas, where α-syn aggregates were particular evident and diffuse (**Figures 5A, B**), resembling the α-syn toxicity in the cerebral cortex of PD patients, which leads to cognitive disabilities and dementia. Initially, we asked whether the overexpressed GCase enzyme encoded by the GBA1 transgene could be properly targeted to the

lysosome and acquire functionality. Thus, GBA1 was tagged with mCherry, a fluorescence tag, which maintains its activity in the acidic lysosomal environment, and transfected into HeLa cells. Co-staining for mCherry and Lamp2 revealed that the exogenous GCase protein was at least in part correctly localized in the lysosomes (**Figure S4A)**. To determine whether the expressed GCase was functional, we assessed the overall enzymatic activity using a quantitative assay with a specific synthetic substrate. GBA1-overexpressing cells exhibited a significant increase in GCase catalytic activity compared with untransfected cells, demonstrating the complete functional maturation of the exogenous GCase (**Figure S4B, C**). Hence, we cloned the GBA1 cDNA upstream of a P2A-GFP cassette driven by the EF1α promoter in a shuttle vector and used it to generate AAV-PHP.B viral particles. Then, 5-month-old A53T-SCNA transgenic mice were infused with either the GFP-(control) or the GBA1-P2A-GFP-expressing virus. A group of animals was subsequently euthanized at 10 months of age, when control mice started to perish, and

brain tissue was isolated for molecular and neuropathological inspection. Since GBA1 antibodies failed to give reliable immunohistochemical staining, we investigated the global pattern of brain transduction in A53T-SCNA mice by GFP reporter analysis. As shown in Figures 5D-G, GBA1-P2A-GFP (hereinafter referred to as GBA1 only) gene transfer was efficient and diffuse in all the forebrain regions, infecting both neurons and glia. Accordingly, the immunoblotting profiling of cortical and hippocampal tissues from GBA1-transduced animals confirmed a robust increase in the overall amount of GCase protein (**Figures 5H, I)**. We then evaluated the levels of GCase activity in control and treated animals. Interestingly, GCase enzymatic activity was strongly reduced in control A53T-SCNA transgenic mice in most of the neural regions tested, in line with previous data suggesting that α-syn pathology affects GCase protein processing and targeting to lysosomes[14]. Conversely, GBA1-transduced animals exhibited a strong rescuing of GCase enzymatic levels, which were at least comparable to those detected in wild-type animals in

all the CNS regions (**Figure 5J)**.

Figure 5

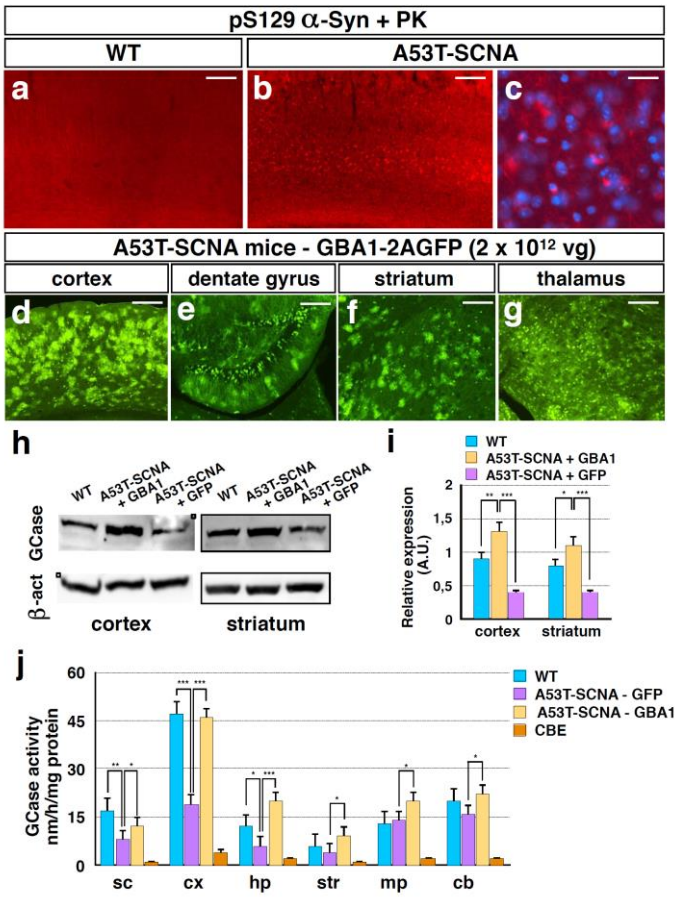


Figure 5. AAV-PHP.B intravenous delivery enables a global stimulation of GCase activity in adult A53T-SCNA mice.

A-C. Immunostaining for phospho-S129-□-syn show the diffuse accumulation of PK resistant □-syn deposits in the somato-sensory cortex of 8 month olds A53T-SCNA transgenic mice mainly localized in the neuronal soma (arrows in **C**). **D-**

G. Representative pictures showing the amount and distribution of GFP transduced brain cells in cortex (**D**), dentate gyrus (**E**), striatum (**F**) and thalamus (**G**) in 10 month old A53T-SCNA mice infused with the AAV-PHP.B-GBA1-P2A-GFP virus. **H**. Immunoblotting analysis showing the total amount of GCase protein in cortical and hippocampal tissues of wild-type (WT) and A53T-SCNA transgenic mice treated with the GFP- or GBA1-expressing AAV-PHP.B. **i.** Bar graph illustrating the quantification of the GCase the immunoblotting signal (n = 3 A53T-SCNA + GFP; n = 3 A53T-SCNA + GBA1; n = 3 WT tested at 3 months after infection; $p < 0.05$). **J.** Direct quantification of total GCase catalytic activity showing a significant recovery of the enzymatic activity in all the nervous system. The GCase selective inhibitor conduritol-B-epoxide (CBE) was included to evaluate the specificity of the reaction. (n = 3 WT; n = 3 A53T-SCNA + GFP; n = 3 A53T-SCNA + GBA1 tested at 3 months after infection). Data are expressed as mean + SEM and analyzed by unpaired Student's *t*-test (* < 0.05, ** < 0.01, *** < 0.001). Scale bars: 100 µm (B,C, D-G); 10 µm (C).

Then, α-syn pathology was specifically assessed by immunostaining for pS129-α-syn in PK-treated brain sections to enable the accurate identification of insoluble intracellular α-syn deposits. In the visual cortex, α-syn aggregates were mainly detected within the somata of neurons, as revealed by both immunohistochemistry and immunofluorescence imaging (**Figures 6A-D**). Remarkably, GBA1 gene transfer elicited a strong reduction of α-syn pathology in the visual cortical areas (**Figure 6A-E**). To extend

this analysis, we performed stereological semi-automatic counting of α-syn inclusions within the visual (anteroposterior position from -3 mm to -4 mm, centered on bregma), cingulate, motor and somatosensory cortical areas as well as the striatum (anteroposterior position from +1 to -0.5 mm, centered on bregma). Accordingly, the overall quantity of PK-resistant deposits was significantly diminished in all these brain domains to a comparable extent, confirming efficacious and widespread protection from α-syn pathology (**Figure 6E**). To confirm the effects of the exogenous GCase activity on α-syn protein processing, the various forms of α-syn were resolved and analyzed by Western blotting of TBS-soluble and TBS-insoluble fractions of forebrain lysates (**Figure 6F**). Indeed, a significant decrease in both monomeric and oligomeric forms of α-syn (including low- (LMW) and high-molecular-weight (HMW) aggregates) was observed in GBA1 compared with GFP transduced tissues (**Figure 6F**).

Figure 6

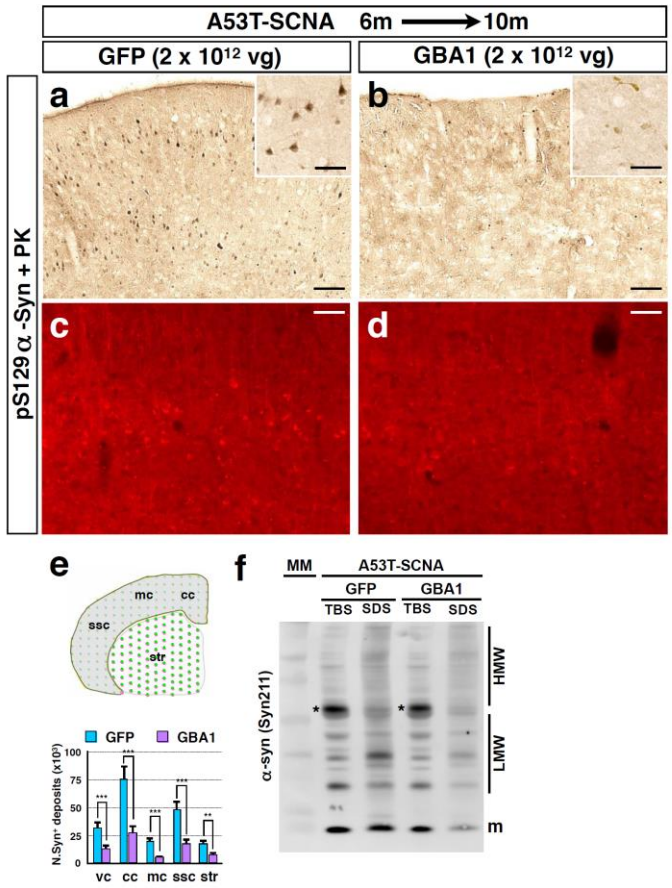


Figure 6. Global brain GCase gene transfer ensures a diffuse protection from □-syn deposits throughout all the forebrain regions in adult A53T-SCNA mice.

A-**D**. Immunohistochemistry (**A**,**B**) and immunofluorescence (**C**,**D**) analysis for pS129-α-syn on PK-treated visual cortical tissue from 10 month old A53T-SCNA transgenic mice treat with GFP (control) or GCase expressing AAV-PHP.B. Insets

in **A** and **B** are high power enlargements showing the α-syn deposits concentrated in the cytoplasm of the cortical neurons. (n = 6 A53T-SCNA + GFP; n = 5 A53T-SCNA + GBA1). **E**. Total number of insoluble α-syn inclusions in different forebrain regions as quantified by semi-automatic stereology counting in selected forebrain areas. Counting was automatically performed in a selected patterning within the brain tissue as highlighted in the drawing. **F**. Immunoblotting with TBS and SDS soluble tissue lysates from GFP and GBA1 transduced brains detecting the monomeric (m) and high- (HMW) and low-molecular weight (LMW) α-syn aggregates. Quantitative analysis showed a significant reduction of α-syn monomeric and aggregated species protein after GCase treatment. (n = 3 A53T-SCNA + GFP; n = 3 A53T-SCNA). Data are expressed as mean + SEM and analyzed by unpaired Student's *t*-test (* < 0.05, ** < 0.01, *** < 0.001). Scale bars: 50 μm (A-D); 10 μm (insets in A, B).

Next, we wondered whether the acute reduction of α-syn pathology in adulthood correlated with any behavioral amelioration. GBA1-transduced animals showed a consistent increase in median survival compared with control treated mice, with a consistent fraction of animals surviving when all control mice had expired (**Figure S5A**). In addition, GBA1-treated, but not control mice exhibited a strong recovery in learning and cognitive performance as revealed by a significant improvement in the novel object recognition test both at 3 and 5 months after treatment

(**Figure 5B**). Overall, GBA1-transduced mice showed a robust reduction of α-syn pathology in the whole forebrain, suggesting that the exogenous GCase provided sufficient supplemental activity to limit and counteract the widespread development and accumulation of α-syn deposits. Hence, these data strongly indicate that AAV-PHP.B-mediated gene transfer in the adult brain is an outstanding system to express a therapeutic gene throughout the brain tissue in order to curb pervasive pathological manifestations often associated with the progression of neurodegenerative diseases.

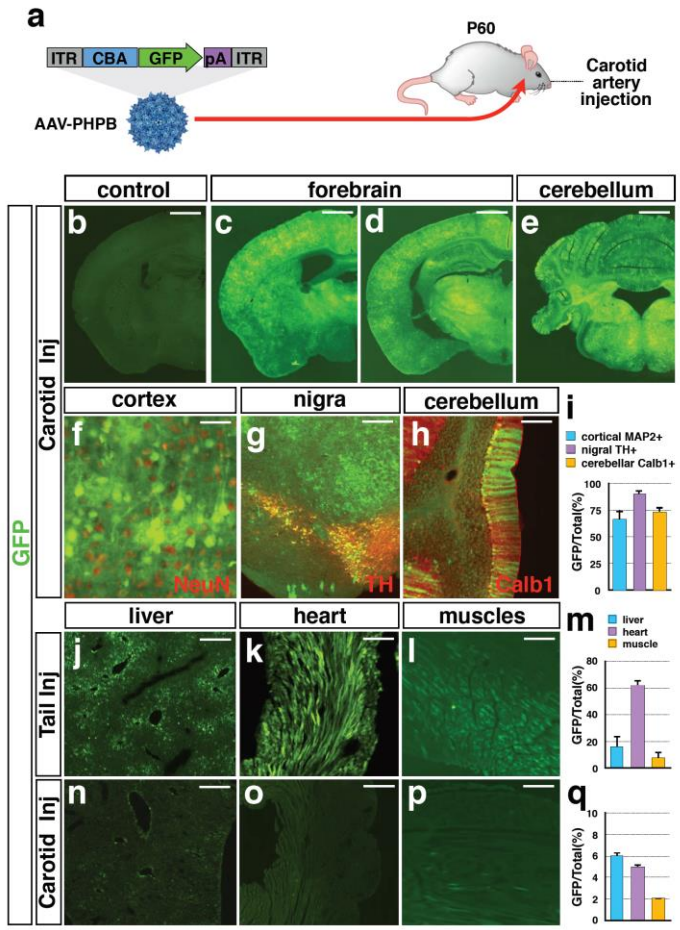
AAV-PHP.B viral brain transduction through the carotid artery route limited viral diffusion in peripheral organs

Systemic i.v. delivery enables the effective spreading of the virus throughout the brain vasculature and subsequently in the neural parenchyma. However, the peripheral venous route diffuses the virus to the whole body and its peripheral organs. Thus, a single i.v.

injection of AAV-PHP.B is sufficient to transduce, beyond the nervous system, a non-marginal fraction of cells in all the peripheral organs[10]. This undesired viral spreading might result in a serious drawback for many potential applications of this system. To restrict the viral delivery to the nervous system, we sought to inject the virus directly into the brain circulation. For this, 2 x 10^{12} vg of AAV-PHP.B-GFP was directly infused into the internal carotid artery via a microcatheter (**Figure 7A**). Brains and peripheral organs were retrieved 3 weeks after injection for immunofluorescence analysis. Notably, GFP transgene expression was detected diffusely throughout the brain with high transduction efficiency (**Figures 7B-D**). Co-labeling between GFP and either NeuN, TH or calbindin-1 showed that a high fraction of cortical and mesencephalic nigral neurons as well as cerebellar Purkinje cells were effectively transduced (**Figures 7F-I**). We then compared the viral GFP gene transfer in peripheral organs after tail vein and carotid artery injections. Analysis of GFP expression in the liver, heart and muscles showed that carotid infusion

substantially reduced the viral distribution in all these peripheral organs (**Figures 7J-Q**). In particular, the viral transduction in the liver and heart was decreased by more than 5- and 10-fold, respectively (**Figure 7Q**).

Figure 7



A. Schematic view depicting the transgenic cassette integrated in the AAV-PHP.B vector and injection of the viral particles into the carotid artery of an adult mouse. **B.** GFP immunofluorescence in brain transduced with an empty AAV-PHP.B (negative control). **C-E.** GFP localization on coronal hemi-sections at different rostro-caudal coordinates of a brain transduced with the AAV-PHP.B-GFP virus. **F-H.** High-magnification images of cerebral cortex (**f**), mesencephalic nigral tissue (**G**) and cerebellum (**H**) showing the double staining for GFP and the neuronal marker NeuN, TH and Calb1, respectively. **I.** Bar graph showing the fraction of cells positive for a specific neuronal marker (NeuN, TH and Calb1) and expressing the viral GFP transgene. **J-Q**. GFP localization in the peripheral organs liver (**J,N**), heart (**K,O**) and muscles (**L,P**) after injection into either the tail vein (**J-M**) or the carotid artery (**N-Q**). **M,Q.** Bar graphs showing the percentage of GFP positive on total cells. Note that the artery route substantially reduces viral targeting in peripheral organs. (n = 3 mice). Scale bars: 500 µm (B-E); 200 µm (J-L, N-P); 100 µm (G); 50 µm (H); 20 µm (F).

These data indicate that the carotid artery route is advantageous since the nervous system targeting is coupled to a reduction in peripheral spread.

AAV-PHP.B brain targeting does not impair blood-brain barrier integrity or selectivity

Considering the extremely efficient brain diffusion of the AAV-PHP.B after i.v. injection, we wondered

whether it could alter blood-brain barrier (BBB) properties. We therefore analyzed BBB permeability and inflammation after AAV-PHP.B transduction *in vivo*. For this aim, mice were intravenously injected with fluorescent-conjugated cadaverine dye, a small (640 Da) BBB permeability marker, together with the virus AAV-PHP.B-GFP (**Figure 8A**). Staining for viral capsids with the AAV-VP3-specific antibody (B1) confirmed the localization of the viral particles within the brain endothelium 24 hrs after viral delivery (**Figure 8B**). However, the transduced brain tissue did not show any evident diffusion of the cadaverine dye (**Figure 8C**). In addition, no signs of astrocytosis were revealed by GFAP staining in the targeted tissue 2 days after viral transduction (**Figure 8C**). As a positive control, diffuse cadaverine staining and astrocyte activation were detected in the brain parenchyma of kainic acid-treated mice that developed seizure-induced BBB permeability and severe inflammation (**Figure 8E-G**). To further assess BBB integrity upon AAV-PHP.B transduction, we employed a simplified *in vitro* BBB model obtained by isolating and culturing

primary mouse brain microvascular endothelial cells (BMVECs). Acutely dissociated BMVECs were cultured to confluence to form an organized epithelial layer and then either infected with AAV-PHP.B-GFP or left untreated for 5 days (**Figure 8H**). In these conditions, untreated cells maintained cell-cell contacts positive for the tight junction markers ZO-1 and claudin-5 (**Figure 8I, J**). Similarly, virally transduced cells, identified by GFP expression, displayed comparable ZO-1 and claudin-5 protein localization at cell junctions (**Figures 8K, L**). Finally, we asked whether the viral infection could perturb the transendothelial electrical resistance (TEER), a key measurement of tight junction resistance in endothelial cells. Notably, there was no significant difference in TEER values between untreated and infected cells as measured up to 5 days from viral loading (**Figure 8M**). Conversely, TEER signal was strongly abolished when EDTA was added to the culture, causing a loss of calcium-dependent cell junctions (**Figure 8M**)[29]. Altogether, these data indicate that AAV-PHP.B targeting to the BBB does not alter the basic properties

of the brain endothelium, maintaining unaltered its barrier selectivity *in vivo* and morphological integrity *in vitro*.

Figure 8

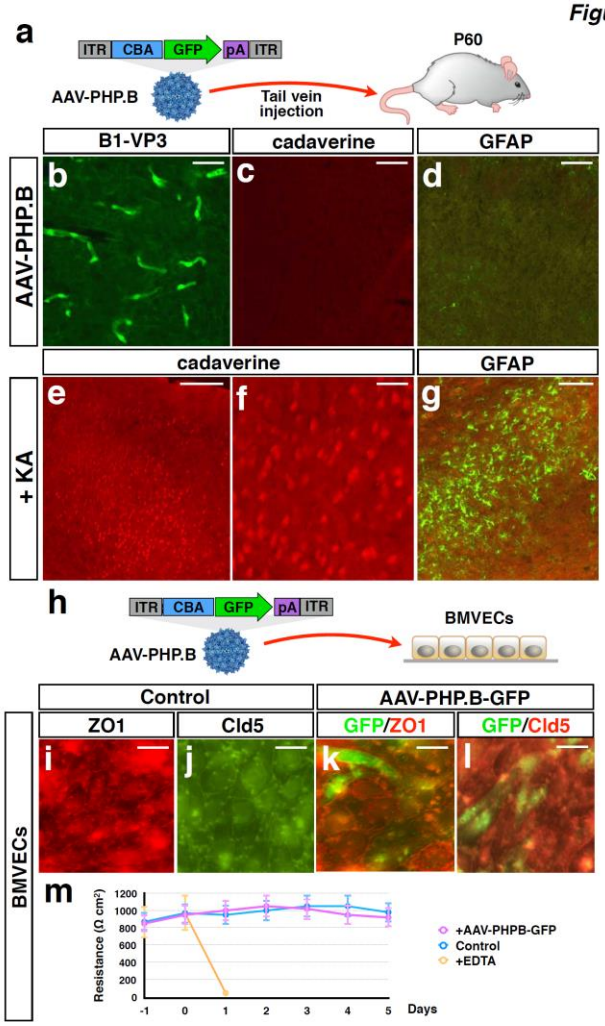


Figure 8. The AAV-PHP.B brain transduction does not affect BBB permeability in vivo and endothelial integrity in vitro.

A. Experimental set up for the tail vein injection of the AAV-PHP.B-GFP in wild-type adult mice. **B.** Viral capsid staining using the B1 anti-AAV VP3 antibody reveals a robust targeting of the AAV-PHP.B-GFP in the brain endothelium 4 hrs after infection. **C.** Alexa Fluor-555 conjugated with cadaverine is undetectable in brain parenchyma 24 hrs after AAV-PHP.B injection. **D.** No evident sign of astrogliosis (GFAP staining) is present 2 days after AAV-PHP.B injection. **E.** Cadaverine staining in the brain parenchyma in kainic acid (KA) treated animals **F.** High magnification of cadaverine staining in the cortical tissue. **G.** Strong GFAP positive astrogliosis in KA injected animals. **H.** Schematic view depicting the infection of brain microvascular endothelial cells (BMVECs) with the AAV-PHP.B-GFP. **i-l**. Immunofluoresce for the cell-cell junction markers ZO1 and Claudin-5 (Cld5) in confluent BMVECs either untreated (**I,J**) or infected with the AAV-PHP.B-GFP (**K,L**). Transduced cells are visible for GFP expression in **K** and **L**. **M**. Transendothelial electrical resistance (TEER) analysis of confluent BMVEC cultures infected with AAV-PHP.B-GFP at time 0 (purple line). Cultures never exposed to virus were used as stable baseline controls (blue line). EDTA was used as a control for the loss of baseline TEER values. All treatments were performed in triplicate. Scale bars: 50 µm (B-G); 200 µm (K-M); 10 µm (I-L).

Discussion

AAV9 is the only AAV serotype able to cross the BBB when delivered through the vascular system. However, this ability is considerably diminished in adulthood, raising significant hurdles for pervasive targeting of the adult brain through a peripheral route. Remarkably, the AAV-PHP.B variant maintains efficient

penetration even in the mature BBB and widely diffuses in the brain parenchyma in adult mice[10]. Herein, we confirmed and extended these data, showing that a single i.v. injection of AAV-PHP.B can globally transduce both the central and the peripheral nervous system. Interestingly, we showed that DRGs and SGs are both efficiently targeted by AAV-PHP.B, which mostly infects the sensory neurons. Given that satellite glial cells or and interneurons were poorly transduced by the virus, it is likely that the infection mainly followed a retrograde route, with initial uptake at the periphery followed by retrograde transport to the neuronal soma. Our results and those of previous studies have shown that the systemic i.v. route is intrinsically associated with widespread transduction of peripheral organs[30,31].

In this work, we employed single-stranded AAVs (ssAAV) exclusively. Recent studies have shown that self-complementary AAV9 (scAAV9) can potentially transduce the adult brain parenchyma[9]. However, in all cases, the efficiency of transduction was far from the

level necessary for supporting the technical approaches herein presented. In addition, scAAV9 facilitates viral transduction by circumventing the limiting step of the synthesis of the viral DNA complementary strand, but it also causes a loss of half of the coding packaging, reducing it to only 2.2 kb for the entire expression cassette including the promoter, coding gene and polyA sequences[32]. Thus, AAV-PHP.B supports the global spread of the virus in the nervous system while maintaining the full packaging space for AAV, providing convenient flexibility in designing the transgene cassette.

Herein, we established straightforward approaches to control gene expression and neuronal activity in the adult mouse brain with a single-step protocol. These procedures will have a strong impact by accelerating functional studies of genes and molecular labeling of neuronal cell types for anatomical tracing. Furthermore, the AAV-PHP.B whole-brain delivery of the chemogenetic DREADD system opens the opportunity to manipulate the activity of selected

neurons in large brain areas and eventually in the entire brain and subsequently evaluate the resulting behavioral response.

We provided a strong proof of concept of this strategy by showing that whole-forebrain inactivation of PV^+ GABAergic interneurons, while not sufficient per se to elicit spontaneous epileptic seizures, creates a strong predisposition to them after a proepileptic insult. However, PV is also expressed in some caudal brain areas and in Purkinje cerebellar neurons. Overall, we could not exclude the possibility that other cell populations might have influenced this phenotype. Thus, the choice of selective genetic tracing, when available, is a crucial prerequisite to subsequently retrieve conclusive functional data. Wide-scale access to the adult mouse nervous system makes feasible to misexpress genes and evaluate their direct impact on brain functions and consequent behavior.

Taken together, these results provide solid evidence that the brain-penetrant AAV-PHP.B is an ideal platform for transducing therapeutic genes to treat

neurodegenerative disorders that globally affect the brain tissue. Herein, we focused on α-syn inclusions, that spread over time throughout large brain areas in PD, LBD and MSA and are responsible for cortical functional decline leading to severe dementia[33,34]. Approximately 5–8% of PD patients are carriers of a heterozygous GBA1 mutation, causing a detectable reduction in GCase global activity[11,12]. PD patients carrying GBA1 mutations often develop more severe symptoms then GBA1 non-carrier patients, including an accelerated cognitive decline associated with increased α-syn accumulation[35,36]. Thereby, stimulating GCase activity in these patients represents a direct and valuable therapeutic approach. Furthermore, GCase activity gradually declines with aging in healthy individuals, and in addition, sporadic patients showed a further reduction in GCase functionality[37,38]. Given this, increasing GCase levels can also be an effective therapeutic strategy for age-related and sporadic forms of PD. Herein, we showed that AAV-PHP.B-mediated global expression of GCase is sufficient to provide robust and long-lasting

protection from α-syn deposits in a mouse model of synucleinopathy. Exogenous virally delivered GCase is targeted to the lysosome and acquires functionality, which resulted in significantly diminished accumulation of insoluble α-syn species in all the forebrain regions. Previous studies have shown that α-syn accumulation is promoted by diminished GCase activity *in vitro* and *in vivo*, which leads to an abnormal accumulation of its glycolipid substrates in the lysosomes[39,40]. Conversely, α-syn inhibits the lysosomal activity of GCase, thereby causing a loss of its catalytic function upon progressive accumulation of α-syn [14]. Hence, pathological conditions establish a vicious cycle between α-syn and GCase that can sustain and progressively worsen the disease[14,15]. Along these lines, stimulating GCase activity has been shown to counteract α-syn pathology in mouse and human neurons *in vitro*[16,17,41]. Our results support this view, showing that GCase is a strong determinant of α-syn accumulation and that increasing its enzymatic levels significantly protects against α-syn pathology

and toxicity. Intriguingly, although GBA1 viral transduction did not target the entire neuronal population, we nonetheless observed a general and even reduction of α-syn inclusions throughout the neural parenchyma. These results are plausible considering that the GCase enzyme has non-cell-autonomous action, reducing α-syn deposits even in cells in which it is not directly stimulated as long as they are placed close enough to virally transduced cells. In support of this hypothesis, classical cell biology experiments have shown that lysosomal enzymes can be released from producing cells, endocytosed by their neighbors, and correctly trafficked to their lysosomes both *in vitro* and *in vivo*[42]. Therefore, our findings imply that it is not necessary to transduce the entire brain neuronal population; GCase overproduction in a partial subset of neural cells (either neurons or glia, or both) is sufficient to achieve widespread protection from α-syn inclusions.

Strategies to reduce α-syn toxicity by active immunization or by stimulating GCase activity

through small-molecule noninhibitory chaperones are currently being explored to establish therapeutic approaches for these diseases[43-45]. However, limited crossing of the BBB, toxic side effects and restricted efficacy to only some disease forms are some of the important hurdles that remain to be fully cleared to translate these treatments to patients. The present results strongly imply that gene therapy should be considered as a further therapeutic opportunity for synucleinopathies with the potential advantage of providing a long-lasting beneficial effect with a single treatment. The introduction of the AAV-PHP.B viral platform, which sustains effective BBB crossing and global spreading of the therapeutic GBA1 gene in the adult brain tissue, fulfills the necessary conditions for the development of an effective and non-invasive gene therapy approach for synucleinopathies. Further studies on wild-type animals will be necessary to address whether the chronic stimulation of the GCase activity might cause any long-term adverse effect on neuronal homeostasis and function, regardless of whether this stimulation will be achieved by a small-

molecule or gene-based approach. However, this might not be a serious hurdle in our approach since the GBA1 viral transduction elicited a strong rescuing of GCase activity in most brain regions of A53T-SCNA transgenic mice, but without causing evident supraphysiological activity compared with wild-type conditions.

The massive penetration of this virus into the brain upon acute delivery in the circulation raises some concerns about altering normal BBB physiology. Thus, we searched for any sign of acute derangement of BBB integrity after virus administration. Importantly, no evidence of loss of functional selectivity was found; our work showed that a small (640 Da) fluorescent dye was continuously excluded from entering the brain space immediately or soon after viral infection. This is in line with the absence in transduced brain tissues of any overt sign of inflammation, which is closely associated with abnormal BBB permeability. Thus, the acute targeting of AAV-PHP.B to the brain appears to be substantially

harmless, at least in regards to major BBB functionality.

To concentrate the viral transduction to the brain, we administered the virus through the carotid artery, thereby maintaining high transduction efficiency within the neural parenchyma while strongly diminishing the spread of the virus in the peripheral organs. Although viral targeting in the liver and heart was strongly restrained, some remaining infected cells were still detectable, indicating that even direct arterial brain delivery can diffuse some viral particles into the systemic circulation. However, this delivery route has important advantages, especially from a therapeutic prospective. In fact, this viral administration is feasible in large apes and human clinical practice, limiting unnecessary viral spreading to peripheral organs while concentrating the viral particles to the therapeutic target, namely, the adult nervous system.

The diffuse penetration of the AAV-PHP.B in the adult mouse brain parenchyma is a unique property among all the recombinant viral strains in current use. Future

studies will address whether this capacity will remain intact and equally efficient when tested in large animals. If so, this viral strain might become the system of choice to deliver therapeutic genes such as GBA1 to the brain, enabling the development of effective and non-invasive gene therapy approaches for synucleinopathies.

Materials and Methods

Generation of the AAV transfer vectors

The pAAV-CBA-eGFP construct was kindly donated by Dr. G. Gonzalez (CIMA, Pamplona, Spain). This plasmid was further modified to express under the control of the CBA promoter: the CreNLS cassette, the multicistronic sequence including GBA1 coding region and GFP and the Flex eGFP cassette. In Flex cassettes the transgene is antisense respect to promoter driven transcription and flanked by two consecutive but different pairs of flox sequences. In presence of Cre recombination the transgene gets reverted and its gene expression activated. The GBA1 coding region was purchased from Origene (RG216061). Subsequently,

GBA1 was cloned in frame with a P2A sequence followed by the eGFP coding region. Finally, for the GBA1-mCherry expression vector, the GBA1 and mCherry coding regions were cloned in frame but separated by a Gly-Ser-Gly linker and inserted downstream to the EF1-alpha promoter.The pAAV-hSyn-DIO-hM4D(Gi)-MCherry plasmid was purchased from Addgene (#44362).

Cell cultures

293T cells were cultured in Iscove's Modified Eagle Medium (Sigma-Aldrich) containing 10% fetal bovine serum (Sigma-Aldrich), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were split every 3-4 days using Trypsin 0.25% (Sigma-Aldrich). Isolation of mouse brain microvascular endothelial cells was performed as previously reported by Liebner et al.[46]. BMVECs were grown in EBM-2 plus bullet kit (Lonza). For immunostaining 2 x 10^5

cells were seeded on 24 well-plate, coated with rat tail collagen type-1 (150 µg/ml; Sigma). The day after, fresh culture medium was added supplemented with LiCl (10mM, Sigma) and the cells were infected 48 hours after seeding in 300 µl of total volume. The medium was changed every 2-3 days. Hela cells were cultured in Dulbecco Modified Eagle Medium – high glucose (Sigma-Aldrich) containing 10% fetal bovine serum (Sigma-Aldrich), 1% non-essential amino acids (Gibco), 1% sodium pyruvate (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were split every 2-3 days using Trypsin 0.25% (Sigma-Aldrich). For transfection Lipofectamine LTX® (Thermo Fisher Scientific) was used, according to manufacturer's protocol.

Virus production and purification

Replication-incompetent, recombinant viral particles were produced in 293T cells by polyethylenimine (PEI) (Polyscience) co-transfection of three different

plasmids: transgene-containing plasmid, packaging plasmid for rep and cap genes and pAdDeltaF6 for the three adenoviral helper genes, The cells and supernatant were harvested at 120 hrs. Cells were lysed in Tris buffer (50mM Tris pH=8,5, 150mM NaCl, Sigma-Aldrich) by repetitive freeze-thawing cycles (3 times) whereas the viral particles present in the supernatant were concentrated by precipitation with 8% PEG8000 (Polyethylene glycol 8000, Sigma-Aldrich), lysed in Tris buffer and combined with correspondent cell lysates. In order to clarify the lysate, Benzonase treatment was performed (250U/mL, 37°C for 30min, Sigma-Aldrich) in presence of 1mM MglCl2 (Sigma-Aldrich) and cellular debris separated by centrifugation (2000g, 30min). The viral phase was isolated by iodixanol step gradient (15%, 25%, 40%, 60% Optiprep, Sigma-Aldrich) in the 40% fraction and concentrated in PBS (Phosphate Buffer Saline) with 100K cut-off concentrator (Vivaspin20, Sartorius Stedim). Virus titers were determined by measuring the number of DNase I-resistant viral particles, using qPCR with linearized genome plasmid as a standard.

Animals

Mice were maintained at San Raffaele Scientific Institute Institutional mouse facility (Milan, Italy) in micro-isolators under sterile conditions and supplied with autoclaved food and water. The following transgenic mouse strains were used: NeuroD6-Cre[47], DAT-Cre[48], Ai9[18], PV-Cre[49], Olig2-Cre[50] and Tsc1 conditional mutants[51]. All procedures were performed according to protocols approved by the internal IACUC and reported to the Italian Ministry of Health according to the European Communities Council Directive 2010/63/EU.

Viral injections

For tail vein injection, 2-6 month old mice were previously warmed under an heat lamp for 10 minutes and, then, placed into a restrainer for further manipulation. Mice were injected with variable viral concentrations depending on experimental set-up in a

total volume 200 µl of PBS (from $1x10^9$ to $2x10^{12}$ vg/each mouse). For injections into mouse neonates, 1 day after birth pups were rested on a bed of ice for anesthetization. 50 µl of AAV viral suspension ($1.5x10^{11}$ vg) was manually injected into the facial vein using a 29 gauge insulin syringe. After injection, pups were rubbed with bedding to prevent rejection before reintroducing the mother into the cage. For carotid artery injections, 8-10 weeks old C57Bl6/J mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) and temperature maintained during the procedure between 36 and 36.5 °C using a feedback-controlled heating system. Under a stereomicroscope a midline neck incision was performed, the common carotid artery exposed and the external carotid artery ligated. A micro-catheter was placed inside the common carotid artery and advanced up to the internal carotid artery. Under a sterile hood, the 50 µl of viral suspension ($2x10^{12}$ vg) in PBS was infused for 5-6 minutes with an infusion pump (World Precision Instruments) [52]. At the end of the procedure the microcatheter was

withdrawn, the incisions sutured and animals allowed recovering.

Immunohistochemistry

Immunohistochemical analysis of the tissue sections was conducted essentially as previously described[53]. Briefly, mice were anesthetized with Ketamine/Xylazine and transcardially perfused with 0.1 M phosphate buffer (PB) at room temperature (RT) at pH 7.4 with freshly prepared, ice-cold 4% paraformaldehyde (PFA) in PB. Tissues were post-fixed in 4% PFA overnight and then soaked in cryoprotective solution (30% Sucrose in PBS). After OCT embedding in dry ice, tissues were sectioned using cryostat. For immunofluorescence, free-floating 30 µm thickness coronal sections were rinsed in PBS, incubated for 10 min with H_2O_2 3% and 10% methanol, then for 20 min with Triton X 100 2%. BSA 3% for 1 hour was used to saturate the unspecific binding site before the overnight incubation with primary antibody (diluted in a solution containing BSA

1% and Triton X 100 at room temperature). Following incubation, sections were rinsed three times in PBS and incubated for 1 hour with the secondary antibody. For immunohistochemistry, free-floating slices were rinsed in PBS and treated for one hour with a blocking solution containing BSA 3% and Triton X 100 0.3% in PBS. After blocking, samples were incubated with the primary antibody diluted with a solution containing BSA 1%, Triton X100 0.3% over night at room temperature. The slices were then incubated with the secondary antibody, followed by Vectastain ABC enhancing reaction, and finally the staining was revealed in DAB solution. After mounting the slices were dehydrated in xylene and the coverslip sealed with Eukitt^{TM} mounting medium. The slices treated with Proteinase K (PK) were incubated for 10 minutes in a solution with 1 μg/mL of PK prior any step and the tissue processed for the immunostaining.

Primary antibodies for the following epitopes were used: GFP (1:500, Molecular Probes), TH (1:1000, Immunological Sciences), Calbindin (1:200, Swant),

Parvalbumin (1:500, Sigma), beta-III-Tubulin (1:1000, Covance), NeuN (1:1000, Immunogical Sciences), human α-Syn (1:200, Syn211, BDbiosciences), PhosphoS129-α-syn (1:100, Abcam). Slices were mounted with fluorescent mounting medium (Dako). For blood-brain barrier integrity evaluation, cadaverine (0.2 mg/animal) conjugated to Alexa Fluor-555 (Life Technologies) was injected intravenously into the tail vein 2 hours before sacrifice. Images were captured with a Nikon Eclipse 600 fluorescent microscope. Images were then imported and processed with the Photoshop Suite applications.

Stereological counting

Unbiased semi-automatic stereological sampling and counting was performed with a Leica DM4000B microscope equipped with MAC 6000 system and Stereo Investigator 9 software (MFB Bioscience, Williston, Vermont, USA). After structure boundaries delimitation, cortical phosphoS129-α-syn positive cells were automatically counted at 40X

magnification. Slices were collected every 180 μm, encompassing about 1.5 mm of cortex (antero-posterior +1 to -0.5 from bregma). The optical fractionator stereological probe (40 X 40 sized, 240 X 240 spaced) was then used to estimate the total number of phosphoS129-α-syn positive neurons.

GCase enzymatic assay

Dissected brain parts were lysates and mechanical homogenized in GCase assay buffer PH 5.4 (Citrate buffer 1×, Triton X-100 0.25% w/w, Taurocholic acid 0.25% w/w, H20) supplemented with 1% protease inhibitor mixture (Roche Diagnostics). After 30 min lysis on ice, samples were then centrifuged at 13,000g for 15 minutes at 4°C. Supernatant was collected, and used for activity assays and Western blots. GCase activity in tissues was measured using 10 μg of protein/well, quantification made with the Pierce BCA protein Assay Kit (Thermo Fisher Scientific). The substrate 4-methylumbelliferyl β-D-glucopyranoside

(4MUG, Sigma M3633) was then added to the plate to a final concentration of 5 mM with tissue lysates for 1 hr at 37°C. After blocking the reaction with 1M glycine solution, the signal was read at the Victor plate reader (Perkin Elmer) with excitation and emission wavelengths at 360 nm and 440 nm, respectively. The standard used for this assay was the fluorescent product 4-Methylumbelliferone (4MU) (Sigma, M1381). The specific activity was calculated with 4MU standard curve by converting the relative fluorescence units (RFUs) to the concentration of the fluorescent cleaved product (GraphPad Prism 5.1). This interpolated value was then used to calculate the GCase enzymatic activity in the lysed tissue, which was expressed as nmol/hr/mg. Specificity of the enzymatic activity was assessed by adding the specific GCase inhibitor Conduritol-B-Epoxide (CBE) at 16 mM (Sigma).

Immunoblotting

Brain lysate samples (~30 μg of protein lysates) were separated using 10% or 15% polyacrylamide gel and, then, transferred to PVDF membranes. Membranes were incubated overnight at 4°C with the following primary antibodies: C-terminal GCase antibody (Sigma, G4171, 1:1,000), anti-actin (Sigma, A3853, 1:10,000), anticalnexin (1:5000; Sigma, Cat. C4731). Subsequently, membranes were incubated with the corresponding horseradish-peroxidase-conjugated secondary antibodies (1:5000, Dako). The signal was, then, revealed with a chemiluminescence solution (ECL reagent, RPN2232, GE Healthcare) and detected with the ChemiDoc imaging system (Bio-Rad). For α-syn immunoblotting, brain homogenates were processed in order to collect the TBS and SDS-soluble fraction of α-syn as described[54]. Briefly, brains were homogenated with TBS (pH 7.4), clarified from non-homogenate residue and submitted to 100000 g centrifugation at 4 °C for 1hr. The resulting supernatant represents the TBS-soluble fraction. Then,

pellets were solubilized in TBS-SDS (SDS 5% w/v) by sonication and centrifuged at 100.000 g for 30 min at 25°C. Supernatants were collected and referred to as the SDS-soluble fractions. Sampled (15 ug/uL of total proteins) were loaded in a gradient gel (Bis-tris gel 4-12%, NP0322BOX, Invitrogen) with MOPS as running buffer (NuPAGE MOPS SDS, NP0001, Invitrogen) at 200V. The transfer was performed for 2hr at 40V on nitrocellulose membrane (nitrocellulose membrane 0.45 μm, 1060003, GE Helthcare). Membranes were, then, blocked in 5% BSA for 1hr and the primary antibody (Syn211) incubated overnight at 4 °C. After the incubation with the appropriate HRP-conjugated secondary antibody for 30 min the signal was then revealed and processed as previously described.

EEG recordings

At least three days before recording, epidural stainless steel screw electrodes (0.9 mm diam./3 mm long) were surgically implanted under ketamine/xylazine

anesthesia and secured using dental cement (Ketac Cem, ESPE Dental AG, Seefeld, Germany). Two active electrodes were placed on right and left parietal areas (2mm lateral to midline, 1mm posterior to bregma) and one over the occipital area (1mm posterior to lambda) as a common reference. Freely moving 12 hour sessions of digital EEG monitoring were performed via a flexible cable connected to the amplifier (Micromed Mogliano Veneto, Italy) in a Faraday cage, with food and water available *ad libitum*. EEG traces were filtered between 0.53 and 60 Hz and sampled at 256 Hz (16 bits). EEG recordings were visually inspected to detect epileptiform discharges and/or seizures, defined as high-amplitude (at least 2 times the baseline) rhythmic discharges lasting at least 5 seconds.

Ex vivo electrophysiological recordings

Mice (60-90 days of age) were anesthetized with an intraperitoneal injection of a mixture of ketamine/xylazine and transcardially perfused with

ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, 1 $MgCl_2$ and 11 D-glucose saturated with 95% O_2, 5% CO_2 (pH 7.3). After decapitation, brains were removed from the skull and mounted in a VT1000S vibratome chamber (Leica Microsystems, Wetzlar, Germany) filled with ACSF at 4°C. Sagittal brain slices were cut at a 300-µm thickness. Individual slices were submerged in a recording chamber mounted on the stage of an upright BX51WI microscope (Olympus) equipped with differential interference contrast optics (DIC) and an optical filter set for the detection of mCherry red fluorescence (Semrock, Rochester, NY). The slices were continuously perfused with ACSF (3-5 ml/min) at room temperature. Fast-spiking interneurons expressing DREADD were visually identified by tdTomato fluorescence. Whole-cell patch clamp recordings were performed using pipettes filled with a solution containing the following (in mM): 124 KH_2PO_4, 2 $MgCl_2$, 10 NaCl, 10 HEPES, 0.5 EGTA, 2 Na_2-ATP, 0.02 Na-GTP (pH 7.2, adjusted with KOH;

tip resistance: 6-8 MΩ). CNO (10 μM) was added through extracellular perfusion. All recordings were performed using a MultiClamp 700B amplifier interfaced with a PC through a Digidata 1440A (Molecular Devices, Sunnyvale, CA, USA). Data were acquired using pClamp10 software (Molecular Devices) and analyzed with Prism 5 (GraphPad Software, Inc., La Jolla, CA). Current-clamp traces were sampled at a frequency of 10 kHz and low-pass filtered at 2 kHz.

Behavioral studies

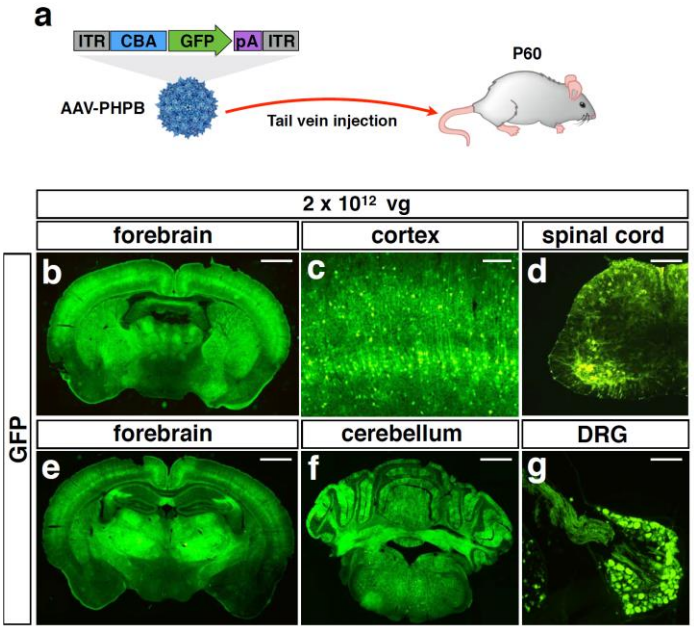
The novel object recognition test was performed in a square arena of 40 × 40 cm. On day 1, mice were habituated to the open-field apparatus in a 5 min session. On day 2, animals underwent the training phase (10 min), in which two identical objects were introduced into the arena before allowing the mouse to explore. The amount of time that the rodents spent exploring each object was scored. Finally, on day 3, mice were tested for their memory (10 min). The

discrimination index (DI) is defined as the difference between the exploration time for the novel object and the one for the familiar object, divided by total exploration time, was calculated.

Statistics

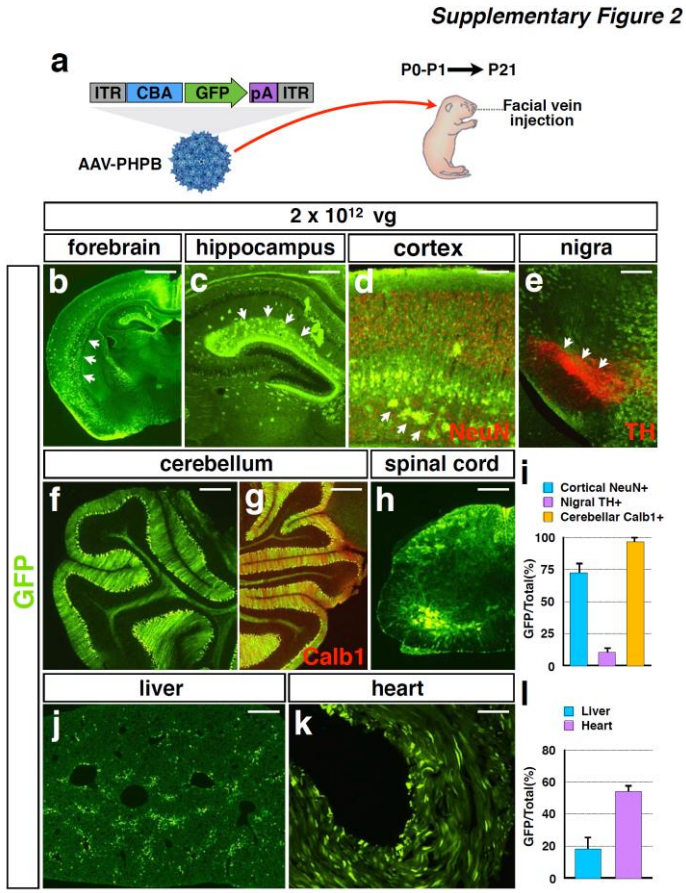
The results were analyzed with GraphPad Prism version 6.0c for Macintosh. Unpaired Student's *t*-test or two-way ANOVA followed by Bonferroni's post-tests, was used in the datasets to be analyzed.

Supplementary Figure 1



Supplementary Figure 1. Intravenous delivery of AAV-PHP.B elicits a diffuse and efficient transduction of the adult central and peripheral nervous system.

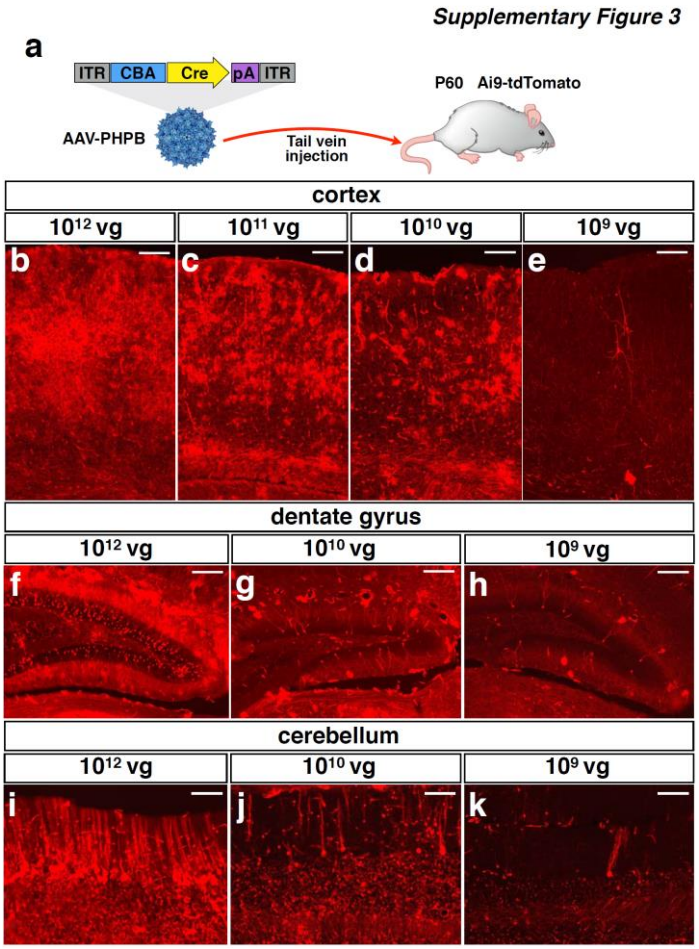
A. Schematic view depicting the transgenic cassette integrated in the AAV-PHP.B vector and injection of the viral particles into the tail vein of an adult mouse. **B-G**. Representative images of GFP immunofluoresce on coronal sections of forebrain (**B,E**), cortical tissue (**C**), thoracic spinal cord (**D**), cerebellum (**F**) and dorsal root ganglia (DRG) (**G**) 3 weeks after *in vivo* transduction. (n = 12 mice). Scale bars: 500 µm (B,E,F); 100 µm (D,G); 50 µm (C).



Supplementary Figure 2. Injection of the AAV-PHP.B into the facial vein of neonatal mice promotes an efficient and global transduction of the neuraxis.

A. Schematic view depicting the transgenic cassette integrated in the AAV-PHP.B vector and injection of the viral particles into the facial vein in a neonatal mouse. **B-H.** GFP localization on coronal sections of forebrain (**B**), hippocampus (**C**), cortical tissue (**D**), substantia nigra (**E**), cerebellum (**F,G**) and spinal cord (**H**). **D**,**E**,**G**. Co-staining between GFP and the neuronal markers NeuN (**D**), TH (**E**) and Calb1 (**G**). **I.** Bar graph showing the fraction of cells positive for a specific

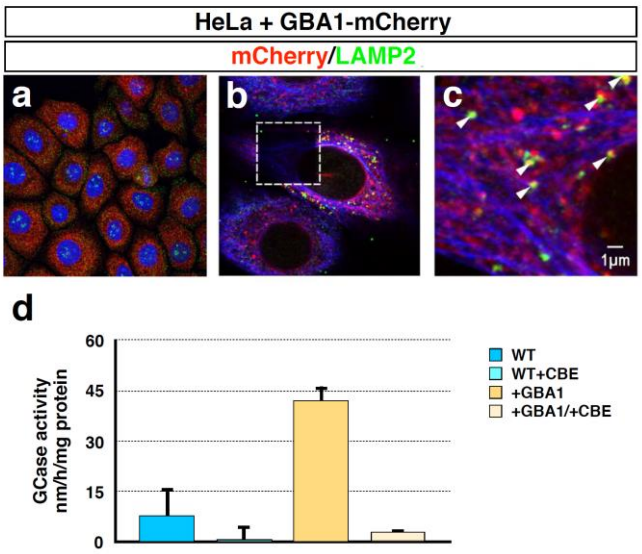
neuronal marker (NeuN, TH and Calb1) and expressing the viral GFP transgene. **J,K.** GFP staining in liver and heart peripheral organs. **L.** Bar graph showing the fraction GFP transduced cells in liver and heart. (n = 6 neonatal pups). Scale bars: 500 µm (B); 200 µm (F-H,J); 100 µm (C-E,K).



Supplementary Figure 3. Low-dosed AAV-PHP.B virus enables spare and single neuronal labeling throughout the brain.

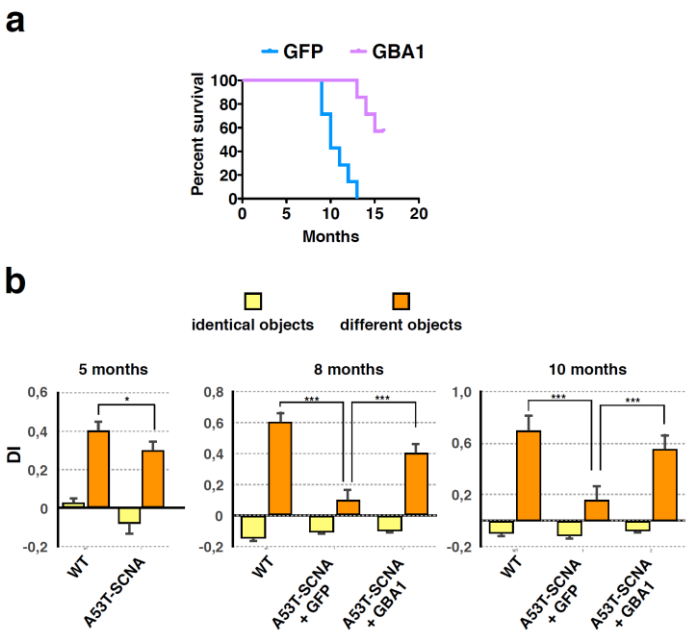
A. Schematic view depicting the transgenic cassette constitutively expressing the Cre recombinase and integrated in the AAV-PHP.B vector employed for injection of the viral particles into the tail vein of an adult Ai9 tdTomato reporter mouse. **B-K.** tdTomato immunofluorescence on coronal sections of cortex (**B-E**), hippocampus (**F-H**) and cerebellum (**I-K**) of Ai9 mice transduced with the Cre-expressing AAV-PHP.B at three (dentate gyrus and cerebellum) or four (cortex) different doses. Note that at lowest dose (10^9 vg) of AAV-PHP.B-Cre, the viral transduction targeted few sparse neurons in these tissues enabling a close morphological inspection of the infected neurons. (n = 3 mice for each viral dose). Scale bars: 100 µm.

Supplementary Figure 4



Supplementary Figure 4. Exogenous GCase is targeted to the lysosomes and acquires functional activity. A. Confocal image of HeLa cells transfected with a GBA1-mCherry expression vector show partial co-labelling between mCherry and LAMP2 (arrows) indicating correct targeting of the exogenous GCase to the lysosomes. **B**. GCase enzymatic activity assay show a substantial increase in total catalytic activity in the GBA1-mCherry transfected as compared to wild-type (WT) cells. The GCase selective inhibitor conduritol-B-epoxide (CBE) was included to evaluate the specificity of the reaction. **C**. Immunoblotting for GCase protein levels in wild-type and GBA1 transfected HeLa cells. Arrowheads point to the faint, but specific, GCase protein signal detectable in wild-type cells. Scale bar: 10 µm

Supplementary Figure 5



Supplementary Figure 5. GBA1 treated A53T-SCNA mice showed a significant extended survival and behavioral improvement.

A. Kaplan–Meier survival curves of GFP and GBA1 treated A53T-SCNA mice showing a significant increase in lifespan and rescue from mortality after global GBA1 gene transduction. **B**. In the novel object recognition test, GBA1 treated A53T-SCNA mice show a significant recovery in learning and memory as revealed by an increase discrimination index (DI) indicating a significantly higher exploration time for the novel object as compared to control GFP transduced A53T-SCNA mice (n = 6 wild-type; n = 8 GBA1 treated A53T-SCNA; n = 8 GFPtreated A53T-SCNA; tests at 3 and 5 months after gene transduction; * < 0.05, *** < 0.001)

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Chapter 3

Conclusions and future perspective

After their discover in the middle of 1960s[1,2], AAVs have been underappreciated by virologists community, because of their replication deficiency and absence of pathogenicity[3]. About twenty years later the first report, AAVs were re-evaluated for their ability to transduce eukaryotic cells, with the AAV-based first clinical trial reported 1996[4]. The AAV vector strengths are numerous and include the flexibility of use in several biological contexts, the low immunogenicity and reduced integration properties[5]. Furthermore, the continuous development of new variants allows to expand the roster of available recombinant AAVs with new unique properties[6]. Remarkably, the targeted selection method allows the genesis of new AAV variants, positively selected for a specific aim[7].

The delivering of drugs, including gene therapy vectors, in the central nervous system represented one of the most challenging topics in neurobiology[8]. The

AAV-PHP.B vector is a variant of AAV9 and was selected for its improved ability to cross adult Blood Brain Barrier (BBB) in vivo[9]; do date it serves as one of the best example of targeted selection method for the generation of AAV variants, and with this strategy other effective vectors were generated[9,10]. Compared to AAV9, whose transduction efficiency in neurons declines with age[11], AAV-PHP.B extensively transduces neuronal cells in a highly reproducible manner, when injected in adult animal models[9,12]. Other newly generated vectors showed enhanced efficiency for this aim. Although comparative studies of the capsid variants are not reported, AAV-PHP.B and its heir, are decisively the best option for brain targeting after systemic administration. This success is mainly due to the capsid selection method (CREATE), which allows to pick capsid variants able to transduce specific Cre recombinase expressing cells[9,10,13]. Furthermore, the same AAV-PHP.B was subsequently reselected, and currently, a new vector descending from it, AAV-PHP.eB showed close but enhanced properties in transduction[10].

AAV-PHP.B applications in neuroscience

This type of brain targeting is a useful strategy for divergent applications in neuroscience including disease-modelling or neuronal activity modulation. This is the case of tuberous sclerosis which is a pathology affecting multiple organs in particular brain and kidney[14]. Mutations of TSC1 and TSC2 genes are responsible of the disease. These two genes encode for a complex inhibitor of mTOR pathway. It is known that patients affected by Tuberous Sclerosis manifest epileptic events that is thought to depend on the presents of and cortical tubers[15], which are benign lesions generated during embryogenesis[16]. In our mouse model, the systemic injection of AAV-PHP.B carrying Cre recombinase allowed the ablation of TSC1 gene in a conditional-like manner. Adult $TSC1^{flox/flox}$ mice injected with AAV-PHP.B-Cre showed the onset of epileptic events starting from three weeks after injection without presence of lesions. Thus, is reasonable to think to a specific function of TSC1 in the control of neuronal activity. Conditional

interneuron specific TSC1 knock out animals showed impairment in development of interneurons and a higher susceptibility to pharmacological epilepsy[17]. Our model however, showed onset of epilepsy in adulthood, without the chance of embryonic lesions or interneuron migration impairment. These evidences highlight a possible and undescribed key role of TSC1 in epilepsy and neuronal function, beyond the inhibition role in mTOR complex, during development.

Although this model is considerable less accurate then typical transgenic models, it allows a selective knock out of a gene of interest in the brain, without crossbreeds which can be time consuming. Moreover, this method resembles tamoxifen-inducible knock out strategy, but it is definitively less toxic then the hormone and requires a single injection[18]. Furthermore, instead of Cre recombinase, a similar strategy can be adopted with Doxycycline-inducible gene systems, to have transient and reversible gene expression[19].

Another interesting application is the possibility to target specific neurons and modulate their activity directly in the brain. This intent can be achieved with the use of transgenic animals or specific promoters[20]. In addition, DREADD receptors are modified proteins which can activated only by clozapine-N-oxide (CNO) administration[21]. This classes of protein are suitable for chemogenetic[22] application and can be expressed in specific manner. In our set up, we use an interneuron specific transgenic mouse, Parvalbumin-Cre, to selectively express DREADD-hM4Di in this neuronal population.

The use of AAVs in our model allowed the target of almost all the Parvalbumin^{+} GABAergic neurons. The overexpression of DREADD-hM4Di receptor, leaded to acute hypoactivation of Parva^{+} interneurons. As result treated mice showed an altered EEG recording and a higher susceptibly to kainic acid treatment compared to controls. Modulation of neuronal populations is an attractive way to prevent or enhance excitotoxicity and neuronal cell death[23,24]. Thus, the use of DREADD receptors can be useful in tuning

neuronal firing, even for therapeutic purposes. Modulation of specific neurons is a novel application that can actively contribute to the study of neuronal activity and its role in behaviour and diseases.

Of note, many drugs and system delivery through the Blood Brain Barrier[25], can cause vascular damage. The same effect is reported for pathogenic viruses[26]. For these vectors no BBB impairment was detected. AAVs are described to interact in a serotype specific manner with surface proteins and can be internalized in the cells through an endocytic pathway[27]. Probably AAV.PHP.B crosses adult BBB through transcytosis[28], but the mechanisms involved are not fully characterized. We propose a combinatorial model consisting in multiple events:

AAV-PHP.B contacts selectively cerebral endothelial cells surface proteins in the apical membrane (Blood facing); subsequently it crosses ECs to the basal membrane (Brain facing), and finally reach glial cells and neurons as final destination. Other pathways can explain this tropism, as other AAVs can target the

nervous system through retrograde/anterograde axonal transport from peripheral organs[29].

Our model, however is supported by various observations. Curiously, AAV-PHP.B is able to transduce weakly brain endothelial cells, where 24 hours after intravenous injection capsid proteins can be detected[9]. Moreover, the PHP.B capsid mutation is in VP1 sequence and can somehow regulate endosome trafficking and escape from nuclear targeting or degradation in brain endothelial cells[30]. Finally, the neural transduction pattern is extraordinarily reproducible and not consistent with axonal transport, since the transduction is not limited to ascending and/or descending pathways while seems to be enriched in areas crossed by the major brain arteries[31]. Future studies will shed light on the mechanism of AAV transport through BBB, identifying new opportunities for pharmacological and genetic therapies for brain disorders.

AAV-PHP.B for human gene therapy

Although it represents the best way to target nervous system through systemic administration, AAV-PHP.B was positively selected in mice, and a subsequent work demonstrated the reproducibility of results and application in other rodent models. Many differences have been found in mouse and human Blood Brain Barrier[32], that can raise doubts on the efficacy of transduction in adult humans. Thus, the tropism in other animal models should be tested. Large mammals, including non-human primates, represent an important model for validation of AAV, since is possible to perform long term longitudinal studies and are more similar in size[33].

However, the use of human cells for AAV validation is definitely more reliable for translational application. Human BBB can be modelled in vitro by the use of physical barrier like transwell assay[34] coupled with cell lines, like hcMEC/D3 cells[35] or human brain vascular endothelium derived from induced pluripotent stem cells (iPS)[32]. Other studies using human models, including endothelial cells[36] and possibly vascularized

brain organoids[37] will help to validate the use of these vectors for human patients.

Certainly, the most important AAV-PHP.B application is the gene therapy. Many CNS diseases are due to specific mutations that cause brain impairment. The single systemic injection is a simple and definitely non-invasive strategy to be used in patients.

In theory, clinical studies using AAV-PHP.B can be designed for virtually all CNS disorders, but there are additional caveats to general AAV drawbacks. Not all the animal models perfectly recapitulate the human diseases. Generally, the animal models used are transgenic mice. Although they share with humans many important similarities, the majority of animal models often reproduce only some aspects of the human diseases; alternatively, they might be more severely affected than human cases, or have no clinical phenotype at all[38]. The availability of animal models can be an obstacle for testing rescue events in vivo.

Another caution regards brain vasculature, since many CNS diseases can damage BBB structure[39], is

reasonable to think that the passage of AAV vectors can be altered.

Extensive changes in BBB molecular structure can occur fallowing the onset of diseases, leading to dysregulation of surface molecules and transport pathways[39]. Many others rAAVs have shown specific interactions with the cerebral blood vessels: AAV-BR1 was shown to target selectively healthy endothelial cells in the CNS in adult mice[40], while AAV8 variants were positively selected to cross BBB only in damaged sites due epileptic foci[41].

In general, in case of BBB alteration, should be recommended to perform pilot analysis of brain transduction by AAV-PHP.B, or as alternative to design a clinic application in a pre-symptomatic phase.

Gene therapy in Parkinson Disease represent an important testing ground for brain gene therapy[42]. The neuronal degeneration is well localized in the Substantia Nigra, and many genetic causes, and risk factors, of the disease have been identified contributing to comprehend the pathogenetic mechanisms[43]. Unfortunately, the α-Syn aggregates can spread

throughout the brain, in a prion-like fashion, leading additional neurological complications, including dementia[44]. In this scenario, all the tested gene therapy strategies for PD failed in restore effectively neurological functions[45].

Many studies reported the crosslink between α-synuclein and GCase, identifying the encoding gene GBA1 as a potential therapeutic target for PD and Synucleinopathy[46]. GBA1 heterozygous mutations are an important risk factor for PD, while homozygous mutations of GBA1 cause the Gaucher disease, a lysosomal storage disorder, affecting the brain in the more severe cases[47].

Our result showed for the first time that systemic injection of therapeutic AAVs, in a PD preclinical model restored the brain pathology, with a clear reduction of Lewy bodies and increased survival. To further validate and increase the consistency of results, we plan to use a second synuclein model, due to directly mutation of GBA1 gene[48]. These mice show the formation of Lewy bodies four months after birth, and are associated with memory impairment[49]. This

additional model will contribute to test therapeutic action of GBA1 in a large period, including pre- and post-symptomatic interventions.

This GBA1 based strategy could be useful not only for PD and synucleinopathies but also for Neuropathic Gaucher's disease, which is the most severe type, with no effective pharmacological treatment and bad prognosis[50] .

AAV-PHP.B and its descendants are helpful tools easily adaptable for many CNS disorders, and can lead the way for the use of therapeutic AAVs and gene therapy in the nervous system in adult patients. Further studies will validate the effectiveness of employment for Parkinson disease and other CNS pathologies before the use for patients.

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