**Commentary article (Invited)**

**Title**

Gene therapy in the CNS - One size does not fit all

Running Title: Gene therapy in the CNS

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Gene therapy in the central nervous system (CNS) has taken centre stage in the past two decades due to significant advancements in lentiviral (LV) and adeno-associated viral (AAVs) technologies. Viral vectors have been utilised for a variety of applications from labelling axons for fluorescent tract tracing to treatment-based strategies such as re-expression of missing proteins. Many viral vectors can retrogradely or anterogradely transport within axons giving rise to neuronal/axonal tract tracing approaches. Rabies virus takes this ‘technology’ one step further giving the user the ability to transynaptically trace neurons/axons to study neuronal connections. The field is continuously evolving with cutting-edge technologies such as optogenetics leading to modulation of gene expression in real time1, inducible viral vectors such as those able to evade the immune system2 and engineered AAV capsids able to cross the blood brain barrier (BBB)3, 4, 5.

Despite the numerous advantages available for gene therapy, fundamental questions still plague this area of research, specifically how best to optimise transduction efficiency in target cells. Research has shown that transduction efficiency varies widely depending on the target cell type, particularly whether dividing or non-dividing, with AAVs being the gold standard for use in the latter. This potential variability has led to the design and optimisation of viral vectors specific for an experimental paradigm that may not equate to efficient expression in other, even similar, cell types within the nervous system. Whilst the field has excelled with potential therapeutic applications, many viral vector studies have instead focused on improving methodology to efficiently transduce specific neuronal subtypes. Although researchers may be looking to modify gene expression within all cell types within the nervous system, neuronal transduction has received significant attention. Therefore, neuronal transduction provides an excellent platform to examine important factors which can be applied to other cells and systems. Some of the main considerations for increasing neuronal transduction efficiency include: 1) viral serotype (for AAVs); 2) promoter type; and 3) method of administering/delivering the virus to the target cell. In addition, a fourth factor should also be included when considering transduction efficiency which includes the experimental species used in these studies, whereas some researchers have noted significant variability in transduction efficiency amongst different species.

In neurodegenerative diseases as well as traumatic injuries such as spinal cord injury, neurons are susceptible to death or damage leading to a significant need for therapeutic intervention. For many years, AAV serotype 2 (AAV2) was the standard serotype used in the CNS as it was the first to be used successfully for transduction in the brain providing an advantageous alternative over immunogenic viral vectors such as adenoviral and herpes simplex viral vectors6. As such, AAV2 was extensively characterised for cellular tropism in neurons7. Since those initial studies, several serotypes have been characterised and tested for neuronal tropism as well as overall transduction efficiency, including but not limited to AAV1, AAV4, AAV5, AAV6, AAV8, AAV9, and AAVDJ. Most results from serotype testing studies have highlighted that neuronal subtypes respond differently to viral transduction making serotype an important consideration when designing CNS gene therapy experiments. Serotype testing studies have mainly used direct vector injection to target a specific neuronal populations with many examining a panel of AAV serotypes (such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV8) with some including lentivirus for comparison. These studies commonly monitor transduction efficiency using GFP-expressing AAVs followed by quantification of GFP-expressing neurons. For example when AAVs (as well as LV) were injected directly into rodent dorsal root ganglia (DRG), a type of sensory neuron, Mason and colleagues demonstrated that the AAV5 serotype transduced the largest number of DRG neurons over a sustained time period (12 weeks)8. This was a key finding for *in vivo* based gene therapy as LV is the optimal vector for *in vitro* based assays on DRG neurons, however it did not result in significant DRG transduction *in vivo*. AAV5 (and AAV8) were subsequently confirmed as optimal serotypes for DRG neuron transduction by another research group using intrathecal administration, an indirect and less invasive approach of accessing the neuronal population9. With another sensory neuron type, retinal ganglion cells (RGCs), a study revealed AAV2 and AAV6 serotypes performed optimally to transduce the largest number of cells, as opposed to AAV5 which resulted in the least neuronal transduction10. Further differences were found when motor neurons were examined. Hutson and colleagues demonstrated that AAV1 was the optimal serotype for corticospinal neurons11 whose results were comparable to a study of red nucleus neurons which also responded best to the AAV1 serotype, with the largest number of transduced neurons at immediate and sustained time periods12.

Many studies including those described above have reported that non-neuronal cells, commonly glia, can be transduced with AAVs making promoter type an important consideration for targeted therapies in the CNS. Significant progress has been made to develop cell-specific promoters for AAVs to target specific cell types, such as the glial fibrillary acidic protein (GFAP) promoter for targeting astrocytes, and the myelin basic protein (MBP) promoter for targeting oligodendrocytes13. As for neurons, general promoters such as human CMV cytomegalovirus (hCMV) or short CMV early enhancer/chicken  actin (sCAG) transduce neurons however with less selectively when compared to neuron-specific promoters such as human synapsin (hSYN)14. Nieuwenhuis and colleagues recently addressed this issue using AAV1 transduction of corticospinal (CS) neurons15. They demonstrated a stark difference of CS neuronal transduction based on promoter type comparing hCMV, sCAG, hSYN, and mouse phosphoglycerate kinase (mPGK)15. Their findings show that hSYN is the optimal promoter to drive high and sustained neuronal specific expression of CS neurons however, the mPGK promoter led to high levels of CS neuronal transduction with a small amount of oligodendrocyte transduction, with sCAG and hCMV lagging far behind in neuronal expression levels15. There are no CS neuron-specific promoters, as is the case for several neuronal populations however, hSYN appears to circumvent this issue especially when using the optimal serotype and direct injection.

Route of administration is a third consideration in CNS gene therapy. Direct injection into the brain/spinal cord/nerve parenchyma is a commonly used approach for *in vivo* experiments. Direct injection has the benefit of targeting specific neuronal subpopulations with less off-target neuronal transduction, however its disadvantages include injuring injected tissue and being less effective in gene targeting across (large) functional groups of neurons. As the goal of gene therapy is eventual clinical application, less invasive approaches that provide a larger distribution profile of transduced cells may be required such as in treating conditions that affect multiple sites in the nervous system. Pietersz and colleagues addressed this in a recent study where they examined transduced neurons in adult rats following AAV5-GFP application using two direct approaches, intrastriatal (IStr) and intrathalamic (ITH), as well as two indirect approaches, intracerebroventricular (ICV) and intrathecal (IT)16. The direct methods, IStr and ITH injections, led to concentrated neuronal transduction within the injected area, striatum or thalamus, respectively16. Both injection routes also led to viral spread and neuronal transduction in surrounding tissues, which others have reported, and in this case led to transduction in the nearby cortex, hippocampus, with ITH injections showing transduction in cerebellar and brainstem neurons. On the other hand, indirect injections resulted in more widely distributed expression found localised to more superficial or surface levels within the tissue. Their results showed the most extensive and widespread transduction was found with ICV injections16. Interestingly, the ICV injections led to nearly complete neuronal transduction in the hippocampus, leading the authors to suggest this as a potential route for AAV-based therapies treating Alzheimer’s Disease. In contrast, the IT injection, performed at lumbar spinal level (L3), led to more localised neuronal transduction in the lower spinal cord and DRGs16. The authors discuss the benefit of cerebrospinal fluid flow which likely led to an enhanced distribution of virus in the ICV group compared to the IT group which was sequestered near to the injection. Bailey and colleagues observed comparable results following AAV9 administration via the intracisterna magna and intrathecal (L4/5) routes in mice17, together asserting that indirect injections are the optimal route for diffuse transduction in the nervous system. On the other hand, if any injection into the CNS can be avoided, this may prove advantageous to clinical gene therapy. Normally the BBB prevents AAV entry into the CNS if introduced systemically, however Gradinaru and colleagues have engineered AAV variants (AAV-PHP.eB and AAV.CAP-B10) that pass through the BBB after intravenous injection in mouse and marmoset3, 4, 5. These variants currently have shown widespread neuronal, and in some cases glial transduction. On the other hand, if these engineered AAV variants were paired with cell-specific promoters, there exists a capacity for non-invasive cell-specific neuronal transduction.

An often overlooked consideration for gene therapy is with the experimental species used. Many researchers consistently use the same species in their experiments, therefore it may not be appreciated that transduction efficiency may vary across species. However, rodent species as well as strain18 can also affect viral transduction levels as cells and tissues may respond differently to viral vector administration. Some have done comparison studies between rodents and non-human primates, and while similarities are observed, there are yet differences in transduction efficiency and cellular tropism in some cases19, 20. Clinically, this is an important consideration when therapies are developed in rodent models, but require translation into higher order species such as non-human primates and eventually humans.

Understanding the limitations as well as how to best optimise viral vector design is vital to guide researchers and potential clinical studies in CNS gene therapy. With advancements in viral vector design, users are inundated with options for experimental design. The above considerations are by no means an exhaustive list. Researchers must also consider further factors such as size of the gene of interest, which may be constrained by the packaging capacity of the viral vector. One thing is clear however, for use of viral vectors in the nervous system, one size does not fit all.

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