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„Generation of a Virus Library by Simultaneous Co-Packaging of Individual AAV-Vectors“

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Christian Hüttmay BSc

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Dr. Simon Rumpel

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1 Zusammenfassung

Die Neurowissenschaft ist ein weites und interdisziplinäres Feld, welches das Nervensystem in molekularen, zellulären, strukturellen, funktionellen, entwicklungsbiologischen, evolutionären, medizinischen als auch mathematischen Aspekten zu beschreiben versucht, wobei große Bedeutung insbesondere der Vernetzung einzelner Neurone und deren Interaktionen zukommt. Um kausale Zusammenhänge zwischen der Struktur und Funktion einzelner Neurone und höherer Gehirnfunktionen wie dem Verhalten herzustellen, ist die spezifische Manipulation definierter Elemente in eben diesen neuronalen Netzwerken unvermeidlich.

In den vergangenen Jahren hat sich hierbei der Einsatz von viralen Vektoren als Standardwerkzeug für die Transduktion von Neuronen in murinen Systemen etabliert und dabei seine Fähigkeit unter Beweis gestellt, dies auch in einer räumlich und zeitlich definierbaren Art und Weise zu vermögen.

Einer der beliebtesten viralen Vektoren ist wegen seiner äußerst niedrigen Pathogenität und seiner Fähigkeit auch nicht teilende Zellen zu infizieren, der rekombinante Adeno-Assoziierten Virus (rAAV). Die Produktion solcher Vektoren ist jedoch immer noch sehr Kost- und Zeitintensiv und viele Anwendungen benötigen die gleichzeitige Transduktion einer Zelle mit mehreren Transgenen, besonders in Anbetracht AAVs stark limitierter Verpackungskapazität von nur rund 4.5 kbps.

In meiner These versuchte ich deshalb 10 unterschiedliche rAAV Vektoren parallel in einem Schritt zu verpacken und dessen Effizienz, sowie das Expressionslevel jedes einzelnen Virus in vivo, mit Hilfe von Deep Sequencing und qPCR zu untersuchen. Im Zuge dessen wollte ich zudem das Potential eines zeitgleichen Verpackens ganzer Gen-Bibliotheken demonstrieren, indem ich versuchte mit Hilfe einer kleinen 10 Vektoren Bibliothek eine Screening Methode für Signalsequenzen, die für den axonalen Transport bestimmter mRNAs verantwortlich sind, zu entwickeln. Hierzu verwendete ich 10 unterschiedliche Lo-

kalisationssequenzen in der 3'UTR der letztlich exprimierten messenger RNAs, deren Fähigkeit zur Induktion eines axonal gezielten mRNA Transports entweder bereits in der Literatur bestätigt werden konnte oder deren transport-inerter Charakter als Negativkontrolle jeglichen axonalen Transport der mRNA verhindern sollte.

Ich fand heraus, dass die gleichzeitige Verpackung multipler rAAVs eine durchaus valide Methode ist, die zu einer ausgewogenen Expression aller seiner Komponenten führt. Somit ist ein Multiplexverfahren in der Untersuchung unterschiedlicher Transgene oder Transkriptions-Elemente möglich, welches wiederum zu einer Einsparung von Zeit- als auch Manufaktorkosten in den Experimenten führt und somit großes Potential für die Zukunft zeigt. Ein mit Hilfe dessen verwirklichter Assay für die Suche nach mRNA Transportsequenzen bedarf jedoch an Überarbeitung.

2 Abstract

The Neurosciences are a broad and interdisciplinary field of study that try to characterize the nervous system in molecular, cellular, structural, functional, developmental, evolutionary, medical and mathematical terms; whereby particular importance is placed on the interconnectivity of individual neurons and their specific interactions. In order to establish a causal relationship between the structure and function of a neuron on one hand and higher order brain functions such as behavior on the other, the specific targeting and manipulation of defined elements within those circuits is absolutely essential.

In recent years, the application of viruses has herein established itself as the customary tool for the transduction of neurons in the rodent brain and proven successful to do so in a spatially and temporally defined manner. Due to its very low immunogenicity and ability to also infect quiescent cells the Adeno-Associated Virus (AAV) has especially proven useful for the in-vivo application in neurons but the production of individual viruses is still cost and time-intensive and many applications call for the simultaneous transduction with various constructs, especially considering AAVs very limited packaging capacity of only about 4.5 kbps.

In the course of my thesis I thereby tried to co-package 10 different rAAV vectors in parallel and characterize the efficiency of packaging as well as their individual expression levels in vivo using both deep sequencing and qPCR. At the same time, I wanted to demonstrate the potential of co-packaging whole libraries by using my small 10 vector library to attempt and establish a novel screen for signaling sequences within mRNAs responsible for axonal targeting. For this purpose, I used 10 distinct localization sequences within the 3'UTRs of certain messenger RNAs, whose ability to facilitate axonal targeted transport has either already been shown in the literature or whose transport inert character would prevent any axonal targeting as part of the negative control.

I found that the co-packaging of multiple rAAVs is a valid method that leads to an even expression of all its components and thereby allows for the multiplexed characterization

of different transgenes or transcription elements, leading to a reduction in time- as well as manufacturing costs and shows great potential for future applications.

The screen for mRNA transport sequences based on these results although is in need of revision and further experiments.

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3 Introduction

The Neurosciences try to answer a broad array of questions about the organization, the development and the general function of the nervous system which is responsible for generating behaviour. These questions are usually explored with a multitude of tools that it shares with various fields such as genetics and genomics, molecular and cell biology, anatomy and systems physiology, behavioural observation, and psychology. The majority of topics that could be successfully investigated to this point were thereby mostly concerned about how individual cells of the basal subset of the nervous system, namely neurons and glia, perform their functions in anatomical, cellular, electrophysiological as well as molecular terms. (Purves, 2012)

3.1 The Brain

The central nervous system (CNS) is generally considered to be made up of seven different components. Along the caudal/rostral axis those are: the spinal cord, the medulla, the cerebellum, the pons, the midbrain, the diencephalon, and the cerebrum with both its hemispheres. The medulla, pons and midbrain are collectively called the brainstem while the diencephalon and cerebrum are jointly called the forebrain. In between those subdivisions are the so-called ventricles, hollow spaces filled with cerebrospinal fluid. They are in fact remnants of the lumen formed during development, during the rounding of the neural plate into the neural tube (Purves, 2012. chapter: survey of human neuroanatomy)

As shown by the recent advance of imaging techniques and a series of experimental methods, each and every of those regions has a unique task. Regions like the Broca- or Wernicke's area for instance are responsible for the production and understanding of written and spoken language. (Kandel, Schwarz and Jessell, 1996. Chapter: Gehirn und Verhalten)

These regions are found in the cortex cerebri which is divided into two hemispheres, each of which is 2 to 4 millimetres thick and contains about 8 billion neurons in the human (Azevedo et al, 2009). Each of those two hemispheres is again divided into the Lobus frontalis, Lobus parietalis, Lobus occipitalis and Lobus temporalis, all of which perform specialized tasks. To be more exact the Lobus frontalis is mostly concerned with the planning of future events and control of movement, while the Lobus parietalis controls tactile perception and proprioception; the Lobus occipitalis controls vision and the Lobus temporalis auditory perception as well as facets of learning, memory and emotion.

The division of the cortex into two separate hemispheres gives rise to two distinct properties of organization. Firstly, each hemisphere controls primarily the sensory and motoric processes of the contralateral side of the body and secondly, neither hemisphere is completely symmetrical or even performs necessarily the same task (Kandel, Schwarz and Jessell, 1996. Chapter: Gehirn und Verhalten). Although they are connected by a bundle of nerve fibres called the corpus callosum.

A lot of what we know about the human speech centres comes from the study of Aphasias, neurologically induced speech disorders. In the second half of the 19th century numerous noteworthy discoveries were made in this field, most notably by Pierre Paul Broca with the discovery of Brocas area and Carl Wernicke who was responsible for the discovery of Wernickes area. Brocas ground-breaking studies eventually sparked off further research into behaviour regulating areas. In 1870 German physiologist Gustav Fritsch and psychiatrist Eduard Hitzig discovered that electrical stimulation of defined cortical areas induces a characteristic movement of the extremities. Based on these discoveries Wernicke formulated a new theory of speech where he found a middle ground between the two prevailing theories, with the Phrenologists who imagined the cortex as a mosaic of defined functions on one side and the supporters of the theory that the brain as a whole was an aggregate of mental functions with no defined specialized regions on the other. Wernickes theory stated that it's more likely that only the most basic

of functions are localized to one area while more complex higher order tasks would require the collaboration of various brain regions, which made Wernicke the first to develop the concept of distributed processing (Kandel, Schwarz and Jessell, 1996. Chapter: Gehirn und Verhalten).

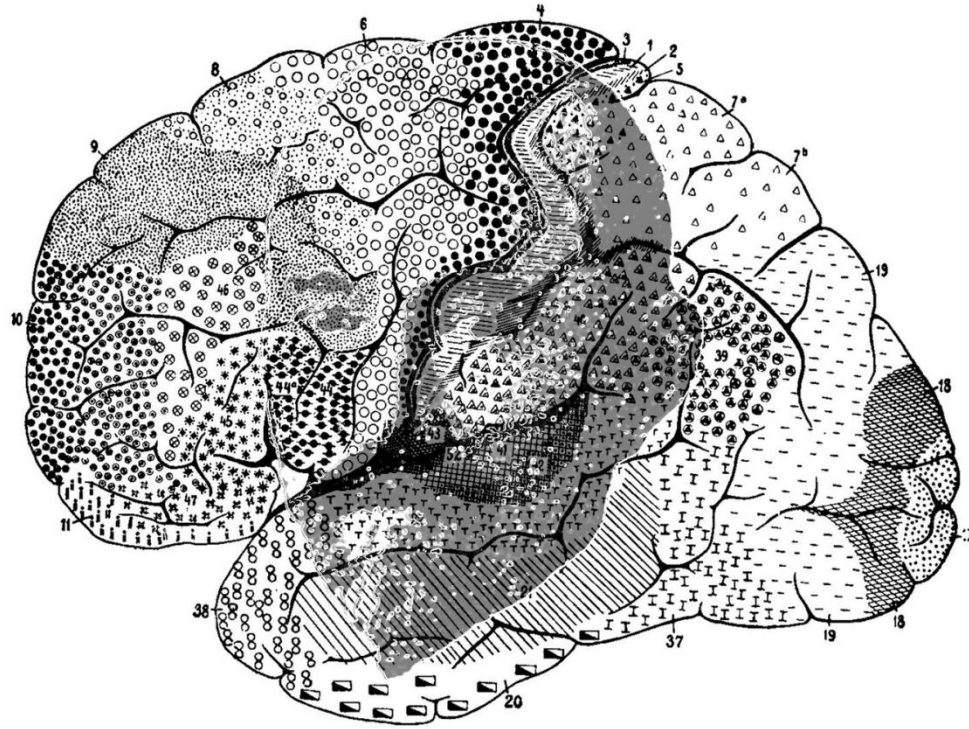


Figure 1: Brodmann's Areas. Brodmann described 52 Areas in the human cortex. This illustration only shows areas that are visible on the outer surface of the cortex. Adapted from *"Portraits of European Neuroscientists"*, by N. Wade, M. Piccolino & A. Simmons, 2012. Retrieved from neuroportraits.eu. Copyright by Nicholas Wade.

At the beginning of the 20th century there emerged a new school of thought concerning cortical localization partially based on Wernickes work. Under leadership of anatomist Korbinian Brodmann they tried to divide the cortex into different layers based on the structure of its cells and their characteristic arrangement. Using these cytoarchitectonic methods Brodmann subdivided the cortex into 52 individual areas (Figure 1).

More and more evidence suggesting localization of certain functions was accumulating over the years and at the end of the 1930s prominent scientists Edgar Adrian, Wade

Marshall and Philip Bard were able to show that tactile impulses could elicit activity in specific cortical regions. Following those experiments many scientists now rigorously examined the concept of architectonic centres and eventually were able to show that cortical regions could indeed be defined by various independent criteria, like cell type and -layering, Input- and Output connections and most importantly physiological function (Kandel, Schwarz and Jessell, 1996. Chapter: Gehirn und Verhalten).

3.2 Neural Circuits

With the development of an increasing amount of sophisticated methods over the years and specifically designed behavioural experiments the scientific community was able to learn a lot about functional localization of behavioural tasks yet there is still a great deal left to discover, this is particularly true for the interconnection and communication between those regions, about how the responsible neural circuits work and what they ultimately compute. In order to reach a deeper understanding of the nervous system and its higher functions, it is therefore imperative to gain a profound understanding of this connectivity and to possess the ability to specifically target and manipulate defined elements in those neural circuits.

Currently there are a variety of methods in use to investigate the relationship between the neuronal structures and functions on one hand and higher order brain functions such as behaviour on the other. There is for example the recently rising field of optogenetics that utilizes the channelrhodopsin-2 system in order to use light impulses for the cell-type specific manipulation of neuronal activity (Alilain & Silver, 2009; Adamantidis, Carter & de Lecea, 2010; Han et al., 2009) as well as a variety of other methods like the manipulation of unique receptors (Nichols & Roth, 2009; Wisden et al., 2009) and ion channels (Hodge, 2009; Holford et al., 2009; White & Peabody, 2009), the reversible blockade of neurotransmission using tetanus toxin (Tessier & Broadie, 2009; White &

Peabody, 2009) or the manipulation via a temperature dependant shibire system in *Drosophila* (Kasuya, Ishimoto & Kitamoto, 2009). Using Cre driven mouse lines many of those techniques can furthermore be used in a region specific and cell-type selective way (Wisden, Williams & Meier, 2009), yet some transgenes might also require a temporal component. To accommodate all those needs many scientists now use viral vectors for the manipulation of neural circuits.

3.3 Viral Vectors

The development of viral vectors has in recent years proven a successful tool to transduce neurons especially of the rodent brain in a spatially and temporally defined manner. One particular strategy to specifically target neurons in a complex tissue consisting of an assortment of different cell-types is thereby to use cell-type specific promoters, whereas all viruses are already inherently modifiable when it comes to the temporal component.

Adenoviral-, retro-/lentiviral-, herpesviral-, and adeno-associated viral vectors are typically the major viral systems used for transduction (Huang & Kamihira, 2013) whereby each system exhibits its characteristic advantages and downsides.

3.3.1 Adenoviral Vectors

All Adenoviruses contain a double-stranded DNA genome of about 36kbps and are encapsidated into an icosahedral protein shell. Group C adenoviruses are able to infect a wide range of host cells and are relatively well investigated, rendering it possible to insert therapeutic genes or remove undesirable viral genes. They are also capable of expressing transgenes directly from their viral chromosome even within non-dividing cells such as cells of the central nervous system, making it an attractive vector candidate in

gene therapy (Kaplitt & Loewy, 1995: Chapter 3). The insert capacity of the viral vector is currently limited to 8 kb of foreign DNA.

3.3.2 Herpesviral Vectors

The HSV contains a 120 to 200 kbp long double-stranded chromosome inside of an icosahedral capsid that is surrounded by an amorphous matrix of proteins named the tegument and enveloped by at least 10 different glycoproteins. In order to replicate the virus usually undergoes a lytic cycle that is typically interrupted during vector design to establish a latent state in the infected neurons. They can infect non-dividing cells and portray a wide range in host cell transfection but show a particular tendency for cells of the central nervous system. It is often chosen as a viral vector for its natural non-pathogenic long term expression within neurons and its large transgene carrying capacity of up to 150 kbp of foreign DNA (Kaplitt & Loewy, 1995: Chapter 1; Heister et al., 2002).

3.3.3 Retroviral & Lentiviral Vectors

The genome of a retrovirus consists of a linear single stranded RNA molecule that is between 7 and 12 kbps long and encodes among other things for its characteristic RNA-dependant DNA polymerase, the reverse transcriptase. Retroviruses are an excellent gene delivery system that have the characteristic property of integrating their viral DNA into the host cell genome and are consequently able to not only offer the advantages of highly efficient gene transfer but also a stable expression in the host, where many other vector systems would only allow for transient gene expression (Mulligan, 1993). They can either be replication-competent or replication-defective, and exhibit a wide host range in both cell types and species but require for cells to divide for infection. The integration of the retroviral provirus genome might, while relatively rare, also entail the

disruption of endogenous genes or the activation of cellular proto-oncogenes by accidentally introducing a promoter/enhancer sequence when integrating in their vicinity. Other limitations that retroviral vector systems bring with them are their relatively high mutation rate per replication cycle that could potentially lead to gene inactivation, as well as their fundamental inability to infect non-dividing cells, effectively excluding their application in cells such as neurons (Kaplitt & Loewy, 1995: Chapter 5).

There is one particular subclass of retroviruses that is responsible for an entirely separate class of retroviral vector though, the lentivirus. These vectors are mostly based on the Human Immunodeficiency Virus (HIV) and possess – much in contrast to their relatives – the fundamental ability to integrate their genome not only into dividing but also into non-dividing cells. Consequently, they also allow for the transduction of neurons and do so with long-term expression and limited mutagenesis as compared to the conventional gamma retroviral Vectors (Torashima et al., 2007; Montini et al., 2009).

Both vector types are capable of carrying a medium sized insert of about 8kb.

3.3.4 Adeno-Associated Viral Vectors

The Adeno-Associated Virus genome consists of a 4.7 kb long encapsidated single-stranded DNA molecule of plus or minus polarity. It is a replication-defective member of the parvovirus family that can either undergo site-specific integration into the host cell genome or enter a lytic cycle to propagate. During integration, the virus generally favours a specific site on the human chromosome 19q13.3-qter (Kotin et al., 1989) with only a negligible amount of integrations into random sites, reducing the risk of mutagenesis that is usually associated with retro viruses. This feature that was lost though during its development into a recombinant gene expression vector following the removal of both its AAV Rep gene as well as its AAV Cap gene located between the two 145nt long inverted terminal repeats (ITR) at the termini (Duan et al., 1998). For the AAV

to replicate during a lytic infection the co-infection of either an adeno- or herpes simplex virus is required, hence the classification as replication defective. In the absence of one such helper virus the virion can still enter the nucleus and release its ssDNA though, thereby allowing it to incorporate into the host genome. As the rep gene has been removed in recombinant adeno-associated virus (rAAV) vectors the genome cannot replicate itself and ends up getting converted into dsDNA by the host's own DNA polymerase during a time consuming step. It eventually forms an episomal concatemer in the nucleus which then facilitates stable expression in the infected cell (Kaplitt & Loewy, 1995: Chapter 4). A method to circumvent this late onset of expression was developed by McCarty et al. in 2001 by packaging both strands simultaneously as a single fused DNA molecule. These so called self-complementary AAVs are created by taking a regular AAV construct and appending its compatible inverted repeat sequence by joining them both with a special linker, all at the expense of half its maximally available packaging capacity. This allows the DNA molecules to re-fold into double stranded DNA templates upon uncoating, essentially making the need for the synthesis of a complementary strand by the host cell machinery redundant and thereby considerably increases the efficiency of the whole transduction process (Figure 2). However, it has to be carefully considered that the requirement of an additional, complimentary construct in the resulting Adeno-Associated Virus will lead to a significant reduction in its transgene carrying capacity available for experiments (McCarty et al., 2001; Day et al., 2014).

During virus production two separate vectors are used. The separation of both transgenes and cis-acting elements responsible for packaging and genome amplification on the one hand and trans-acting elements involved in genome replication and virion assembly on the other is thereby quite similar to the retroviral system. Generally, an expression plasmid containing the desired transgenes flanked by the respective AAV ITR sites, and a helper plasmid providing the essential viral rep and cap genes in trans and exclusively during the virus production, are used. They are jointly transfected into a common AAV production cell line such as HEK293, where together they are then able to

interact in a successful virion production. The virus is at a later point purified by a gradient, leaving the final product devoid of any trans-acting elements.

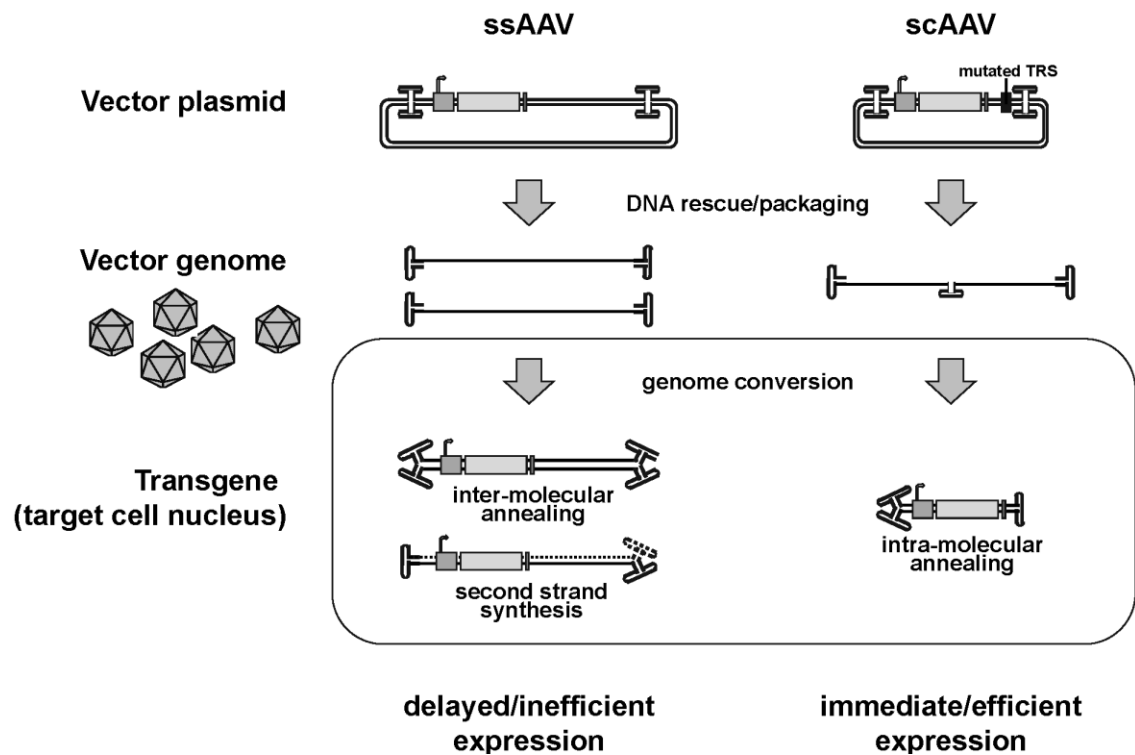


Figure 2: Self-Complementary AAV Vector. Comparison of genome rescue and subsequent genome conversion between single stranded and self-complementary Adeno-Associated Virus Vectors. ssAAVs package either a plus or minus stranded genome copy and rely on the co-transfection and inter-molecular annealing of a complementary strand or on the time consuming process of second strand synthesis in order for gene expression to occur. scAAVs on the other hand have a mutation in their TRS to form a genome with wild-type ITRs at both ends and a mutated ITR in the centre. Although this version of the genome has only have the size, it can readily fold into its double-stranded transcriptionally active form via intra-molecular annealing in the cell nucleus, leading to a more efficient expression. Adapted from “Efficient AAV Vector Production System: Towards Gene Therapy For Duchenne Muscular Dystrophy, Gene Therapy - Tools and Potential Applications” (p.433), by T. Okada, 2013, InTech, DOI: 10.5772/53023. Copyright 2013 by The Author(s).

Yet even though the ability to infect non-dividing cells combined with a stable expression of transgenes, low immunogenicity and lack of insertional mutagenesis make the rAAV one of the most popular expression vectors in neuroscience, there is also a significant weakness that has proven difficult to work around and that is their severely limited packaging capacity of merely 4.5 kbps of recombinant DNA.

To date the only viable options to increase its cargo capacity are either to slightly over-size the rAAV vector with severe loss of transduction efficiency (Grieger et al., 2005) or to make use of the split AAV vector system (Yan et al., 2000; Duan, Yue & Engelhardt, 2001; Ghosh & Duan, 2007) which does, however, require the production and simultaneous delivery of several distinct AAV vectors.

3.4 Co-Transfection with Multiple Vectors

Reaching the limits of rAAVs utilization due to its characteristic size constriction, researchers began to bypass those barriers by splitting their transgenes over multiple AAV vectors and thereby managed to artificially increase the effectively feasible Insert length (Yan et al., 2000; Duan, Yue & Engelhardt, 2001; Ghosh & Duan, 2007).

One such method that compensates for a lack of transgene capacity by directly increasing the overall supportable cargo limit was developed in 2000 by Duan et al. who used an overlapping vector approach. Based on their previous findings that confirmed their believes that the circularization and subsequent concatemer formation of the viral genome is a particularly vital event during transduction and requires recombinatorial events between the ITRs of separate viral genomes, they developed a method that uses two distinct vectors that each carry a different part of the same expression cassette. As a proof of principle they used a construct comprising both a reporter gene, as well as a suitable enhancer and divided those elements onto separate vectors. Provided then that a cell received one of each vectors during transfection this would allow for the two DNA molecules to interact and recombine during the concatemerization process, effectively consolidating the two DNA molecules. With both enhancer and reporter gene now within each other's reach, a positive effect on the reporter signal could be observed. In 2010 they could improve upon the method even further by introducing a highly recombinatorial bridging DNA sequence to both vectors that would then serve as a target

gene independent mediator for the two molecules during homologous recombination, effectively increasing the efficacy of the whole technique (Ghosh, Yue & Duan, 2010). Yet all of these methods that rely on the allocation or splitting of its transgenes over multiple vectors share one decisive drawback, namely that under current practice for each utilized construct a separate virus has to be produced in a laborious and cost intensive procedure. It also has to be considered that for such methods any given batch of AAV cannot be used for experiments until the production of each individual vector is finished and combined in an appropriate ratio.

This led us to the conclusion that a new approach to fundamentally improve this production procedure was in order, that would not only provide a myriad of new possibilities to any scientist employing multiple AAV vectors during transfection but also considerably simplify the simultaneous analysis of transgenes, possibly even in high throughput range.

Such high throughput techniques are already available for the analysis of transgenes in the model organism *Drosophila Melanogaster*. In early 2013 for example Arnold et al. developed a technique to directly and quantitatively characterize the enhancer activity of millions of candidate sequences on a genome-wide level in *Drosophila*.

By utilizing multiple rAAV vectors a genomic screen of this scale might even be attempted in a murine system.

3.5 STARR-Seq

STARR-seq is a method to directly and quantitatively determine the activity of several millions of enhancer candidates from an arbitrary source of DNA. This even makes the screening of entire genomes at once possible as Arnold et al. were able to demonstrate in the model organism *Drosophila Melanogaster*.

Although two other methods that allow for genome wide enhancer prediction were published just recently, they both lack a direct quantitative readout of enhancer strength (Heintzman et al., 2009).

To achieve such a quantitative result Cosmas et al. placed their respective candidate sequences downstream of a minimal promoter in order to cause every activated enhancer to drive its own transcription, whereby their respective abundance among cellular RNA would consequently be reflective of their relative strength (Figure 3). By directly coupling the candidate sequence to the enhancer activity they were thereby able to develop a method that allows the parallel assessment of millions of DNA fragments simultaneously (Cosmas et al., 2013).

This form of regulatory element screening eventually gave us the idea to use our own multiple vector library to implement a small proof of principle experiment for a high throughput screen in parallel but in our case aiming at specific transport sequences responsible for the axonal targeting of messenger RNA such as the Actin Beta Zipcode.

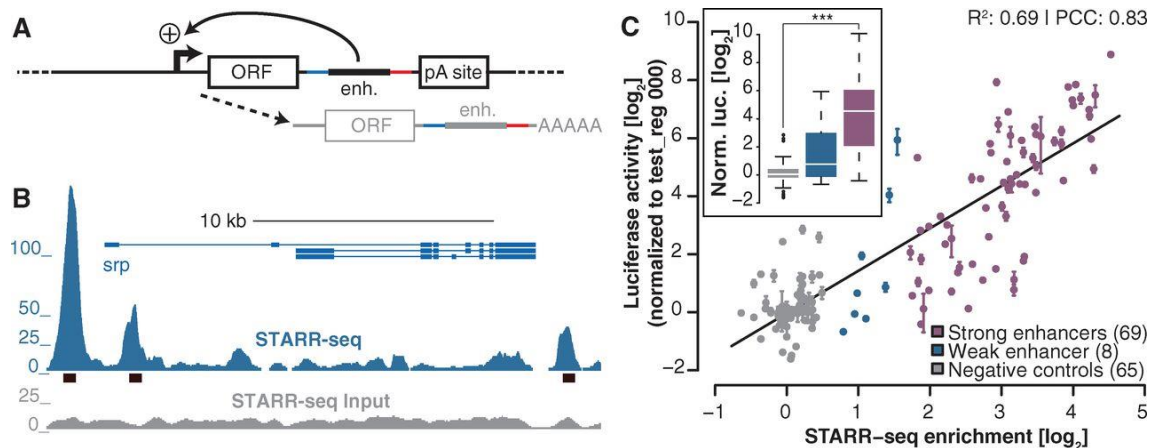


Figure 3: STARR-Seq. STARR-seq genome wide enhancer assay. (A) Reporter setup [+ , transcriptional activation; ORF, open reading frame; enh., enhancer candidate; pA site, polyadenylation site]. (B) STARR-seq (blue) and input (grey) fragment densities in *srp* locus. Black boxes indicate possible enhancers. (C) Linear correlation between STARR-seq and luciferase signals [R², coefficient of determination; PCC, Pearson Correlation Coefficient]. Taken from “Genome-wide quantitative enhancer activity maps identified by STARR-seq” (p. 1074), by Cosmas et al., 2013, New York, NY: American Association for the Advancement of Science. Copyright 2013 by American Association for the Advancement of Science.

3.6 mRNA transport

Messenger RNA consists of three major constituents, the 5'-UTR (untranslated region), the coding region and the 3'-UTR. For the regulation of mRNA concentration and localization within a cell, post-transcriptional regulation is an essential process. It has been shown that signalling sequences involved in such regulatory processes like polyadenylation, degradation and localization are located within the 3'-UTR. The localization of mRNA in particular has been extensively studied in neurons, due to their intrinsic polarity with dendrites on one side and axons on the other. To keep this polarity intact against the force of diffusion the cell uses its microtubular system which shuttles mRNAs and proteins each to their respective regions (Aranda-Abreu et al., 2005). Post transcriptional regulation thereby depends largely on a number of RNA binding proteins (RBPs) that recognize and bind to a number of consensus sequences or structural features which are mainly localized in the 3'-UTR but can occasionally also be found in the 5'-UTR or coding sequence. Many such RBPs for example bind to the primary transcript after transcription and initiate 3'-end cleavage, followed by the addition of a poly(A) tail, thereby not only terminating the transcription process but at the same time also forming the spliceosome, a complex of snRNPs and various proteins that is responsible for the removal of introns and the appropriate joining of exons (Will, Lu, 2011; Liegro, Schiera and Liegro, 2014).

The furthermore now necessary nucleo-cytoplasmic traffic of RNPs through the nuclear pore complex (Figure 4) is accomplished with nuclear export signals present on the RBPs. Upon reaching the cytoplasm, mRNPs are able to bind to motor proteins which are in turn bound to the cytoskeletal filaments and enable the mRNPs to reach their destination in a translationally inactive form. Other notable forms of localization are achieved by either localized protection from degradation or by simple diffusion in combination with local anchoring. The mRNA's localization and stability is thereby also influenced by post-translational modifications such as the phosphorylation of serines/threonines and ubiquitination of lysines, the methylation of arginines/lysines and proline hydroxylation,

as well as SUMOylations and lipidations, all of which lead to different forms of remodeling of the RNP complexes.

The subcellular localization of mRNA and consequentially it's localized translation provides the cell with a variety of advantages: an increased efficacy through the bypassing of a more energy intensive transport of the eventual proteins by shifting translation directly to the site of need; the prevention of potentially unwanted protein interactions by separating the products of translation in advance; and finally as a means for differential translation regulation, with translation of a specific mRNA being restricted to a specific compartment in response to a local signal (Liegro, Schiera and Liegro, 2014).

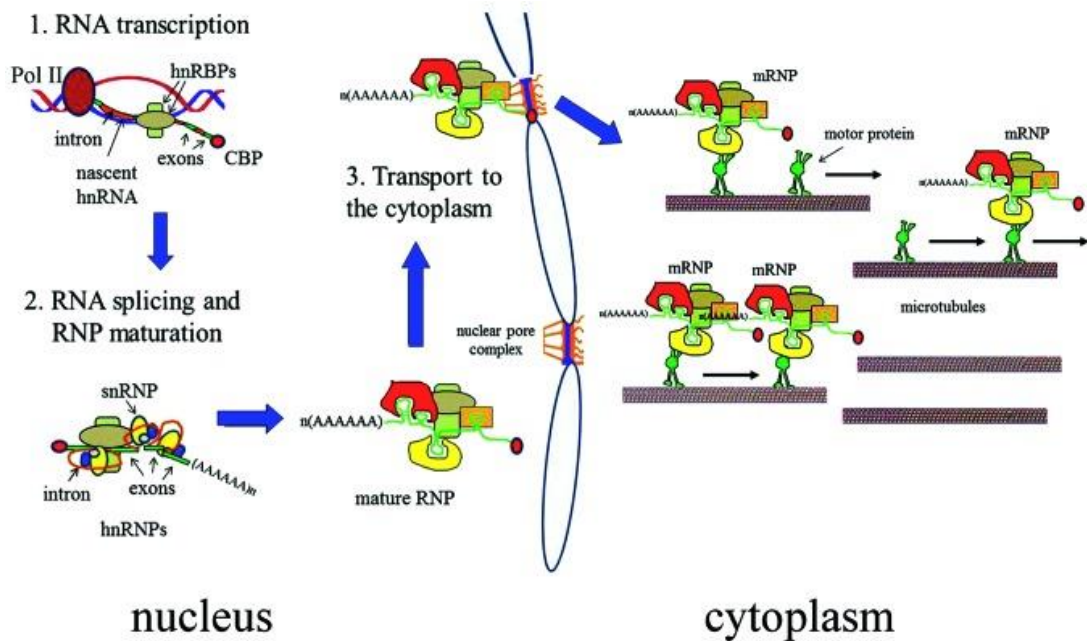


Figure 4: Post-Transcriptional Fate of mRNA. During transcription by Polymerase II (Pol II) the nascent heterogeneous nuclear primary transcript (nascent hnRNA) is combined with RNA-binding proteins (RBPs) and small nuclear ribonucleoproteins (snRNPs) to form the splicosome which in turn removes introns and joins exons, creating mature mRNA in the process. By interacting with proteins of the nuclear pore complex the transport into the cytoplasm is prompted. Having arrived there RNPs attach to cytoplasmic motor proteins that mediate the microtubule-dependant transport of mRNA. Taken from "Regulation of mRNA Transport, Localization and Translation in the Nervous System of Mammals (Review)" (p.749), by Liegro, Schiera & Liegro 2014, Italy: Spandidos Publications. Copyright 2014 by Spandidos Publications UK LTD.

While mRNA transport into dendrites has always been studied quite extensively, the transport into axons was for a long time neglected. With a seemingly insignificant level of translational machinery inside the average axon itself and an already efficient means for anterograde protein transport being present in the cell anyhow, the importance of axonal mRNA transport had long been underestimated (Liegro, Schiera and Liegro, 2014). However, several previous studies were able to show the axonal localization of a number of mRNAs, including β -actin (Willis, 2007) the most prominently studied among them. The β -actin mRNA was thereby found to depend on the recognition of its zipcode by the so called ZBP1 protein in order for it to be localized (Kislauskis, Zhu & Singer, 1994; Sundell & Singer 1990; Welshhans & Bassel, 2011). Axonal mRNA transport has furthermore been reported for β -tubulin (Eng, Lund & Campenot, 1999), tau (Litman et al., 1993; Aronov et al., 2001) calreticulin (Vuppalachchi et al, 2010), oxytocin & vasopressin (Jirikowski, Sanna & Bloom, 1990), the κ opioid receptor (Bi et al., 2006) and the microtubule-associated proteins 1b (MAP1b) (Antar et al., 2006). The number of mRNAs transported into the axon increases even further if external stimuli are applied, such as axonal injury, including thereafter a number of additional transcripts encoding transmembrane receptors and proteins for the translational apparatus (Willis et al., 2007; Liegro, Schiera and Liegro, 2014).

There is still a lot to learn about the localization and functionality of mRNAs but at the same time there is also a lot to gain from this knowledge. The transport of certain mRNAs as well as their regulated translation in neurons e.g. have among other things been shown to be involved in the long-lasting changes related to memory formation, as well as in axonal pathfinding during both development and nerve regeneration (Liegro, Schiera and Liegro, 2014). The ability to screen for the specific mRNAs involved in those processes might subsequently help us shed a new light on these findings.

3.7 Central Question

In the past years the application of viruses has established itself as the standard tool for the transduction of neurons in the rodent brain and proven successful to do so in a spatially and temporally defined manner. The various virus systems used for transduction each have different advantages and downsides; one disadvantage affecting especially the Adeno-Associated Virus (AAV) though is its very limited packaging capacity of about 4.5 kbp.

To date the only viable options to increase cargo capacity though is either slightly oversizing the AAV vector with severe loss of transduction efficiency (Grieger et al., 2005) or using a split AAV vector (Yan et al., 2000; Duan, Yue & Engelhardt, 2001; Ghosh & Duan, 2007) which depends on the delivery of several AAV vectors simultaneously.

However, it has to be considered that by increasing the number of utilized vectors in a single application by merely one already doubles the total expenditure of the necessary high-titre virus production, a procedure that already happens to be very time- and cost-intensive as it is. And it is not the only method that suffers from the work intensity of serial AAV packaging, other instances of applications that depend on the utilization of multiple vectors at once would be methods that rely on e.g. the transduction of more than one subunit at once, an expression in different cell-types using differential promoters, or screens that try to compare varying expression properties for an otherwise identical vector.

I therefore spent the course of my thesis on trying to find a way to improve the serial AAV production by attempting to co-package 10 distinct viruses in parallel and subsequently characterize both the efficiency of the packaging as well as the expression level of each individual virus in vivo, using deep sequencing and qPCR. At the same time I wanted to demonstrate the potential opportunities the parallel packaging of dissimilar viruses would offer by designing my small test-library in a way that would already allow me to try a simple screen for the axonal transport of mRNA. I therefore decided upon

10 different localization signals that the vectors would carry in their 3'UTR, these sequences have either already been shown to facilitate axonal targeting or are simple inert sequences that are expected to have no effect on mRNA Transport, leaving the molecules at the nucleus. By putting the two callosally connected hemispheres of the auditory cortex (ACx) to use in form of a screening device, I wanted to test both the packaging efficiency as well as the axonal mRNA transport in a single experiment.

With this I hypothesised that:

- The co-packaging of 10 distinct viruses has a negligible effect on the total packaging efficiency when using a self-complementary rAAV vector
- The bias introduced during packaging will be negligible to non-existent and all 10 viruses will be expressed with an equal efficiency in vivo
- The endogenous localization signals introduced into the 3'UTR of the vector will show the same localization property for the artificial mRNA
- The expression of this artificial mRNA in one of the hemispheres of the ACx will lead to the contralateral transport into the other.
- No transport will take place to an unconnected region such as the bulbus olfactorius.

3.8 Outline of Experiment

The goal of the experiment was to see if I could simultaneously package a small DNA Library into an AAV Vector and use that vector to test ten different localisation sequences on successful transport of their mRNA.

The individual steps of the experiment can be structured as:

3.8.1 Creating a plasmid library

First an appropriate plasmid was created that contained the following features:

- An Ampenicilin Resistance gene for selection
- Two ITR sites for self-complementary folding of the virus
- A reporter gene (iCRE)
- A human Synapsin Promoter to drive expression of our reporter gene
- A beta globin intron inserted into the reporter gene
- An SV40 PolyA site
- Two Restriction Enzyme Sites (BamHI & KpnI) between the iCRE gene and the PolyA site

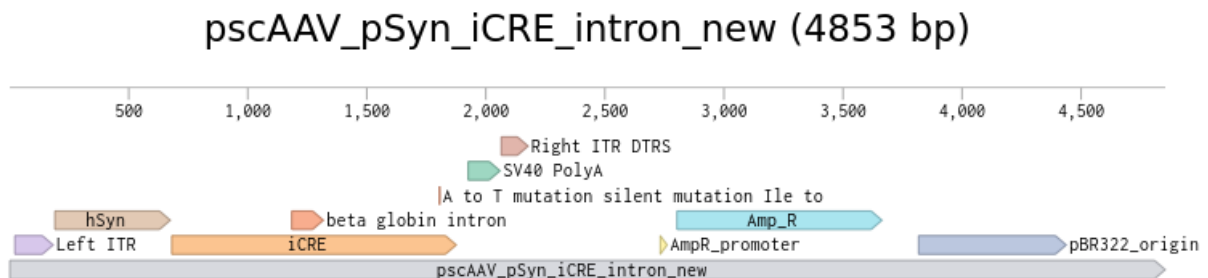


Figure 5: Expression Plasmid for Library Packaging. A self-complementary AAV Expression Plasmid used to create a Library containing 10 different localisation sequences.

Labels from left to right. ITR: Inverted Terminal Repeats, hSyn: human Synapsin Promoter, iCRE: Codon-improved Cre recombinase gene, SV40 PolyA: Simian-Virus 40 Polyadenylation site, ITR DTRS: Inverted Terminal Repeats Direct Terminal Repeats, Amp_R: Ampenicilin Resistance gene, pBR322_origin: origin of replication

The Restriction Enzyme Binding Sites were then used to insert ten different localisation sequences, immediately after the reporter gene and the plasmids were simultaneously packaged in HEK293 cells.

3.8.2 Injection of Virus into Auditory Cortex of adolescent mice

The virus was now injected into the auditory cortex of the right hemisphere of young C57BL/6 mice.

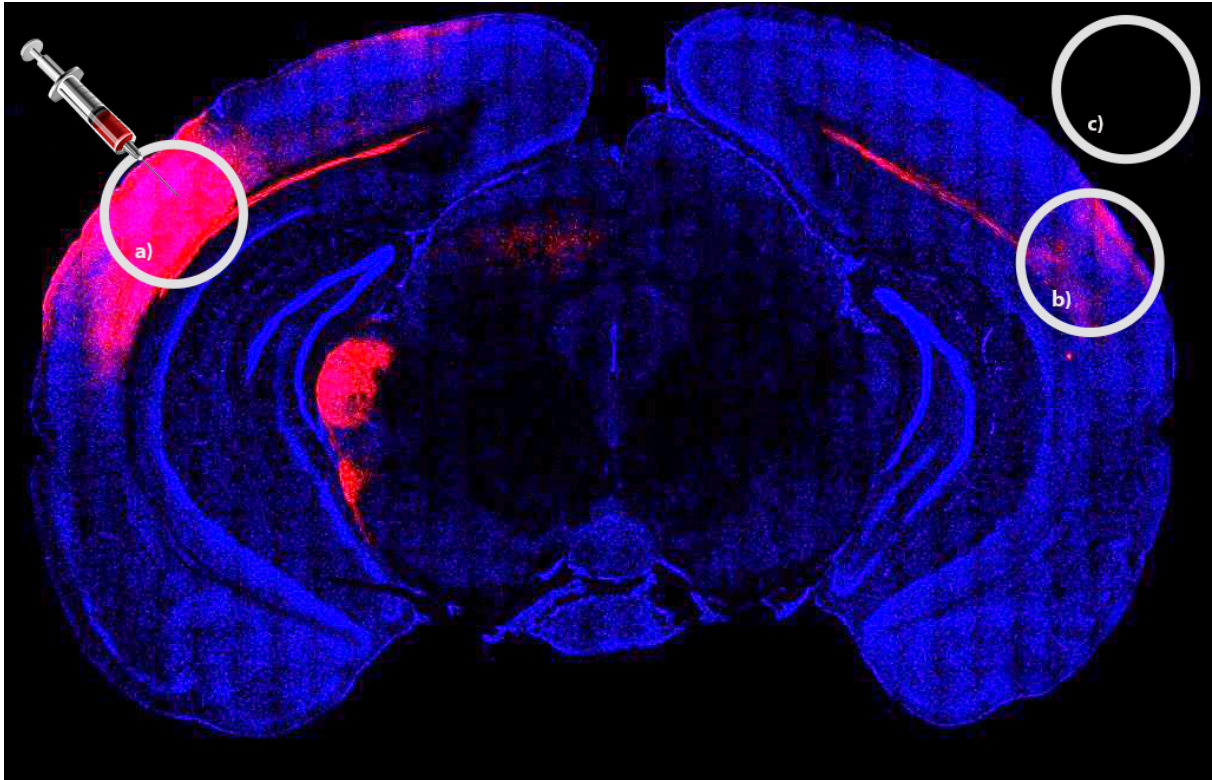


Figure 6: Callosal Connections of the murine auditory cortex. Coronal section of the murine brain, showing an injection into the auditory cortex. Axons project to the contralateral auditory cortex and ipsilateral thalamus. Marker used: tdTomato. White circles indicate samples taken during experiment: a) Ipsilateral site; b) Contralateral site; c) Negative Control (Bulbus Olfactorius)

Since the auditory cortex has axonal connections to its counterpart on the contralateral side of the brain (Figure 6) we can reasonably expect that a successful transsynaptic transport of our expressed mRNA would lead to an increased expression of our reporter gene on the other hemisphere.

3.8.3 Retrieval of mRNA & Sequencing

After an incubation time of 4, 8 and 16 days respectively, the ipsilateral- and contralateral auditory cortex, as well as the unrelated bulbus olfactorius are removed and an mRNA extraction is performed.

As the extracted mRNA has undergone splicing, the betaglobin intron of the iCRE gene will be absent from the cDNA. By using the resulting Exon-Exon Border as a Primer Binding Site (Figure 7), we were able to evenly amplify all of our sequences, without also amplifying the viral DNA, containing those same sequences.

Finally another PCR was performed on our cDNA library, a DNA extraction of our viral vector as well as our initial expression plasmid library in order to add the necessary Illumina HiSeq Adapters for Next Generation Sequencing

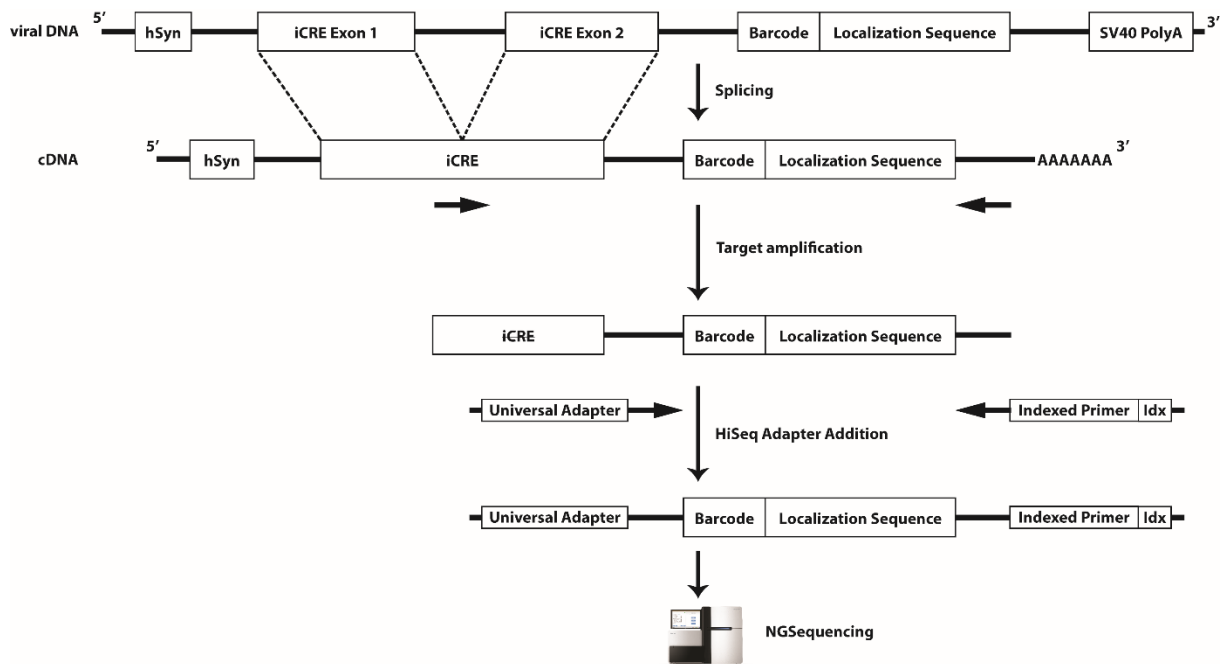


Figure 7: HiSeq Preparation. After extraction of mRNA from our regions of interest, it gets transcribed into cDNA and prepared for NGS. An initial amplification using the Exon-Exon Border as a Primer Binding Site amplifies all localisation sequences using the same primer pair, while excluding viral DNA. A second amplification attaches the necessary HiSeq Adapters for Next Generation Sequencing.

4 Materials

4.1 Instruments/Devices

Eppendorf ThermoMixer (Eppendorf AG)

Vortex-Genie 2 (Scientific Industries Inc.)

PowerPac™ Basic Power Supply (Bio-Rad Laboratories Inc.)

CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc.)

PerfectBlue Gelsystem Mini L & Midi S (VWR International GmbH)

C1000 Thermal Cycler (Bio-Rad Laboratories Inc.)

Heraeus™ Megafuge 40R Refrigerated Centrifuge (Thermo Fisher Scientific Inc.)

Sorvall™ RC 6 Plus Centrifuge (Thermo Fisher Scientific Inc.)

Vibrating Blade Microtome Leica VT1000 S (Leica Biosystems Nussloch GmbH)

NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.)

UV Transilluminator (INTAS Science Imaging Instruments GmbH)

Rotomax 120 Tiltshaker (Heidolph Instruments GmbH & Co KG)

Precellys®24 High Throughput Tissue Homogenizer (Bertin Technologies)

Isotemp 210 Water Bath (Thermo Fisher Scientific Inc.)

Forma™ Series II Water-Jacketed CO₂ Incubator (Thermo Fisher Scientific Inc.)

Automatic Digital Refractometer AR200 MESS-MODUS: nD-TC (20°C) (Reichert, #13950000)

70Ti Fixed Angle Rotor (Beckman Coulter Inc.)

4.1.1 Stereotaxic Surgery

Stereotaxic Alignment System (David Kopf Instruments, Model 1900)

Non-rupture Earbars (David Kopf Instruments, Model 1922)

Electrode Holder (David Kopf Instruments, Model 1970)

Micro Manipulator (David Kopf Instruments, Model 1940)
Alignment Indicator (David Kopf Instruments, Model 1905)
Stereotaxic Drill Unit (David Kopf Instruments, Model 1911)
Centering Scope 40x (David Kopf Instruments, Model 1915)
SYS-Mirco4 Micro Syringe Pump Controller (World Precision Instruments, #UMP3-1)
Nanoliter 2000 Injector (World Precision Instruments)
Pipette Puller (Sutter Instruments, Model P-97)

4.2 General material

Gilson pipettes: P2, P10, P20, P200, P1000
Eppendorf Tubes: 1.5 mL, 2 mL
LB Agar Ampicillin-100 Plates (In-house)
Corning® Tissue-culture Treated Culture Dish, 150mm (Sigma-Aldrich Co. LLC., #CLS430599)
Corning® Cell Culture Flask, 75cm² (Sigma-Aldrich Co. LLC., #CLS430641)
OptiSeal tubes (Beckmann Coulter #361625)
Omnifix® Syringes (Braun)
Sterican™ Needles (Braun)
20Gx1 ½ needle, 0.90x40mm BI/LB (Braun)
Slide-A-Lyzer Dialysis Cassette G2, 20,000 MWCO, 70 mL Capacity, 6/pkg (Thermo Fisher Scientific, #87738)
Slide-A-Lyzer Dialysis Cassette G2, 20,000 MWCO, 30 mL Capacity, 6/pkg (Thermo Fisher Scientific, #87737)
AMICON ULTRA-15 (max. 15 mL), MWCO 50kDa, 24/pkg (Milipore, #UFC905024)
Millex-GV Filter Unit: Durapore Membran (PVDF), 0.22 µl, Filter-diameter 13mm, sterile (Milipore, #SLGV013SL)
Roti® Coll 1: Cyanoacrylate Glue (Carl Roth GmbH, #0258.1)
Curity™: Steril Cotton Tipped Applicator (Covidien, #541400)
Vaseline (BIPA)

Glass Capillaries for Nanoliter 2010: 3.5 inch (World Precision Instruments, #4878)

DynaMag™-2 Magnet (Thermo Fisher Scientific Inc., #12321D)

Gel Loading Tips, standard, round (Thermo Fisher Scientific Inc., #LC1001)

Sterile Single Use Stainless Surgical Blades (Lance Paragon Ltd.)

4.3 Media

LB (Low Salt Luria Bertani): 10g/L Tryptone, 5g/L Yeast Extract, 5g/L NaCl, autoclaved

DMEM growth medium: DMEM, 10% FCS, 1% 2mM L-Glutathione, 1% Penicilin-Streptomycin

Antibiotics were added after autoclaving if needed.

4.4 Buffers and Solutions

4.4.1 Solutions for surgery

4.4.1.1 Ketamine/Medetomidine Anesthesia Cocktail

Table 1: Ketamine/Medetomidine Anesthesia Cocktail

Ingredient	Final Concentration	Stock	10 ml
Ketamine	10 mg	100 mg/ml	1 ml
Medetomidine	80 µg	1 mg/ml	0.8 ml
Aqua dest.			8.2 ml

4.4.1.2 Cortex Buffer

Table 2: Cortex Buffer pH 7.4

Ingredient	Final Concentration
NaCl	125 mM
KCl	5 mM
Glucose	10 mM
HEPES	10 mM
CaCl ₂	2 mM
MgSO ₄	2 mM

Eventually adjust pH with NaOH or HCl to pH 7.4

4.4.1.3 Others

Ketasol: 100mg/ml Ketamine (OGRIS Pharma Vertriebs-GmbH)

Xylasol: 20mg/ml Xylazine (OGRIS Pharma Vertriebs-GmbH)

Dormitor: 1mg/ml Medetomidine Hydrochloride (Orion Corporation)

Isoflo: 100% w/w Isoflurane (Abbot Laboratories)

Rimadyl: 50mg/ml Carprofen (Pfizer Corporation Austria)

Xylanaest: 1% Lidocaine (Gebro Pharma)

Antisedan: 5mg/ml Atipamezolhydrochloride (Pfizer Inc.)

Paraformaldehyde (PFA): 16% dilution (Sigma-Aldrich, P6148)

4.4.2 Solutions for Virus Production

4.4.2.1 *Dulbecco's Modified Eagle Medium (DMEM) High Glucose HEPES Modification*

DMEM High glucose HEPES modification obtained from the IMP media kitchen, pH 7.1-7.3

Table 3: Dulbecco's Modified Eagle Medium (DMEM) pH 7.2

Ingredient	Final Concentration
Glucose	4,500 mg/L
HEPES	25 mM
L-glutamine	0.584 mg/L
NaHCO ₃	3.7 g/L

4.4.2.2 DMEM Growth Medium

Table 4: DMEM Growth Medium

Ingredient	Final Concentration
DMEM High Glucose	
HEPES Modification	
Fetal Calf Serum (FCS)	10 %
Glutamine	0.02 mM

4.4.2.3 2xHBS, pH 7.1

Table 5: 2xHBS

Ingredient	Final Concentration
HEPES	50 mM
NaCl	280 mM
Na ₂ HPO ₄	1.5 mM

Adjust to pH 7.1

4.4.2.4 Lysis Buffer, pH 8.5

Table 6: Lysis Buffer, pH 8.5

Ingredient	Final Concentration
TRIS	50 mM
NaCl	150 mM
MgCl ₂	2 mM
Complete EDTA-free tablets (Roche #11836170001)	1 piece

4.4.2.5 Polyethylene Glycol 8000 Solution(PEG-8000)

Table 7: Polyethylene Glycol 8000 Solution

Ingredient	Final Concentration
PEG-8000	40%
NaCl	1.915 M

4.4.2.6 Resuspension Buffer, pH 7.4

Table 8: Resuspension Buffer, pH 7.4

Ingredient	Final Concentration
HEPES	50 mM
NaCl	150 mM
EDTA	25 mM

4.4.2.7 Others

Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific Inc., #11668019)

L-Glutamine Solution, 200mM (Sigma-Aldrich Co. LLC., #G7513)

Penicillin-Streptomycin, 10,000 U Penicilin, 10mg/mL Streptomycin (Sigma-Aldrich Co. LLC., #P0781)

Gibco® Fetal Bovine Serum (FBS) EU-approved (Thermo Fisher Scientific Inc., #10270-106)

Gibco® Trypsin-EDTA (0.05%), phenol red (Thermo Fisher Scientific Inc., #25300-062)

300 mM CaCl₂ (In-house)

0.5M EDTA (In-house)

DPBS, 1x (In-house)

Ultrapure CsCl: Optical grade (Invitrogen, #15507-023)

Dialysis Buffer 1x and 1.111x (Invitrogen, #14200-067)

Glycerine (In-house)

4.4.3 Solutions for Dynabeads Oligo-dT

DEPC treated water: 1ml DEPC/500ul MonoQ water incubated o/n, autoclaved

4.4.3.1 2x Binding Buffer

Table 9: 2x Binding Buffer

Ingredient	Final Conc.	Stock	10 ml	100 ml	500 ml
TrisHCl pH 7.5	20 mM	1 M	200 ul	2 ml	10 ml
LiCl	1 M	5 M	2 ml	20 ml	100 ml
EDTA pH 8	2 mM	0.5 M	40 ul	400 ul	2 ml
DEPC treated MonoQ			7.76 ml	77.6 ml	388 ml

4.4.3.2 Washing Buffer

Table 10: Washing buffer

Ingredient	Final Conc.	Stock	10 ml	100 ml	500 ml
TrisHCl pH 7.5	10 mM	1 M	100 ul	1 ml	5 ml
LiCl	0.15 M	5 M	300 ul	3 ml	15 ml
EDTA pH 8	1 mM	0.5 M	20 ul	200 ul	1 ml
DEPC treated MonoQ			9.58 ml	95.8 ml	479 ml

4.4.3.3 Storage Buffer

Table 11: Storage buffer

Ingredient	Final Conc.	Stock	10 ml	100 ml	500 ml
TrisHCl pH 7.5	250 mM	1 M	2.5 ml	25 ml	125 ml
EDTA pH 8	20 mM	0.5 M	400 ul	4 ml	20 ml
Tween-20	0.1 %		10 ul	100 ul	500 ul
NaN ₃ (Sodium azide)	0.02 %	10%	20 ul	200 ul	1 ml
DEPC treated MonoQ			7.07 ml	70.7 ml	353.5 ml

4.4.3.4 10mM TrisHCl

Table 12: 10mM TrisHCl

Ingredient	Final Conc.	Stock	10ml	100ml
TrisHCl pH 7.5	10mM	1M	100 ul	1 ml
DEPC treated MonoQ			9.9 ml	99 ml

4.4.3.5 Reconditioning Solution

Table 13: Reconditioning Solution

Ingredient	Final Conc.	Stock	10 ml	100 ml	500 ml
NaOH	0.1 M	2 M	500 ul	5 ml	25 ml
		5 M			10 ml

4.4.4 Alkaline Gel Electrophoresis Buffers

4.4.4.1 Alkaline Agarose Gel Electrophoresis Buffer, 10x

Table 14: Alkaline Agarose Gel Electrophoresis Buffer, 10x

Ingredient	Final Conc.
NaOH	500 mM
EDTA	10 mM

4.4.4.2 Neutralization Buffer

Table 15: Neutralization Buffer

Ingredient	Final Conc.
Tris-HCl	1 M
NaCl	1.5 M

Adjust pH to 7.6

4.4.5 General Buffers & Solutions

Phusion HF Buffer, 5x (Thermo Fisher Scientific Inc., #F-518)

Phusion GC Buffer, 5x (Thermo Fisher Scientific Inc., #F-519)

Tango Buffer with BSA, 10x (Thermo Fisher Scientific Inc., #BY5)

T4 DNA Ligase Buffer, 10x (Thermo Fisher Scientific Inc., #B69)

O Buffer with BSA, 10x (Thermo Fisher Scientific Inc., #BO5)

ThermoPol Reaction Buffer, 10x (New England Biolabs, #B9004S)

FastDigest Green Buffer, 10x (Thermo Fisher Scientific Inc., #B72)

Desoxynucleoside Triphosphate (dNTP) Mix, 10 mM (Thermo Fisher Scientific Inc., #R0192)

FastAP Buffer, 10x (Thermo Fisher Scientific Inc., #EF0651)

First Strand Buffer, 5x (Thermo Fisher Scientific Inc., #18080)

GeneRuler 1kb DNA Ladder, Ready-to-Use 250 to 10,000 bp (Thermo Fisher Scientific Inc., #SM0314)

GeneRuler 100bp DNA Ladder (Thermo Fisher Scientific Inc., #SM0241)

GeneRuler 50bp DNA Ladder (Thermo Fisher Scientific Inc., #SM0371)

RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific Inc., #AM9780)

Incidur® Spray (Ecolab Deutschland GmbH, #30 117 30)

Agarose Broad Range Pulver (Fa. Roth, #T846.3)

DNA Loading Dye, 6x (Thermo Fisher Scientific Inc., #R0611)

Agarose Gel Loading Dye, 6x, Alkaline (bioWORLD, #700821)

SYBR®Gold Nucleic Acid Gel Stain, 10,000x in DMSO (Thermo Fisher Scientific Inc., #S-11494)

Ethidium Bromide (EtBr) Dye for DNA and RNA Detection (Thermo Fisher Scientific Inc., #15585011)

Tris-Acetate-EDTA (TAE) Buffer, 1x (In-house)

Isopropyl Alcohol ≥99.5% (Sigma-Aldrich Co. LLC., #437522)

Chloroform, ≥99.5% (Sigma-Aldrich Co. LLC., #C2432)

Ampicillin Solution (In-house)

4.5 Enzymes

4.5.1 Restriction Enzymes

AvrII, 10U/μl, 5'-C[^]CTAGG-3'

EcoRI, 10U/μl, 5'-G[^]AATTC-3'

BamHI, 10U/μl, 5'-G[^]GATCC-3'

KpnI, 10U/μl, 5'-G[^]GTACC-3'

NotI, 10U/μl, 5'-GC[^]GGCCGC-3'

4.5.2 Polymerases

Phusion High-Fidelity DNA Polymerase, 2 U/μl (Thermo Fisher Scientific Inc., #F-530)

Phusion U Hot Start DNA Polymerase, 100 U (Thermo Fisher Scientific Inc., #F-555)

ThermoPrime Taq DNA Polymerase, recombinant, 5 U/μl (Thermo Fisher Scientific Inc., #AB-0301)

KAPPA HiFi PCR HotStart ReadyMix (Kapa Biosystems, #KK2602)

iQ[™] SYBR[®] Green Supermix (Bio-Rad Laboratories Inc., #170-8880)

4.5.3 Other Enzymes

T4 DNA Ligase, 5 U/μl (Thermo Fisher Scientific Inc., #EL0014)

FastAP Thermosensitive Alkaline Phosphatase, 10 U/μl (Thermo Scientific Inc., #EF0651)

SuperScript[®]III Reverse Transcriptase (Thermo Fisher Scientific Inc., #18080093)

RNaseOUT[™] Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific Inc., #10777-019)

TURBO[™] DNase, 2 U/μl (Thermo Fisher Scientific Inc., #AM2238)

RNase A, DNase and protease-free (Thermo Fisher Scientific Inc., #EN0531)

Benzonase: 200U Nuclease (Novagen, #70664)

4.6 Software

Lasergene 9.0 Core Suite/ DNASTAR Inc., Madison, WI, USA

PerlPrimer/ Owen Marshall (©2003-2011)

Adobe Creative Suit 5 Design Standard / Adobe Systems Software Ireland Ltd. (© 2010)

4.7 Kits & Reagents

QIAGEN Plasmid Mini Kit (QIAGEN, #12123)

QIAGEN Plasmid Maxi Kit (QIAGEN, #12162)

PureYield™ Plasmid Maxiprep System (Promega GmbH, #A2392)

Wizard® SV Gel and PCR Clean-Up System (Promega GmbH, #A9282)

RNeasy Lipid Tissue Mini Kit (QIAGEN, #74804)

RNeasy MinElute Cleanup Kit (QIAGEN, #74204)

MinElute PCR Purification Kit (QIAGEN, #28004)

Agencourt AMPure XP PCR Purification (Beckman Coulter Inc., #A63880)

Dynabeads® Oligo(dT)₂₅ (Thermo Fisher Scientific Inc., #61002)

Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, #27-9264-01)

TRIzol® Reagent (Thermo Fisher Scientific Inc., #15596026)

DNA/RNA Extraction Reagent ViralXpress (EMD Millipore, #3095)

4.8 Strains

4.8.1 Cell Lines

HEK293: ATCC-LGC (#CRL-1573)

DH5α Strain *E. Coli* (In-house)

4.8.2 Mouse strains

In house bred male C57BL/6J wild type mice were used for stereotaxic injections. The animals were between 10 and 12 weeks old at the start of the experiment.

4.9 Vectors

Helper plasmid (Serotype 8): dp8.ape, plasmid factory, #PF478

4.10 Primers

Table 16: Primers used during project

No.	Name	Sequence 5'→3'
1	Psyn_fw_AvrII	TGACCTAGGgtgtctagactgcagagggc
2	Psyn_rv_EcoRI	GACGAATTcttctcgactgcgctctcag
3	iCre_fw_EcoRI	gaaGAATTCGTCAGTaccATGgtgcccaagaagaag
4	iCre_rv_NotI	CTAGCGGCCGcgttgcttcaGATATCtgtgaagtccccatcctctagcagc
5	ActB_full_fw_BamHI	TGAGGATCCGTGAGCAAGGGCGAGGAGGA- TAACATGGCCATCATCAAGGAGCGGACTGTTACTGAGCTGCG
6	ActB_full_rv_KpnI	GCGGCCGCGGTACCGGTCTCAAGTCAGTGTACAGGCCAG
7	ActB_54nt_fw_BamHI	TGAGGATCCGTTTCATGCGCTTCAAGGTG- CACATGGAGGGCTCCGTGAACGCGGACTGTTACTGAGCTGCG
8	ActB_54nt_rv_KpnI	GCGGCCGCGGTACCCAAAGCCATGCCAATGTTGTCTCTTA
9	Tau_fw_BamHI	TGAGGATCCGGCCACGTCTTCGA- GATCGAGGGCGAGGGCGAGGGCCCGCTTTGGTTCAC- TCCATTACTTCT
10	Cal1_fw_BamHI	TGAGGATCCCCTACGAGGGCACCCAGACCGCCAA- GCTGAAGGTGACCAAAAGCCAAGGATGAGCTGTAGAGG

11	Cal2_fw_BamHI	TGAGGATCCGGGTGGCCCCCTGCCCTTCACTTGG- GACATCCTGTCCCCTTTCATCTGCTCTCCTTCCTGC
12	ActG1_fw_BamHI	TGAGGATCCCAGTTCATGTACGGCTCCAACGCCTACGTGAA- GCACCCCGATGGACTGAGCAGGTGCCAGG
13	p5_fw_BamHI	TGAGGATCCCCGACATCCCCGACTACTTCAA- GCTGTCCTTCCCCGAGGGGTGCCAC- CTAAATTGTAAGCGTTAAT
14	p19_fw_BamHI	TGAGGATCCCTTCAAGTGGGAGCGCGTGATGAAGTTCGAG- GACGGCGGCGGTCTGACGCTCAGTGGAACG
15	p26_F1_fw_BamHI	TGAGGATCCGTGGTGACCGTGACCCAGGACTCCTCCCTG- CAGGACGGCGGGTGGTTACGCGCAGCGTG
16	p26_E1_fw_BamHI	TGAGGATCCAGTTCATCTACAAGGTGAAGCTGCGCGGCAC- CAACTTCCCCCCCCTGACGAGCATCACAAAAAT
17	p26_lacZ_fw_BamHI	TGAGGATCCCTCCGACGGCCCCGTAATGCAGAAGAAGAC- CATGGGCTGGCCATTCGCCATTCAGGCTGC
18	Tau_rv_KpnI	GCGGCCGCGGTACCAAACTGGGGTGTGGAGACTGATTA
19	Cal1_rv_KpnI	GCGGCCGCGGTACCAACCTGCCTGGAAAAAATGAAAA
20	Cal2_rv_KpnI	GCGGCCGCGGTACCTGCTAGAACTGGCTGCCCCACA
21	ActG1_rv_KpnI	GCGGCCGCGGTACCCTAAGGACTAAATCAAGATATGCACAGG
22	p5_rv_KpnI	GCGGCCGCGGTACCGGTTACGTAGTGGGCCATCG
23	p19_rv_KpnI	GCGGCCGCGGTACCCTCGCGGTATCATTGCAGCAC
24	p26_F1_KpnI	GCGGCCGCGGTACCCAGTTTGAACAAGAGTCCAC- TATTAAAG
25	p26_E1_KpnI	GCGGCCGCGGTACCGATAGTTACCGGATAAGGCGCAGC
26	p26_lacZ_KpnI	GCGGCCGCGGTACCCGTCGTGACTGGGAAAACCTGG
27	p5c_seq_BamHI_fw	ATGGTGAGGCTGCTAGAGGATG
28	p5c_seq_KpnI_rv	AAGCATCGAGATCGCAGGTGAG
29	fw1_Cont_qPCR	ATGCCAGGATATCAGGAACC
30	fw2_Cont_qPCR	TGATGAGGAGAATCAGAAAGGAG
31	rv1_ActBf_qPCR	GATGATGGCCATGTTATCCTC

32	rv2_ActBf_qPCR	GGAGGATAACATGGCCATCATC
33	rv3_ActBf_qPCR	GAGGAGGATAACATGGCCAT
34	rv4_ActBf_qPCR	TCCTTGATGATGGCCATGTTA
35	rv5_ActBf_qPCR	ATGTTATCCTCCTCGCCCT
36	rv1_ActB54_qPCR	CACCTTGAAGCGCATGAAC
37	rv2_ActB54_qPCR	CTCCATGTGCACCTTGAAG
38	rv3_ActB54_qPCR	CCCTCCATGTGCACCTTG
39	rv4_ActB54_qPCR	CCTTGAAGCGCATGAACG
40	rv1_Tau_qPCR	CCTCGATCTCGAAGACGTG
41	rv2_Tau_qPCR	CCCTCGATCTCGAAGACG
42	rv3_Tau_qPCR	CGCCCTCGATCTCGAAGA
43	rv4_Tau_qPCR	TCGCCCTCGATCTCGAA
44	rv1_Calr1_qPCR	GGTCACCTTCAGCTTGGC
45	rv2_Calr1_qPCR	GCTTTTGGTCACCTTCAGCT
46	rv3_Calr1_qPCR	CTTCAGCTTGGCGGTCT
47	rv4_Calr1_qPCR	TCTGGGTGCCCTCGTAG
48	rv1_Calr2_qPCR	ATGTCCCAAGTGAAGGGC
49	rv2_Calr2_qPCR	GACAGGATGTCCCAAGTGAAG
50	rv3_Calr2_qPCR	GGACAGGATGTCCCAAGTG
51	rv4_Calr2_qPCR	GTCCCAAGTGAAGGGCAG
52	rv1_ActG1_qPCR	TTGGAGCCGTACATGAACTG

53	rv2_ActG1_qPCR	CTTCACGTAGGCGTTGGAG
54	rv3_ActG1_qPCR	GTGCTTCACGTAGGCGTT
55	rv4_ActG1_qPCR	CGTTGGAGCCGTACATGAAC
56	rv1_p5_qPCR	CTTGAAGTAGTCGGGGATGTC
57	rv2_p5_qPCR	GCTTGAAGTAGTCGGGGATG
58	rv3_p5_qPCR	GGAAGGACAGCTTGAAGTAGTC
59	rv4_p5_qPCR	GGGAAGGACAGCTTGAAGTAG
60	rv1_F1_qPCR	GGAGGAGTCCTGGGTCAC
61	rv2_F1_qPCR	TCCTGCAGGGAGGAGTC
62	rv3_F1_qPCR	AGTCCTGGGTCACGGTC
63	rv4_F1_qPCR	TCCTGGGTCACGGTCAC
64	rv1_ColE1_qPCR	CAGCTTCACCTTGTAGATGAAC
65	rv2_ColE1_qPCR	GCAGCTTCACCTTGTAGATG
66	rv3_ColE1_qPCR	GCGCAGCTTCACCTTGTAG
67	rv4_ColE1_qPCR	GCGCAGCTTCACCTTGTA
68	rv1_LacZ_qPCR	CCATGGTCTTCTTCTGCATTAC
69	rv2_LacZ_qPCR	CAGCCCATGGTCTTCTTCTG
70	rv3_LacZ_qPCR	CATGGTCTTCTTCTGCATTACG
71	rv4_LacZ_qPCR	CCCATGGTCTTCTTCTGCAT
72	iCre_no-In- tron_EcoRI_fw	AAGAATTCGTGTCCAACCTGCTGACTGTG
73	iCre_no-In- tron_BamHI_rv	AGGGATCCTCGACTCAGTTCCAGTCCCCATCCTCGAGCAG

74	junction_1.1	CAGATGCCAGGATATCAGGA*A*C*C*T*G
75	junction_1.2	GAACCTCTGACAGATGCCAGG*A*T*A*T*C
76	junction_1.3	CTCTGACAGATGCCAGGATA*T*C*A*G*G
77	junction_1.4	GACAGATGCCAGGATATCAG*G*A*A*C*C
78	junction_2.1	GAGATCGCAGGTGAGGCC*T*A*G
79	junction_2.2	CATCGAGATCGCAGGTGA*G*G*C
80	junction_2.3	ATCGCAGGTGAGGCCT*A*G*C
81	GSP-RT_01	CATCGAGATCGCAGGTGA
82	GSP-RT_02	AAGCATCGAGATCGCAGG
83	GSP-RT_03	ATCGAGATCGCAGGTGAG
84	before_splice_fw	GAGAACTCTGACAGATGCC*A*G*G*A*T
85	after_splice_fw	ATCAGGAACCTGGCCT*T*C*C*T*G
86	PE_1.0	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACAC- GACGCTCTTCCGATCTATGGTGAGGCTGCTAGAGGATGG
87	PE RV 1	CAAGCAGAAGACGGCATAACGAGATCGTGATGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
88	PE RV 2	CAAGCAGAAGACGGCATAACGAGATACATCGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
89	PE RV 3	CAAGCAGAAGACGGCATAACGAGATGCCTAAGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
90	PE RV 4	CAAGCAGAAGACGGCATAACGAGATTGGTCAGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
91	PE RV 5	CAAGCAGAAGACGGCATAACGAGATCACTGTGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC

92	PE RV 6	CAAGCAGAAGACGGCATAACGAGATATTGGCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
93	PE RV 7	CAAGCAGAAGACGGCATAACGAGATGATCTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
94	PE RV 8	CAAGCAGAAGACGGCATAACGAGATTCAAGTGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
95	PE RV 9	CAAGCAGAAGACGGCATAACGAGATCTGATCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
96	PE RV 10	CAAGCAGAAGACGGCATAACGAGATAAGCTAGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
97	PE RV 11	CAAGCAGAAGACGGCATAACGAGATGTAGCCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
98	PE RV 12	CAAGCAGAAGACGGCATAACGAGATTACAAGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
99	PE RV 13	CAAGCAGAAGACGGCATAACGAGATTTGACTGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
100	PE RV 14	CAAGCAGAAGACGGCATAACGAGATGGAAGTGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
101	PE RV 15	CAAGCAGAAGACGGCATAACGAGATTGACATGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
102	PE RV 16	CAAGCAGAAGACGGCATAACGAGATGGACGGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
103	PE RV 17	CAAGCAGAAGACGGCATAACGAGATCTCTACGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
104	PE RV 18	CAAGCAGAAGACGGCATAACGAGATGCGGACGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
105	PE RV 19	CAAGCAGAAGACGGCATAACGAGATTTTACGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC

106	PE RV 20	CAAGCAGAAGACGGCATAACGAGATGGCCACGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
107	PE RV 21	CAAGCAGAAGACGGCATAACGAGATCGAAACGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
108	PE RV 22	CAAGCAGAAGACGGCATAACGAGATCGTACGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
109	PE RV 23	CAAGCAGAAGACGGCATAACGAGATCCACTCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
110	PE RV 24	CAAGCAGAAGACGGCATAACGAGATGCTACCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
111	PE RV 25	CAAGCAGAAGACGGCATAACGAGATATCAGTGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
112	PE RV 26	CAAGCAGAAGACGGCATAACGAGATGCTCATGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
113	PE RV 27	CAAGCAGAAGACGGCATAACGAGATAGGAATGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
114	PE RV 28	CAAGCAGAAGACGGCATAACGAGATCTTTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
115	PE RV 29	CAAGCAGAAGACGGCATAACGAGATTAGTTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
116	PE RV 30	CAAGCAGAAGACGGCATAACGAGATCCGGTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
117	PE RV 31	CAAGCAGAAGACGGCATAACGAGATATCGTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
118	PE RV 32	CAAGCAGAAGACGGCATAACGAGATTGAGTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
119	PE RV 33	CAAGCAGAAGACGGCATAACGAGATCGCCTGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC

120	PE RV 34	CAAGCAGAAGACGGCATAACGAGATGCCATGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
121	PE RV 35	CAAGCAGAAGACGGCATAACGAGATAAAATGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
122	PE RV 36	CAAGCAGAAGACGGCATAACGAGATTGTTGGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
123	PE RV 37	CAAGCAGAAGACGGCATAACGAGATATTCCGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
124	PE RV 38	CAAGCAGAAGACGGCATAACGAGATAGCTAGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
125	PE RV 39	CAAGCAGAAGACGGCATAACGAGATGTATAGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
126	PE RV 40	CAAGCAGAAGACGGCATAACGAGATTCTGAGGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
127	PE RV 41	CAAGCAGAAGACGGCATAACGAGATGTCGTCGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
128	PE RV 42	CAAGCAGAAGACGGCATAACGAGATCGATTAGTGACTGGAG- TTCAGAC- GTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
129	PE RV 43	CAAGCAGAAGACGGCATAACGAGATGCTGTAGTGACTGGAG- TTCAGACGTGTGCTCTTCCGATCTAGATCGCAGGTGAGGCCTAGC
130	fw1_Nested_qPCR	GAAACCTGGACTCTGAGACTG
131	new_qPCR_fw_150pb	GTCCATCCCTGAAATCATGC

5 Methods

5.1 Plasmid amplification and isolation

For retransformation of a desired plasmid, DH5 α strain *E. coli* were defrosted on ice to assure a considerate thawing of cells and an appropriate amount of Plasmid was added. The bacteria and plasmid mixture was afterwards subjected to a heat shock for 10 seconds on 42°C to permeate the cell walls and allow the uptake of the plasmid. Immediately afterwards the cells were put on ice for 2 minutes to ensure the recovery of the now transfected cells.

500 μ l of LB medium was added to the transformation reaction and incubated on 37°C for about 30 minutes while being lightly shaken. This provides enough time for regeneration and amplification of transfected as well as untransfected cells. The cells were then plated onto an Ampicilin containing agar plate and incubated on 37°C over night to select for plasmid and therefore Amp-resistance containing cells.

On the next day, a colony was picked and about 8mL of LB Amp was inoculated with it. The sample was again incubated at 37°C over night and a Miniprep (QIAprep Spin Miniprep Kit) was performed the next day following the manufacturers protocols. DNA was eluted in 50 μ l.

5.2 The Polymerase Chain Reaction (PCR) and DNA Purification

The polymerase chain reaction is a technique used in molecular biology to amplify between usually 100bp and 10kbp long strands of RNA or DNA in vitro. It utilizes a thermostable Polymerase like Taq which has the special property of still being active even at high reaction temperatures. The template DNA is added to a reaction comprising of the appropriate buffer, a forward and reverse primer specific for the sequence that is to be

amplified, a 10 mM mixture of desoxynucleoside triphosphates (dNTPs) which will consequently get incorporated into the freshly synthesised DNA strands, and the Polymerase. The Buffer serves the purpose to promote a working environment for the polymerase as even slight changes in the pH could possibly hinder the enzyme from working. It also usually contains MgCl_2 , as Magnesium has several roles in the reaction, it acts as a cofactor for the polymerase, forms soluble complexes with the dNTPs and influences not only the specific but also the unspecific binding of the Primers. Lastly, another optional content of the buffer is Dimethylsulfoxide (DMSO) as it lowers the melting point and therefore improves the primer annealing.

The PCR reaction now undergoes several heating and cooling steps inside a PCR Thermal Cycler. It starts with an initial denaturation at 98°C for about 30 seconds, this ensures not only that all primers and templates are denatured and are at hand as single stranded molecules but also that they're free of secondary structure that could inhibit proper amplification.

Afterwards comes a desired amount of cycles each consisting of three phases: denaturation, primer annealing and elongation. The denaturation step is performed again at 98°C but this time only for 5-10 seconds since now only the shorter amplified DNA fragments are of importance. The Primer annealing varies from $45\text{--}72^\circ\text{C}$ depending on the length and GC-content of the designed primers as well as various other reaction conditions (secondary structures, DMSO content, ect.) and lasts between 10-30 seconds. Finally, the elongation step is performed at the optimal working condition of the chosen polymerase, which is 72°C for the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc.) as chosen mostly in my experiments.

After the appropriate amount of cycles there's a final extension step again at 72°C for another 5-10 minutes after which the cycler will hold on 4°C for an indefinite amount of time.

The desired DNA fragment will now be exponentially amplified corresponding to the number of cycles used in the amplification reaction. To the finished PCR reaction was

now added a few μl of 6x DNA Loading Dye (Thermo Fischer Scientific Inc.) and loaded onto a 1% Agarose Gel containing Ethidium Bromide next to an appropriate DNA Ruler (Thermo Fisher Scientific Inc.). Gel Electrophoresis settings were adjusted corresponding to gel size and required degree of separation.

The gel was then removed from the Buffer Chamber (PEQLAB Biotechnologie GmbH) and put onto a UV-Transilluminator (INTAS Science Imaging Instruments GmbH) to locate the relevant DNA bands and check for their size with the help of the DNA Ruler. Later, a carbon steel scalpel blade was used to cut out the respective band representing the PCR amplicon and put into a 1.5 mL Eppendorf Tube.

Using the Wizard® SV Gel and PCR Clean-Up System (Promega GmbH) the gel piece was melted in Membrane Binding Buffer at 55°C and later purified via the provided SV Minicolumns. Subsequently samples were eluted in the appropriate amount of Nuclease-Free Water. 50 μl were used most of the time for the most efficient elution.

5.3 Restriction Enzyme Digest

Both vector and PCR amplified insert were digested with the same restriction enzymes, e.g. EcoRI and AvrII. For the vector digest reaction, additionally 1 μl FastAP (Thermo Fisher Scientific Inc.) Thermosensitive Alkaline Phosphatase per μg of Plasmid DNA was added to prevent vector re-ligation.

Table 17: Restriction Enzyme Digest

Component	Volume		
	Plasmid DNA	Un-purified PCR product	Genomic DNA
Water, nuclease-free	14 μl	16 μl	25 μl
10x FastDigest Buffer	2 μl	2 μl	5 μl
DNA	(up to 1 μg) 2 μl	(~0.2 μg) 10 μl	(5 μg) 10 μl
FastDigest Enzyme 1	1 μl	1 μl	5 μl
FastDigest Enzyme 2	1 μl	1 μl	5 μl
Total volume	20 μl	30 μl	50 μl

Fast Digests were performed at 37°C for 30 min.

Resulting fragmented DNA was put onto a 1% Agarose Gel and separated via Gel electrophoresis. The desired bands were cut out and again purified via the Wizard® SV Gel and PCR Clean-Up System.

Resulting Vector and Insert DNA should now be linearized and have congruent overhangs needed for a successful ligation.

5.4 Ligation and Transformation

For the successful ligation of two DNA-Fragments they need to have the same overhangs. This can either be blunt ends or sticky ends resulting from the digest of both DNA-Fragments with dissenting restriction enzymes.

50 ng of a Vector are combined with an Insert in a 1:1.5 molar ratio. This is calculated with the formula

$$ng(insert) = \frac{kb(insert)}{kb(vector)} \times ng(vector) \times 1.5$$

Equation 1: Ligation

To this reaction 1 µL of a 10x T4 DNA Ligase Buffer and 1 µL of an 5 U/µl T4 DNA Ligase (Thermo Fisher Scientific Inc.) is added and combined with nuclease-free water to a total volume of 10 µL. At the same time one reaction with water instead of an Insert is produced as a negative control.

Those reactions are put on 20°C for 1h while 200 µL per Ligation reaction of a DH5α E.coli strain (In-house) are being defrosted on ice.

Subsequently those 200 µL DH5α E. coli are added to each Ligation reaction and put under heat shock for 10 seconds on 42°C, then immediately put on ice for 2 minutes to grant the bacteria some time to regenerate.

Now 500 μ L LB medium (containing no antibiotics) are added to each reaction and incubated on 37°C for another 30 min to let the bacteria proliferate.

After this the cultures can be plated on Agar plates containing an appropriate resistance (in most cases Ampicillin) in order to pick single colonies after overnight incubation on 37°C.

5.5 Cloning strategy

For the co-packaging experiment 10 different expression vectors had to be cloned using self-complementary AAV ITRs. We decided to implement a small test screen for the mRNA translocation ability of different sequences. Five of those sequences have already been described and proven to work in current literature, the other five were taken from the backbones of different plasmids to function as non-genomic negative controls, meaning they are not expected to be translocated. Additionally, each construct was provided with a unique 40 base pair barcode sequence specific for this construct. The sequence for the bar code was also taken from an inert region of a plasmid backbone, they were checked to be non-overlapping with each other and a BLAST search was performed on the mouse genome to check the uniqueness of those sequences.

As a base for cloning the self-complementation inducing psc_AAV_MCS plasmid was used. It contains an Ampicillin resistance gene and a Multiple Cloning Site (MCS) right after a CMV promoter. For our purposes the CMV promoter was exchanged with a human Synapsin promoter (hSyn) that drives expression specifically in neurons. Into the MCS a reporter gene (iCre) containing a betaglobin Intron was cloned.

This Intron is important to later differentiate between viral DNA and cDNA made from virally expressed mRNA.

The reporter gene iCre itself serves the purpose of driving the expression of an improved version of the Cre-recombinase. This Cre-recombinase provides the ability to specifically remove DNA-sequences in a living organism. This is achieved by flanking regions in the

genome with so called loxP sites facing either same or opposite direction. Those loxP sites are recognised by the Cre recombinase and induce a recombination event. If the two loxP sites face the same direction the floxed (flanked by loxP) region will be excised, on the other hand if the two loxP sites face each other the region within the loxP sites will be inverted. This technique is important to either knock-out genes in the adult stadium of an organism where a normal knock-out during a foetal state would otherwise be lethal or to induce gene transcription at a desired point during development by removing strategic placed Stop-Codons. Additionally, by driving the Cre recombinase with a tissue specific promoter it is possible to create a tissue specific knock-out. While this reporter gene serves no purpose but to provide an Intron in the immediate experiments, it will provide a simple way to label individual cells during follow up experiments.

9 bp after the reporter gene is the 40 bp barcode sequence, immediately after which the individual translocation sequences are located. They range in size between 54bp and 685bp and were cloned in with a BamHI and a NotI restriction enzyme. After the restriction site another 20 bp are there as a spacer before the polyA sequence starts.

5.6 Virus Production

To simultaneously package those 10 expression-constructs, the standard AAV production procedure with expression in a stable producer cell line followed by a CaCl₂ gradient purification was slightly modified.

Production of this virus was carried out in the HEK293 cell line. Stock cells of this line were stored in cryovials kept in liquid nitrogen. For proper freezing at those temperatures a cryoprotectant i.e. DMSO had to be used which has the property of being toxic if the cells are above 4°C, thus special care had to be taken at the thawing process. The screw-cap cryocvials were taken from the liquid nitrogen freezer and immediately put into a 37°C water bath where they were thawed for 1-2 minutes under constant agitation. After the cells were completely thawed the vial was wiped with 70% alcohol and transferred into the cell culture hood.

There the cells were slowly put into the beforehand prepared pre-warmed growth medium inside a 15 mL Eppendorf tube and centrifuged for 10 minutes at 200xg.

The supernatant containing the now toxic DMSO was discarded and replaced with fresh growth media (complete medium/10% FBS/1% L-Glutathin/1% Penicilin-Streptomycin). Cells were now transferred into a culture plate with the appropriate amount of medium and put into the incubator at standard conditions (37°C humidified condition, 5% CO₂) for about 24h to give the cells time to regenerate and properly attach to the plate.

After 24h the condition of the cells was checked under a phase contrast light microscope to check for proper adhesion, absence of bacterial or fungal contamination and cell confluence, suitable cells were then subcultured.

For splitting growth medium, PBS and Trypsin were prewarmed to 37°C. The old media was removed from the culture plates and cells were washed with prewarmed PBS (about half the volume of the growth medium). This step is necessary in order to remove the entire growth medium because of its property to inactivate the Trypsin. After its removal the cell monolayer was treated with the appropriate amount of Trypsin. The flask was rotated until all the surface was covered in Trypsin and put back into the incubator for another 5 minutes. If cells still aren't properly detached the sides of the flask are gently tapped until all cells are released. The cells are now resuspended and carefully rinsed in a small amount of growth to inactivate the Trypsin. After another cycle of centrifugation and resuspension the cells are again plated in the appropriate ratio onto new flasks.

For the virus production about 10-11 x 175 cm² flasks with a confluence of 70-90% are needed, cells were split every other day until this amount was reached.

24 hrs before the transfection, 9 x 10⁶ HEK293 cells were seeded out in 20 mL DMEM growth medium inside a 150 mm culture dish to ensure 70 – 90 % confluency at the time of transfection.

The amount of the mixed AAV Plasmids, Cap Plasmid and Helper plasmid was calculated so that the ratio between them was equimolar and totaled a DNA amount of 78.62 µg / 150mm dish.

Therefore an equimolar mixture of all AAV Plasmids that are desired to be co-packaged had to be made. This was achieved by calculating firstly for each plasmid its proportional share per plasmid size. So for an arbitrary chosen total of 100 µg that would be

$$\mu g(Plasmid\ x) = \frac{100\mu g}{total\ plasmid\ length\ of\ all\ plasmids\ x\ individual\ plasmid\ length}$$

Equation 2: Equimolar Mixture

Each expression plasmid that is to be co-packaged was now combined in the calculated amounts to get the equimolar mixture, the new total concentration is thereby dependent on the individual concentrations of the plasmid used.

The mixture is now treated as a single pseudo plasmid with a length of the average of all combined expression plasmids. Using the same calculation as in [Equation 2](#) now an equimolar mix of the pseudo plasmid, the Helper plasmid and the Cap plasmid was made and with this solution preceded as follows:

Per 150mm culture dish 78.62 µg of the combined Plasmid DNA was put into a 50ml falcon tube and combined with 2.62 mL of a 300mM CaCl₂ solution where it was carefully mixed without vortexing. Meanwhile a second 50ml falcon tube containing 2.62 mL of a 2xHBS solution was prepared; with the consideration that one 50ml falcon tube can only contain enough transfection solution for 5x150mm culture dishes. To this 2xHBS solution the DNA/CaCl₂ mixture was now applied in a dropwise manner with one Pipettboy while the reaction was carefully mixed with a second Pitpettboy. Afterwards the reaction was again one final time mixed by inversion and then incubated for 2 minutes. The mixture should be light and cloudy at this point. The transfection mixture was then added to a dish in a dropwise manner under constant agitation. After 6 hours the medium can be changed to fresh DMEM growth medium and will continue to incubate until about 48-72 hours until the cells are ready for virus purification.

For cell harvesting 0.5M EDTA was added to the dish - that is 12.5µl/ml medium - so that it reaches an end concentration of 6.25Mm. The dish was then incubated for 3-15

minutes at RT after which cells were transferred to 50ml falcon tubes. The remains were collected by washing the dishes once with a small amount of Medium or 1xDPBS. The cells were now centrifuged for 15 minutes at 500xg and 4°C. After the supernatant was removed the pellet was resuspended in lysis buffer (3ml/150mm-dish).

Cell lysis was performed by three consecutive Freeze/Thaw-Cycles with a maximum amount of about 30-35ml Lysate per 50ml-Tube. The Freezing step was done for 15 minutes in Liquid Nitrogen (N₂) while the thawing step was performed for another 15 minutes in a 37°C waterbath.

Cell lysate was treated with 200U Benzonase per 1 mL of Lysis Buffer for one hour on a shaker (100 rpm) at 37°C. Subsequently the reactions were centrifuged at 2500xg for 15 minutes at RT and supernatant was collected.

For precipitation 1M CaCl₂ was added to the supernatant in 1/39th of the volume so that the solution ends up with a final concentration of 25mM of CaCl₂. After careful mixing it was incubated on ice for 1 hour and then centrifuged inside of 50 mL Tubes at 2500xg and 4°C for 15 minutes. The supernatant was collected and 1/4th of the volume PEG-8000 Solution was added. The solution was again mixed and incubated on ice for a minimum of 3 hours. After another centrifugation step at 2500xg and 4°C for 30 minutes the supernatant was discarded and the pellet resuspended in resuspension buffer (26.5 mL Resuspension Buffer/10x150mm-dishes). The solution was incubated overnight (16-24 h) at 4°C on a rotator.

After incubation the tubes were shortly vortexed and centrifuged (2500xg, 30 min, 4°C). The supernatant was collected and prepared for the first CsCl-Gradient. After measuring the volume of the supernatant the refractive index of the lysate was measured by adding 200-300 µl of it to the refractometer.

The refractive index of the virus suspension was adjusted to 1.3710 by adding CsCl or 1xPBS respectively and then transferred to 29.9mL OptiSeal tubes. All bubbles were carefully removed, the tubes properly balanced and their necks properly dried. The Centrifugation was performed in a 70Ti angle rotor for 18-23 hours at 45,000rpm and 21°C.

To get to the fraction containing the virus a hole was put into the tube using a 20G x 1 ½ needle and the respective fractions were collected in 1.5mL eppendorf tubes. Usually, the virus can be found between fractions 3 and 12, correct fractions were determined by measuring the refractive index which should be between 1.3775 and 1.3700. The correct fractions were pooled together.

The combined fractions were now transferred into a Slide-A-Lyzer Dialysis Cassette with a Braun Omnifix Syringe and put inside a beaker containing Dialysis Buffer. This beaker was then put into the Cold Room onto a magnetic rotator. Every 2 – 24 hours the Dialysis Buffer was changed in a 6 step regiment, whereas 1xDPBS was used for step 1-4 and 1.111xDPBS for step 5 & 6.

After the final dialysis step the virus suspension was collected from the cassettes and Glycerin was added to an end concentration of 10%.

At last the AAV was concentrated by Ultrafiltration. To prepare the Ultrafiltration Unit the membrane was equilibrated three times using 1xDPBS, after each time the equilibration buffer was removed by centrifugation at 2.500xg (1 min, RT) without letting the membrane dry out completely. Afterwards the AAV solution was applied to the Ultrafiltration Unit (25-35 mL per unit) and again centrifuged at 2.500xg and room temperature. It was concentrated dependant on the amount of 150mm-dishes of HEK293 cells used for virus production with approximately 375 µl per 10 x 150mm dishes.

After the concentration the AAV was sterile filtered using a Millex-GV Filter Unit.

To determine the virus titre DNA was extracted from 50 µl of the viral prep using the DNA/RNA Extraction Reagent ViralXpress (EMD Millipore), thereby 200 µl of ViralXpress extraction reagent was transferred to a nuclease-free tube and the 50 µl of virus were added. The resulting solution was vortexed for 10 seconds and incubated for 5 minutes at RT. Afterwards 250 µl of isopropyl alcohol were added to the tube and the solution was vortexed for an additional 10 seconds. After a 10 minute centrifugation at 16,000xg at RT the supernatant was discarded and the pellet was washed by adding 400 µl of 70%

on top of the pellet. The vortexing and centrifugation was repeated with the same conditions after which the supernatant could be removed and the pellet left out to air dry. After resuspension in 50 µl of nuclease-free water the DNA was ready for downstream applications like the quantitative PCR to determine the yield, as described farther below.

5.7 Alkaline Gel Electrophoresis for the comparison of double- and single stranded viral DNA

Each virus sample, including a positive and negative control gets treated with the ViralXpress DNA/RNA Extraction Reagent (EMD Millipore) to extract its DNA. In the meanwhile a 0.8% alkaline gel is casted by weighing 0.48g Agarose in an Erlenmeyer flask and filling it up to 54mL of H₂O. The solution is put into a microwave until the Agarose has dissolved and is then swirled under running cold tap water until it cools down to about 55°C, now 6mL of an 10x alkaline Gel-electrophoresis buffer is added and a small 10cm gel is cast with an appropriate comb.

After the Gel hardened each sample combined with 6x alkaline loading buffer as well as 15 µl of the Gene Ruler 1kb Plus (Thermo Fisher Scientific Inc.) are loaded onto the gel and let run for 2 hours at 50V while the gel was weight down with a glass plate.

After the 2 hours the gel was treated for 45 minutes with the Neutralizing Buffer on a tilt shaker (Heidolph Instruments GmbH & Co KG). During the neutralization a SYBR-Gold staining solution was created by combining 150mL of 1xTAE with 15 µl of SYBR-Gold (1:10000). The gel was eventually transferred to a staining tray together with the SYBR-Gold solution and covered with aluminium foil. Pictures were taken after 3h and 20h.

5.8 Stereotactic Injections

In order to place a pipette into the brain of a living mouse for consequent virus injection, corresponding to the coordinates depicted in a brain map such as “The Mouse Brain in

Stereotaxic Coordinates” by Paxinos and Franklin, a 3-axis manipulating device like the 1900 Stereotaxic Alignment System is needed. The system is moving along 3 mutually perpendicular planes named X, Y and Z to a predetermined point in 3-dimensional space. Before use the 1900 Stereotaxic Alignment System has to be calibrated. For this the head holder has to be removed with a 7/64” hex key wrench to expose the base plates’ precision-ground top surface as a precision reference plane to check the perpendicularity of the electrode manipulator.

After installing the Model 1910 Dial Test Indicator into the tool holder block readings are taken at the positions of 12 o’clock and 6 o’clock and the corresponding joints are adjusted until the readings align, afterwards readings are taken at 9 o’clock and 3 o’clock and the procedure is repeated, so as to only adjust one plane at a time. After sweeping the surface plate the head holder was re-attached and the sweeping was done in a Medial-Lateral Slide movement, using the edge of the head holder as a reference plane. After adjusting the joints the setup was calibrated and ready for surgery (Stereotaxic Operation Manual, Model 1900).

In the immediate preparation for the surgery mice were anesthetised by intraperitoneal (i.p.) injection of 0.2 ml ketamine/medetomidine solution (final concentration of 10 mg and 80 µg per mL respectively) and kept on a heating pad until full narcosis. Depth of anesthesia is thereby monitored by a lack of response from toe-pinch or tail-pinch. In the meanwhile the surgery setup and tools were cleaned with 75% Ethanol and the 3-axis manipulating device was set up. The Centering Height Gauge was installed into the socket provided in the head holder base and the Centering Scope into the tool holder. The Centering Scope was then subsequently focused onto the markings on top of the Centering Height Gauge in order to set a central reference point for the Stereotaxic device. At this position the DRO (Digital-Read-Out) scales were set to zero. Now the Centering Scope was replaced with the Stereotaxic Alignment Indicator and properly fas-

tened. It was lowered until the touch probes touch the top of the gauge and the indicator needles point approximately straight up. If the needles did not point at zero the indicators were adjusted by rotating the indicator bezel (Stereotaxic System Operation Manual, Model 1900).

The mouse was now injected with 0.2 ml Xylanaest (Gebro Pharma) under the scalp and 0.02 ml of Dexamethasone intramuscularly (i.m.) in order to avoid inflammation and post-surgical pain. After a ten minute incubation period the mouse was put onto a heating pad to keep its body temperature steady at 37°C and installed into the adaptor by inserting the palate bar into its mouth while making sure to keep the tongue under the palate bar. Now the nose clamp was carefully slid back over the nose and tightened. As an additional fixation measure the ear bars were inserted into the ear canals for optimal stability.

To protect its eyes from drying out and possible damage by light irradiation Vaseline was applied to them and a small piece of aluminium foil was put on top of them. Afterwards its head was cleaned and disinfected with 75% Ethanol whereby the hairs on top of its head were combed unidirectionally to the back. Now a midline skin incision was made from the front to the back of the skull and the skin was pulled laterally to the side. The exposed skull was then carefully cleaned with in Cortex Buffer soaked q-tips and subsequently dried with an air syringe to avoid view obstructing reflections when viewing through the macroscope.

After the clean up the Model 1915 Scope was installed into the tool holder and the head of the mouse was brought under focus. Thereafter Bregma, defined as the anatomical point of the skull where the coronal suture intersects the sagittal suture, was aligned with the cross-hair of the scope by using the lateral shift knob, A-P shift knob and the vertical shift knob, with the DRO scales still indicating zero (Stereotaxic System Operation Manual, Model 1900).

From this point the electrode manipulator was used to align the cross hair over Lambda while looking down through the scope, whereby Lambda was defined as the point on

the skull where the two parietal bones and the occipital bone come together. The value depicted by the DRO should be around 4.0, but a certain variation between individuals and different age groups should be beard in mind.

At this position the DRO of the Y-axis was read and noted down, and the thereby implied distance between Bregma and Lambda was set on the Stereotaxic Alignment Indicator. The electrode manipulator was positioned at exactly half the distance between the two points and retracted along the Z-axis to a safe height approximately 1.0 mm above Bregma. The centering scope was removed and the Stereotaxic Alignment Indicator installed instead. By lowering it slowly onto the skull the probes were carefully positioned to cover both Bregma and Lambda.

Now the head was levelled between the two points in the Dorsal plane by lowering the Stereotaxic Alignment Indicator until at least one of the indicators read "0". If the other indicator deviates from that value the head is adjusted to level with the Dorsal Control Knob. The same is now repeated for the Coronal plane. The Stereotaxic Alignment Indicator is retracted to a safe height above the skull and the indicator assembly is rotated clockwise by 90 degrees. Now it is again lowered onto the skull until one of the indicators reads "0" and the head again levelled by using this time the Coronal Tilt Adjust Knob. With this the head is now level in both Dorsal and Coronal planes. At last the alignment in the sagittal plane is refined. This is done by first installing the centering scope into the tool holder and bringing it into focus. Using the cross hair the alignment between Bregma and Lambda is now adjusted by unlocking the Sagittal locking screw and using the Sagittal Alignment Knob. This concludes the head adjustment and the skull is now ready for drilling.

Since the DRO is still set for Bregma as "0" for all axes, the drilling unit can be installed without further adjustments and directed toward the desired drilling site. To open the skull over the Auditory Cortex drilling is performed with the approximate coordinates of:

Medial-Lateral:	4.500 mm
Posterior:	-2.500 mm – -3.500 mm
Dorsal/Ventral:	2.500 mm

Thereby the drill is brought down and moved along the anterior-posterior axis to drill a line, going deeper in little steps without breaking through the skull completely. If there is only a small layer of skull left the drill is retracted a bit and removed from the tool holder (Stereotaxic System Operation Manual, Model 1900). The last layer of bone is removed carefully with a scalpel as to not injure the dura mater. After this is achieved the hole in the skull is temporarily covered with a small, in Cortex Buffer soaked piece of cotton, while a long-tapered, thin glass cannula is pulled with a P-97 Sutter Pipette Puller (Sutter Instruments). The fresh cannula is now filled with oil and inserted into the electrode holder, which in turn is inserted into the tool holder. Owing to the properties of cannula production the tip of the cannula is still closed, for this reason a small part of the tip is removed with a surgical scissor and some of the beforehand inserted oil is removed via the SYS-Micro4 Micro Syringe Pump Controller (David Kopf Instruments), while at the same time flow and speed of liquid release is surveyed to guarantee appropriate efflux for the injection. Now the Virus is administered to a small piece of parafilm and drawn up into the cannula. The tip of the cannula is again centred over Bregma and the DRO set to zero. From this point the same coordinates as above are used for the injection. Starting from Posterior -2.500 mm 4-5 injections are made with 150 µl each while going about 0.250 mm more posterior with each injection. Each injection of 150 µl is set for 5 minutes and the cannula is kept inside the brain for an additional 3 minutes after the injection is finished to guarantee proper diffusion of the virus before retracting the needle and preparing it for the next injection site.

After the last injection is done, the two skin flaps from the incision are pulled back over the open skull and glued together using Roti® Coll 1 Cyanoacrylate Glue (Carl Roth GmbH). When the glue had enough time to dry the mouse is released from the holders

and the Vaseline is cleaned from its eyes. To speed up the recovery from the anaesthesia 20 µl of Antisedan (Pfizer Inc.) are injected intramuscularly as well as 200 µl of Carprofen (Pfizer Corporation Austria) as a treatment for post-surgical pain and inflammation. Eventually the mouse is put back into its cage onto a heating pad until it had enough time to regenerate from the surgery procedure.

5.9 Slice Preparation

The brain was carefully removed from the storage buffer put onto an even surface. Using a sterile surgical blade (Lance Paragon Ltd.) the cerebellum was removed in a coronal manner. Afterwards another coronal section was made and part of the frontal lobe was removed, enough so that the complete auditory cortex is still intact. The brain was now tilted onto its rostral/frontal end and in that fashion glued onto the specimen holder, using Roti Col1 cyanoacrylate glue (Carl Roth GmbH&Co KG), while trying to keep the specimen as plain as possible. A fresh razor blade was now put into the vibrating head with the vibrating speed set to maximum. The blade was carefully positioned directly in front but still above the brain and the starting as well as the ending point of the section was set. The Vibratome (VT-1000 Leica) was set to a high vibrating speed, a low advance speed and the desired thickness of the slice and the cutting process was started. Brain sections covering the desired region were collected inside a 24-well plate (BD Bioscience) filled with PBS.

5.10 RNA extraction and cDNA conversion

In preparation of the dissection, the workspace and all tools were thoroughly treated with RNaseZap (Thermo Fisher Scientific Inc.) to remove any RNase decontamination from all surfaces that come into contact with the tissue. Next, 3 ceramic beads were

added to 2mL Screw Cap Microcentrifuge Tubes and filled with 1mL Qiazol Reagent (QIAGEN) each. The tubes were put on ice for the dissection.

The previously injected mice were now decapitated and the brain removed from the skull in a quick manner. The desired tissue samples were taken with a scalpel in as little time as possible and each was put into one of the Screw Cap Tubes containing the now ice cold Qiazol. The tubes were homogenized 2x30 seconds at 5000 rpm in the tissue homogenizer (Bertin Technologies).

During the homogenization the workspace and tools were again cleaned with RNaseZap and Filter Tips and ice was prepared for an RNase free work environment.

To the homogenized sample 200 μ l of chloroform was now added and vigorously shaken for 15 seconds. After a 3 minute incubation period at room temperature the sample was centrifuged for 15 minutes at 12.000g and 4°C. The sample should now be separated into 3 separate phases, an upper aqueous phase containing RNA; an interphase containing DNA; and a lower organic phase containing protein. The upper phase was now transferred into a new tube, while the other phases were frozen away for a possible later DNA or Protein extraction. To the aqueous phase was now added 1 volume of freshly prepared 70% Ethanol and thoroughly mixed by vortexing. Up to 700 μ l of sample were subsequently transferred to an RNeasy Mini spin column placed into a 2mL collection tube. The washing steps were then performed as per protocol of the RNeasy Lipid Tissue Mini Kit. After the last wash with an ethanol containing buffer solution the spin column was carefully removed as to avoid ethanol contamination and placed into a new 2 mL collection tube, in which it was spun down for 1 minute in the centrifuge at full speed to eliminate possible buffer carryovers.

The column was now put into a new RNase-free 1.5 mL collection tube and 50 μ l RNase-free water was added directly onto the spin column membrane. The column was now centrifuged for 1 minute at 8.000g to elute the RNA.

RNA concentration and purity of sample was now checked on a Nano Drop UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.). If the results were satisfactory the sample was used for the cDNA conversion.

Therefore 34 µl of the sample were transferred to a new micro centrifuge tube, considering that for each sample there should be at least 1-5 µg of RNA in those 34 µl. The tube was then heated to 65°C for 10 minutes and then chilled on ice for another 2 minutes. Next the RNA solution was transferred to one of the Ready-to-Go You-Prime First-Strand Beads reaction mix tubes (GE Healthcare) and 0.5 µg of Oligo(dT) primers and DEPC-treated Water was added to a final volume of 35 µl. The RNA solution was now left at room temperature for about 1 minute before it was gently mixed by vortexing and centrifuged to collect all contents of the reaction at the bottom. After one hour at 37°C the first-strand reaction is now finished and ready for further handling.

5.11 qPCR

In a standard Polymerase chain reaction specific DNA sequences from relatively small amounts of starting material are amplified using biochemical processes. It therefore mimics the DNA replication in vitro using one key enzyme, the thermophilic DNA polymerase.

Real-time PCR is based on this fundamental principle but shifts the focus from the end product to the entire process of the PCR (Dorak, 2006). In order to detect specific DNA sequence the addition of a marker such as Ethidium Bromide or Sybr-Green is necessary; this detection is possible without opening the reaction tube. And since those markers bind only to double stranded DNA, an increase in fluorescence will indicate a positive amplification. The quantitative PCR is therefore able to amplify a specific DNA Fragment and simultaneously quantify its product (Higuchi, et al. 1992).

In order to find appropriate primers that display optimal properties namely:

- G/C content between 50-60%

- Length between 18-24 bp
- Amplicon size should be between 70-150 bp but may not exceed 200 bp
- Melting temperature aimed at 60°C
- Gene specificity
- No primer dimers or secondary structures
- If possible one primer should stretch across an exon-exon border

Different candidates were created for each target with the PerlPrimer Software by Owen Marshall (©2003-2011). Special attention in design was paid to the attributes that the targeted amplicon size did not exceed 70-150 bp, that the melting temperature of both primers did not stray far from 60°C in either direction and that their GC content was between 40-60%

For optimal performance a test qPCR was performed for each primer combination on a plasmid containing the appropriate target sequence. A known amount of plasmid was therefore diluted in three consecutive 1:10 steps and a curve was produced from the results. The efficiency was calculated using the following formula

$$E = -1 + 10^{\frac{-1}{slope}}$$

Equation 3: qPCR Efficiency

Taking those results into account the primer pair with the efficiency closest to 1 was then chosen. To evaluate the quality of results also the R² value was examined, excepting only values >0.95 and aiming towards >0.99 for good confidence in correlating two values.

Directly after the PCR also a Melt Curve Analysis was performed by heating the reactions from 55°C to 95°C in 0.5°C increments with 5 seconds per step. The results were analysed to assess possible primer dimers.

For the actual experiments 1:4 dilutions of the cDNA and reference plasmid were made in 15 aliquots and the appropriate dilutions were used for the qPCR. The reference plasmid was diluted from 100ng to 0.1 fg, to be in the range of the target gene dilutions 8-15 were used in the experiment. For the cDNA dilutions 1-6 were used.

The PCR reactions consisted of 12.5 µl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.), 0.75 µl of each primer (30 µM), 5 µl of the Template and 5 µl of H₂O to reach a total volume of 25 µl in each well.

The Protocol for the CFX Connect Thermal Cycler (Bio-Rad Laboratories Inc.) was as follows:

Polymerase Activation and Denaturation:	95°C	3 min
→ 40 cycles:		
Denaturation:	95°C	15 sec
Annealing/Extension:	60°C	30 sec

The results of the reference gene were plotted on a curve and values for y at the linear region were noted down and used to calculate the number of copies of the cDNA using the formulas

$$y = c(t) \text{ value}$$

$$x = \log(\text{conc.})$$

$$\text{Concentration} = 10^{(\log \text{conc.})}$$

Equation 4: qPCR Concentration.

The concentration was eventually multiplied with the amount of input and the resulting total copy number compared between individual samples.

5.12 Sequencing

After the total RNA extraction with the RNeasy Lipid Tissue Mini Kit (QIAGEN) RNA concentration was measured and 10 µl of each sample were saved for gel analysis.

The rest of the sample was adjusted to a concentration of 750 ng/µl with DEPC-treated water and used for mRNA Isolation via Oligo-dT₂₅ Beads (Dynabeads® by Thermo Fischer Scientific Inc.)

To prepare the Dynabeads they were resuspended in the vial by vortexing and the desired volume of beads was transferred to a fresh RNase free tube. 2x volume of beads were thereby used for 1x volume of RNA solution.

To the new tube 1x volume of 2x Binding Buffer (Materials; Buffers and Solutions) was added and resuspended, afterwards the tube was placed in a DynaMag-2 magnetic rack (Thermo Fisher Scientific Inc.) for 1 minute; the supernatant thereby cleared from the beads was discarded. The tube was now removed from the magnet and the beads were resuspended in the same volume of 2x Binding Buffer as the initial volume of Dynabeads taken from the stock.

To the RNA 100 µl of Binding Buffer was added, if the total RNA was more dilute an equal volume of Binding Buffer was also added to the Dynabeads. In order to prepare the RNA to bind to the Dynabeads it was heated for 7 minutes to 65°C to disrupt secondary structures and afterwards immediately placed on ice for at least 3 minutes. Now the 200 µl of total RNA were combined with the 100 µl of washed beads for each sample. The intended calculation hereby is to use 1 mg of beads for every 75 µg of total RNA. The RNA/beads solution was now thoroughly mixed and allowed to bind to each other by rotating continuously on a platform shaker for 5 minutes at room temperature. Now the tube was again placed on a magnet for 2 minutes after which the supernatant was carefully removed. Instead 200 µl of Washing Buffer B (Materials; Buffers and Solutions) was added aside from the magnet and carefully mixed by pipetting up and down. Now the tube was put onto the magnetic rack again and the washing procedure was repeated once more. To eluate the mRNA from the beads, Washing Buffer B was removed and 50

μl of 10 mM Tris-HCl added instead. The beads were heated to 80°C on a heat block for 3 minutes and then immediately transferred onto the magnetic rack where it again incubated for 1 minute. Finally the now solute mRNA was quickly transferred to a new RNase-free tube.

Again the RNA concentration was measured on a Nanodrop Spectrophotometer (Thermo Fisher Scientific Inc.) and 10 μl of sample were saved for gel analysis while the beads were stored in Storage Buffer (Materials; Buffers and Solutions) at 4°C.

To remove any residual genomic DNA from the freshly extracted mRNA, the samples were treated with Turbo DNase I (Thermo Fisher Scientific Inc.)

88 μl of mRNA with a max concentration of 200 ng/μl were combined with 10 μl of Turbo DNase Buffer (Thermo Fisher Scientific Inc.) and 2.4 μl of Turbo DNase I (scale to concentration) and incubated for 30 minutes at 37°C.

Afterwards the samples were cleaned up using the Qiagen RNeasy MinElute clean up kit (QIAGEN) to inactivate the Turbo DNase I and to concentrate the RNA. All steps of the clean-up were performed per manufacturers' protocol.

The mRNA was now converted to cDNA using the SuperScript®III (Thermo Fisher Scientific Inc.) Reverse Transcriptase.

A maximum of 4-5 μg of RNA per sample were combined with 1 μl of dNTPs (Thermo Fisher Scientific Inc.) and 1 μl of a 2 μM Gene specific primer (ATCGAGATCGCAGGTGAG) and then filled up to 13 μl with RNase free Water. The RNA was heated to 65°C for 5 minutes and then put on ice for an additional minute.

Meanwhile a Mastermix of 4 μl FS5x Buffer (Thermo Fisher Scientific Inc.), 1 μl 0.1M DTT (Thermo Fisher Scientific Inc.), 1 μl RNaseOUT (Thermo Fisher Scientific Inc.) and 1 μl SSIII per sample were made and then added to the previously made 13 μl RNA mixture to a combined volume of 20 μl. Hereby special attention is paid to a fast handling since

both RNaseOUT and SSIII are unstable enzymes and can only be removed from the freezer with a ThermoBlock for a small amount of time.

The samples were then incubated at 50°C for 1 hour and afterwards at 70°C for an additional 15 minutes.

Now that the mRNA has been converted to cDNA, the samples were treated with RNase A (Thermo Fisher Scientific Inc.) to degrade as much of the starting product as possible. Therefore 1 µl of a 10mg/ml RNaseA solution was added to each sample and then incubated for 1 hour at 37°C.

The resulting product was then purified via the MinElute PCR purification kit (QIAGEN) as per manufacturers' protocol and eluted in 30 µl of Buffer EB.

To further increase the divergence between the expressed gene product and the viral input a junction PCR was performed, spanning with one Primer over an artificial Intron strategically placed in the viral construct.

Therefore the following reaction was pipetted for each sample into a separate PCR tube.

X µl	cDNA (max 400 ng)
25 µl	KAPA 2xHiFi (Kapa Biosystems)
2.5 µl	junction_1.1 fw 10 µM (CAGATGCCAGGATATCAGGA*A*C*C*T*G)
2.5 µl	junction_2.3 rv 10 µM (ATCGCAGGTGAGGCCT*A*G*C)
to 50 µl	H ₂ O

* = phosphorothioate bond

For the PCR the following settings were used:

Denaturation:	98°C	45 sec
→ 15 cycles:		
Annealing:	65°C	30 sec
Elongation:	72°C	70 sec
Final Elongation:	72°C	60 sec

Subsequently the PCR products were purified using the QIAquick PCR purification kit (QIAGEN) as per manufacturers protocol and eluted in 100 µl of Buffer EB.

For the test sequences to bind to the sequencing chip an adapter sequence has to be added to the cDNA. In my case this was achieved through a second PCR, with the Adaptor sequence joined to the forwards primer and a second adaptor sequence as well as a barcode unique to each sample added to the reverse primer.

To test how many cycles are necessary for the PCR product to show up on a gel, three separate PCRs were carried out, with 15, 20 and 25 cycles respectively.

20 µl	junction PCR product
25 µl	KAPA 2xHiFi Mix (Kapa Biosystems)
2.5 µl	PE_1.0 (10 µM)
2.5 µl	PE RV 1 (10 µM)

Denaturation:	98°C	45 sec
→ 15, 20 & 25 cycles		
Annealing:	65°C	30 sec
Elongation:	72°C	30 sec
Final Elongation:	72°C	60 sec

The resulting PCR product was analysed via gel electrophoresis on a 1% agarose gel, 95V, 45 min. Based on the results the number of cycles for the final seqReady PCR was determined.

The seqReady PCR used the same conditions as the PCR above, with the now determined number of cycles. For each sample a different PE RV primer was used to give them all a different barcode and thereby permit multiplexing of samples during Illumina Next-Generation Sequencing (Illumina Inc.).

Finally the PCR product was purified using AMPure beads (Beckman Coulter Inc.) to remove primer dimers resulting from the previous adapter addition.

Therefore AMPure beads were thoroughly mixed by vortexing before 0.8 volume of beads were added to 1 volume of DNA, which were then mixed by pipetting the solution up and down 20 times. After a 15 minute incubation period at room temperature they were put onto a magnetic separator (Thermo Fisher Scientific Inc.) where they incubated for another 15 minutes.

The beads should now be collected to the wall of the tube while the liquid should be clear, at this point all the liquid is removed from the samples while the tubes are kept on the magnet until elution. The beads are now washed 2 times with 1 ml of 80% Ethanol in which they incubate for 2 minutes. Afterwards the beads are air dried for 10 minutes at room temperature, therefore the ethanol is removed and the beads are kept on the magnetic rack with open lid.

To now eluate the DNA from the beads 20 μ l of RNase free H₂O is added per reaction and mixed by pipetting up and down 25 times. Afterwards the beads are put onto a heat block at 37°C where they shake at 300 rpm for about 3 minutes. Immediately following this step they are put back onto the magnet and incubated for 1 minute in a backwards tilted position. The DNA solution is now ready to be transferred into a new tube where they can be stored at -20°C or eventually be used for deep sequencing.

Oligo-dT₂₅ Dynabeads can be used several times, in order to recover them the following washing steps were carried out after each mRNA extraction.

Dynabeads were pooled together and put onto the magnetic separator for 1.5 minutes until the solution is clear. Afterwards the solution is removed completely using gel loading tips (Thermo Fisher Scientific Inc.) and instead 1 volume of Reconditioning Solution (Materials: Buffers and Solutions) is added. The beads were placed on a heat block at 65°C and shaken at 300 rpm for about 10 minutes. Afterwards they were washed 2 times

with Reconditioning Solution, incubating them each time for 1.5 minutes on the magnetic separator. The Reconditioning Solution was removed entirely and the beads were now washed with Storage Buffer (Materials: Buffers and Solutions) instead. The procedure was repeated a total of 3 times again with an incubation period of 1.5 minutes. Eventually a final volume of Storage Buffer was added and the beads were stored at 4°C until the next use.

6 Results

6.1 Cloning of a reporter construct for the investigation of axonal transport sequences

The goal of this study was to multiplex the production of rAAV vectors and use the resulting library in an assay to test 10 different mRNA sequences for axonal targeting. In order to create a construct that was able to perform such an assay, the viral expression plasmid needed at least the following contents: an origin of replication and an Ampicillin Resistance gene, two AAV ITR sites with a mutated terminal resolution site (TRS) that promotes self-complementation of the viral DNA, a promoter limiting the transcription of the viral DNA to neurons, a reporter gene containing an Intron, a site for the insertion of the desired localization sequence and a polyadenylation site.

For this reporter construct we used the human synapsin I promoter, as it confers highly neuron-specific transgene expression over several months (Kuegler, Kilic & Baehr, 2003). The promoter was cloned out of the “131_pAAV_hSyn-3NLS-RGECO” plasmid (Figure S 4) (Material and Methods; PCR) and inserted into the vector “pscAAV_CMV-GFP” (Figure S 2) where it replaced the existing CMV promoter (Material and Methods; Restriction Enzyme Digest, Ligation) since the plasmid already had the required ITR site for the self-complimentary AAV production as well as a streptomycin resistance gene and an SV40 polyA site. The utilization of an expression boosting self-complimenting AAV was necessary to compensate for the proportionately lower viral count of each virus in the final vector mix compared to the results of single packaging.

As a reporter gene I used the improved Cre recombinase (iCre). Although the gene itself is not explicitly necessary until a possibly ensuing follow-up experiment, the reporter gene also contains a Betaglobin Intron, which is indeed necessary if the transported mRNA is to be extracted and analysed as it will provide the ability to differentiate between the administered viral DNA itself and the cDNA during sequencing. As cDNA undergoes RNA maturation during which the RNA gets spliced and hence all Introns are

removed, it possesses an Exon-Exon border which provides us with a specific site on our gene of interest, which will be used to design a cDNA specific primer (Figure 8).

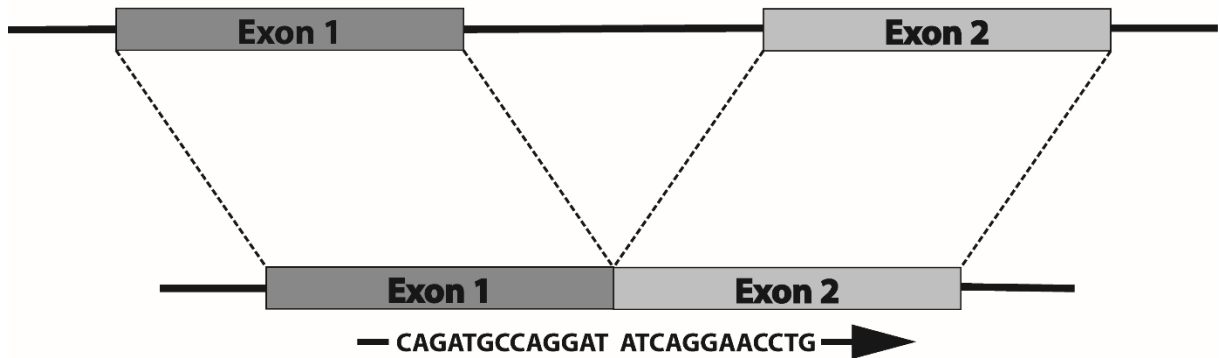


Figure 8: New Primer Binding Site by RNA splicing. The Splicing of the iCRE Intron provides a new Primer Binding Site. This site is only present on cDNA but not on viral DNA.

iCre was cloned out of the “46_pCMV-MCS-iCRE-mKO-betaglobinintron” plasmid (Figure S 3) (Material and Methods; PCR) and cloned into the modified “pscAAV_CMV-GFP” vector to replace the existing GFP gene (Material and Methods; Restriction Enzyme Digest: Ligation).

Between said iCre gene and the terminal polyA site 50 nucleotides of the inert backbone sequence were deliberately left in place. This sequence contains two restriction enzyme sites (BamHI and KpnI) and acts as a cloning site for our array of localisation sequences

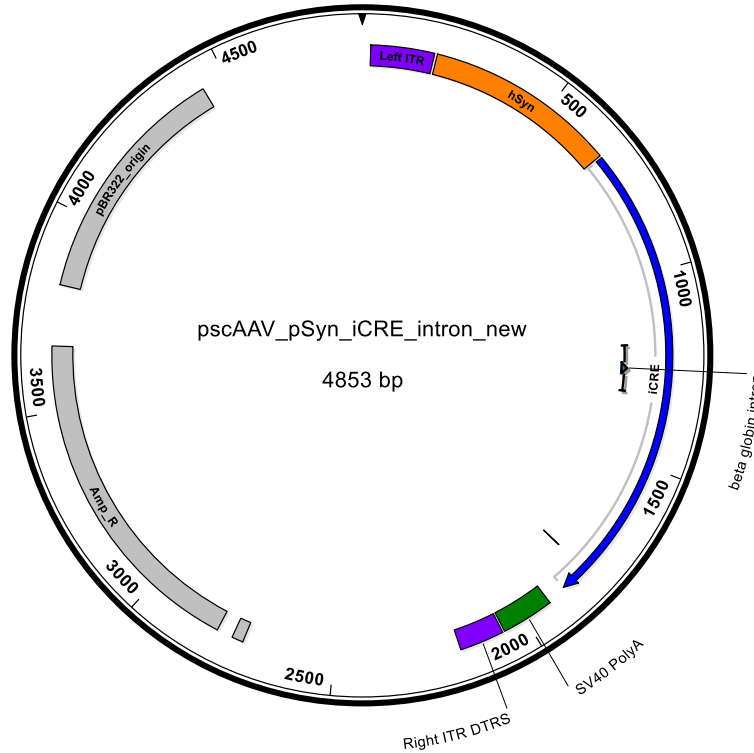


Figure 9: Plasmid Map 143_pscAAV_pSyn_iCRE_Intron. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance

whose effectivity and efficiency will be tested during the ensuing experiments. The resulting reporter template construct and viral expression vector “143_pscAAV-hSyn-iCRE-intron” is shown in Figure 9.

6.2 Cloning of a Synaptic Targeting Library

Based on the previously cloned Expression Construct p143 (Figure 9) I created a small cDNA library as a basis for the co-packaging experiment by cloning ten different versions of p143 varying only in their Localization Sequence and a small individual barcode.

Those ten sequences consisted of five positive controls whose ability to facilitate mRNA transport has already been shown in the literature, namely the complete Actin Beta

3'UTR, the singled out 54 nucleotides of the Beta Actin zipcode, the tau mRNA zipcode and both calreticulin zipcodes as well as five negative controls taken from inert regions of different plasmid backbones (Table 18).

Table 18: Localisation Sequences

<i>Positive Control</i>	Abbreviation	Length [bp]	Sequence
<i>Actin Beta full 3'UTR</i>	ABfull	651	gcggactgttactgagctgcgttttacacccttctttgacaaa acctaacttgcgcagaaaaaaaaaaaaataagagacaacatt ggcatggccttggtttttaaatTTTTTaaagTTTTTTTTTTT TTTTTTTTTTTaaagTTTTTgtttgtttggcgctttgactca ggatttaaaaactggaacggtgaaggcgacagcagttggttg gagcaaacatccccaaagtctacaaatgtggctgaggact ttgtacattgtttgtttttttttttgtttgtctttttaata gtcattccaagtatccatgaaataagtggttacaggaagtccc tcacctcccaaagccacccccactcctaagaggaggatgg tcgctccatgccctgagtcaccccggggaaggtgacagca ttgcttctgtgtaaattatgtactgaaaaattttttaaatcttc cgcttaataacttcatTTTgttttaatttctgaatggcccaggt ctgaggcctccctTTTTTgtccccaacttgatgtatgaagg cttggctccctgggagggggtgaggtgttgaggcagccag ggctggcctgtacactgacttgagacc
<i>Actin Beta Translocation Sequence (TS)</i>	AB54nt	96	gcggactgttactgagctgcgttttacacccttctttgacaaa acctaacttgcgcagaaaaaaaaaaaaataagagacaacatt ggcatggcctt
<i>Tau TS</i>	Tau	306	gtttggttcactccattacttctatcctggatgggaactg gtgtgtgcctgcctggggatgaccttgacctctgccttt tctttatctaagtggatgcctcctaggcctgactcctgt gttgagctggaggcagccaagtcaaggcgccaatgtcttg gcatcagtaagaacagtcaagagtcccagggcagggc cacacttctccatcttctgctccacccagcttgtgatc gtagcctcccagagctcagctgccattaagtcccatg cacgtaatcagtctccacacccagttt
<i>Calreticulin TS-1</i>	Cal1	98	aagccaaggatgagctgtagaggccacaccctgccttcag ggctggactgaggcctgaacacctgccgcagagctggctgc tccaataatgtctctatgagactcaagaacttttcatttttc aggcaggtt

<i>Calreticulin TS-2</i>	Cal2	113	ttcatctgctctccttctgcatctcagaggagggcagga gaaggggggtggtgtttccctccccgcactgaggaag aatggggctcttctcatcccccttcccttgccccagg actgggccacttggtggggcagccagttctagca
<i>Negative Control</i>	Abbreviation	Length [bp]	Sequence
<i>Actin G1 full 3'UTR</i>	ActG1	685	atggactgagcaggtgccaggcatctgctgcatgagctg atattgaagtatcaatttgcctggcaaatgtacacacc tcatgctagcctcatgaaactggaataagccttgaaaa gaaatttgccttgaagcttgatctgatatcagcactgg atcgtagaacttggtgctgattttgaccttgattcaagt taactgctcccttggtatatgttaataccctgtgcatatc ttgatttagtccttagttcatgtggctcggctcacttggggg ctggggagagcacgctgtagatgagaaagccccagcct ggtggatctctgtgagcaccactgagtgatctgtgcagg gtattaaccaacagcagacttccaggatttctgaggct ggcaaggggttctgaaccagttaccactccttcttgcca gtctaacaggggtgggaaagtcgagccttaggaccag tttcagttctggttcttccctcctgaccaccatcggttgtt agttgccttgagttgggaacggttgcacacacctgta aatgtattcattctttaattatgtaaggtttttgtactca attctttaagaaatgacaaatttggttttctactgttcaa tgagaacattaggccccagcaacacgtcattgtgtaaa gaaataaaagatcttctagaagatctcctacaatattc tcagctgccatggaaaatcgatgttctt
<i>p5 Helper Plasmid</i>	p5	241	gtgccacctaattgtaagcgtaatatatttgttaaaattc gcgttaaattttgttaaatcagctcatttttaaccaata ggccgaaatcggcaaaatcccttataaatcaaaagaat agaccgagataggggtgagtggttccagtttgaaca agagtccactattaaagaacgtggactccaacgtcaaa gggcgaaaaaccgtctatcagggcgatggcccactacg tgaacc
<i>F1 ori</i>	F1	281	ggtggttacgcgcagcgtgaccgctacacttgccagcgc cctagcgcgcgctccttctgcttcttcccttcttctcgc cacgttcgcccgtttcccgtcaagctctaaatcgggg gctccctttaggggtccgatttagtgctttacggcacctc gaccccaaaaaacttgattagggatggttcacgtagt gggccatcgccctgatagacgggttttcgccctttgacgt

			tggagtccacgttctttaatagtggaactcttgttccaaac tg
<i>E.Coli1 ori</i>	E1	289	ccccctgacgagcatcacaaaaatcgacgctcaagtca gaggtggcgaaacccgacaggactataaagataccag gcgtttcccctggaagctccctcgtgcgctctcctgttcc gacctgccgcttacggatacctgtccgcctttctccctt cgggaagcgtggcgctttctcatagctcacgctgtaggt atctcagttcgggtgtaggtcggtcgctccaagctgggctg tgtgcacgaacccccggttcagcccgaccgctgcgcctt atccgtaactatc
<i>LacZ alpha</i>	LacZ	138	ccattcgccattcaggctgcgcaactgttgggaagggcg atcgggtcgggcctcttcgctattacgccagctggcgaa agggggatgtgctgcaaggcgattaagttgggtaacgc cagggtttccagtcacgacg

The sequences were cloned out of a C57BL/6 mouse cDNA library and the backbone of the plasmids “5_pHelper”, “10_pLECYT(TetO)” and “26_pBluescripts” (Figure S 7 - Figure S 9) respectively (Material and Methods; Plasmids) and cloned into the Localization Sequence Site of the plasmid “143_pscAAV-hSyn-iCRE-intron” using the restriction enzymes BamHI and KpnI (Material and Methods; PCR, Restriction Enzyme Digest, Ligation). Additionally each plasmid got a 40 base pair long individual barcode put immediately in front of the localization sequence in order to be able to design primers specific to each plasmid while still guaranteeing optimal primer conditions. The 40 base pairs were again taken from an inert region of the backbone of plasmid “54_pAAV-Pam-Cherry1-NLS” (Figure S 6), resulting in the final DNA library. The final design of each plasmid can be seen in Figure S 10 – Figure S 19.

6.2.1 Test of barcode specificity

To make sure that the primers used for an eventual qPCR analysis give no false positive signal, by binding either to another mRNA construct, its own viral DNA or an endogenous

version of the translocation signal sequence, primer pairs that targeted each barcode as well as the exon-exon border of iCre had to be designed and tested for their specificity. As the viral mRNA would undergo splicing during the experiment, one primer used for the qPCR needs to span the exon-exon border of our reporter gene in order to differentiate between the viral genome and the transcribed mRNA.

The primers from Table 19 were hence ordered but before we could start to test for the most efficient primer combinations we had to generate a copy of our plasmid library that would represent such post-spliced versions of our constructs. This was achieved with conventional cloning by exchanging the iCre gene containing the artificial betaglobin intron with the conventional version of iCre.

The primers from Table 19 were then ordered and all possible combinations were tested individually on each of the intron-less plasmids as well as on a C57BL/6 mouse genome cDNA library using a Phusion Polymerase PCR (Material and Methods; Primers, PCR).

Table 19: pPCR Primer Candidates

Name	Sequence
fw1_Cont_qPCR	ATGCCAGGATATCAGGAACC
fw2_Cont_qPCR	TGATGAGGAGAATCAGAAAGGAG
rv1_ActBf_qPCR	GATGATGGCCATGTTATCCTC
rv2_ActBf_qPCR	GGAGGATAACATGGCCATCATC
rv3_ActBf_qPCR	GAGGAGGATAACATGGCCAT
rv4_ActBf_qPCR	TCCTTGATGATGGCCATGTTA
rv5_ActBf_qPCR	ATGTTATCCTCCTCGCCCT
rv1_ActB54_qPCR	CACCTTGAAGCGCATGAAC
rv2_ActB54_qPCR	CTCCATGTGCACCTTGAAG
rv3_ActB54_qPCR	CCCTCCATGTGCACCTTG
rv4_ActB54_qPCR	CCTTGAAGCGCATGAACG

rv1_Tau_qPCR	CCTCGATCTCGAAGACGTG
rv2_Tau_qPCR	CCCTCGATCTCGAAGACG
rv3_Tau_qPCR	CGCCCTCGATCTCGAAGA
rv4_Tau_qPCR	TCGCCCTCGATCTCGAA
rv1_Calr1_qPCR	GGTCACCTTCAGCTTGGC
rv2_Calr1_qPCR	GCTTTTGGTCACCTTCAGCT
rv3_Calr1_qPCR	CTTCAGCTTGGCGGTCT
rv4_Calr1_qPCR	TCTGGGTGCCCTCGTAG
rv1_Calr2_qPCR	ATGTCCCAAGTGAAGGGC
rv2_Calr2_qPCR	GACAGGATGTCCCAAGTGAAG
rv3_Calr2_qPCR	GGACAGGATGTCCCAAGTG
rv4_Calr2_qPCR	GTCCCAAGTGAAGGGCAG
rv1_ActG1_qPCR	TTGGAGCCGTACATGAACTG
rv2_ActG1_qPCR	CTTCACGTAGGCGTTGGAG
rv3_ActG1_qPCR	GTGCTTCACGTAGGCGTT
rv4_ActG1_qPCR	CGTTGGAGCCGTACATGAAC
rv1_p5_qPCR	CTTGAAGTAGTCGGGGATGTC
rv2_p5_qPCR	GCTTGAAGTAGTCGGGGATG
rv3_p5_qPCR	GGAAGGACAGCTTGAAGTAGTC
rv4_p5_qPCR	GGAAGGACAGCTTGAAGTAG
rv1_F1_qPCR	GGAGGAGTCCTGGGTCAC
rv2_F1_qPCR	TCCTGCAGGGAGGAGTC
rv3_F1_qPCR	AGTCCTGGGTCACGGTC
rv4_F1_qPCR	TCCTGGGTCACGGTCAC
rv1_ColE1_qPCR	CAGCTTCACCTTGTAGATGAAC

rv2_ColE1_qPCR	GCAGCTTCACCTTGTAGATG
rv3_ColE1_qPCR	GCGCAGCTTCACCTTGTAG
rv4_ColE1_qPCR	GCGCAGCTTCACCTTGTA
rv1_LacZ_qPCR	CCATGGTCTTCTTCTGCATTAC
rv2_LacZ_qPCR	CAGCCCATGGTCTTCTTCTG
rv3_LacZ_qPCR	CATGGTCTTCTTCTGCATTACG
rv4_LacZ_qPCR	CCCATGGTCTTCTTCTGCAT

A gel electrophoresis was performed on the PCR reactions and only primer pairs that did neither cross react with one of the other plasmids nor with the whole C57BL/6 genome cDNA library itself were chosen. The chosen primer pairs are marked in bold in [Table 19](#). PCR reactions were eventually repeated for the final selection of primer pairs. The gel with their results can be seen in Figure 10.

After confirming these ten specific primer pairs and thereby ensuring distinct barcodes, the plasmids were retransformed and a Maxiprep was performed using the QIAGEN Plasmid Maxi Kit (#12163) in order to gain enough plasmids for the virus packaging.

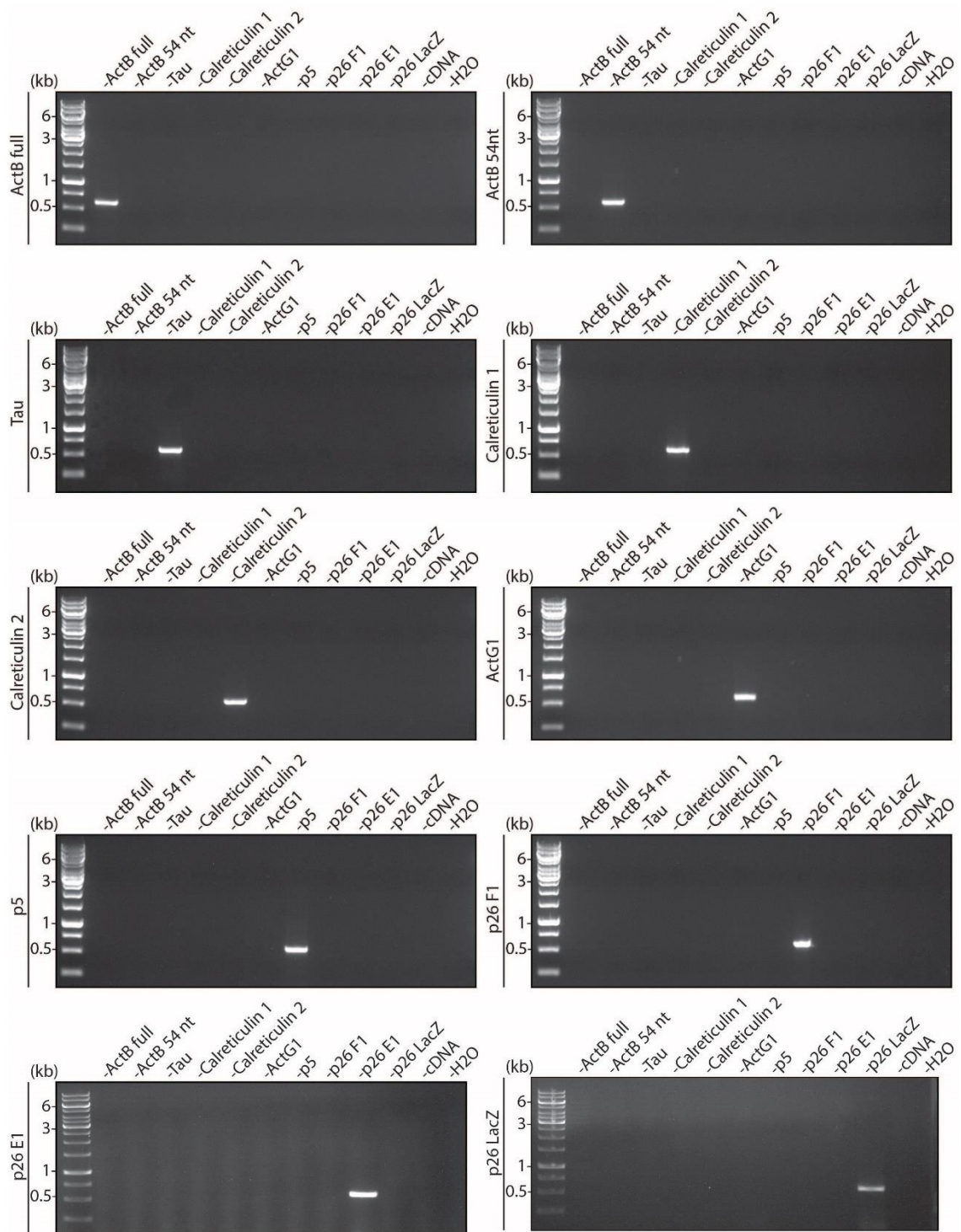


Figure 10: Primer Specificity Control. Control PCR for each sequence specific primer pair using as input their individual expression plasmid, cDNA from a C57BL/6J mouse and H2O as a negative control. Left side: Name of Plasmid who's Primers were tested for cross reactivity against all other Plasmids. Top: DNA Samples that were tested for binding of Primers.

6.3 Virus Co-Packaging

The virus production was performed as described in more detail in the Material and Methods section (Material and Methods; Virus). For the transfection an equimolar mixture of all 10 AAV plasmids was made, the calculated amounts are shown in Table 20.

	ActBfull	ActB54nt	Tau	Calreth1	Calreth2	ActG1	p5	F1	E1	LacZ	total
length [bp]	5,539	4,984	5,193	5,025	5,039	5,629	5,128	5,168	5,176	5,025	51,906
conc. [$\mu\text{g}/\mu\text{l}$]	0.285	0.17	0.398	0.357	0.423	0.459	0.162	0.405	0.221	0.297	
μg	10.67	9.60	10.00	9.68	9.71	10.84	9.88	9.96	9.97	9.68	100.00
μl	37.44	56.48	25.14	27.12	22.95	23.63	60.98	24.58	45.12	32.60	356.04
total conc.											0.281

Table 20: Multi-transfection of expression plasmids. To guarantee uniform packaging, all 10 GOI plasmids were combined in an equimolar manner before transfection.

This mixture of all 10 constructs was now for further steps treated as a single pseudo plasmid with an averaged length of 5.191bps and a concentration of 0.281 $\mu\text{g}/\mu\text{L}$. During the triple-transfection this pseudo-plasmid was then again combined in an equimolar fashion with the pHELPER and the pAAV_cap8 Plasmid using the calculations as seen in Table 21 .

To subsequently determine the yield of the virus production, an absolute qPCR had to be performed. DNA was therefore extracted from 50 μl of Virus prep using the RNA/DNA Extraction Reagent ViralXpress (EMD Milipore, #3095) following manufacturers protocol. To get the total virus count without differentiation between individual constructs, primers aiming at the human Synapsin promoter – that all 10 constructs share in common – had to be used (Material and Methods; qPCR).

Growth surface:	Growth surface: 152.00 cm ²	
Plasmid DNA amount for transfection:	0.52 µg/cm ²	
Total-Plasmid DNA for transfection	(All Plasmids are utilized in an equimolar manner, whereby larger plasmids require higher amounts than smaller plasmids.)	78.62 µg
pHELPER	Conc. [µg/µL]:	1.000 µg/µL
	Size [bp]:	11635 bp
	Needed amount of plasmid [µg]:	37.87 µg
	Volume used [µL]:	37.87 µL
pAAV_cap	Conc. [µg/µL]:	1.000 µg/µL
	Size [bp]:	7330 bp
	Needed amount of plasmid [µg]:	23.86 µg
	Volume used [µL]:	23.86 µL
pAAV_transgene_cassette	Conc. [µg/µL]:	0.281 µg/µL
	Size [bp]:	5191 bp
	Needed amount of plasmid [µg]:	16.90 µg
	Volume used [µL]:	60.13 µL
300 mM CaCl₂ [mL]:	Needed volume per area [mL/cm ²]:	0.0172 mL/cm ²
	Volume used [µL]:	2.62 mL
2x HBS [mL]: (50 mM HEPES, 280 mM NaCl, 1.5 mM Na ₂ HPO ₄ , pH 7.10)	Needed volume per area [mL/cm ²]:	0.0172 mL/cm ²
	Volume used [µL]:	2.62 mL

Table 21: Tripple-Transfection. Co-transfection of pHELPER, pAAV_cap8 and the expression plasmid mixture for simultaneous packaging of all 10 constructs.

For the titre calculation a standard curve was made using the qPCR measurements of a 1:4 dilution series of an initial 100ng/µl p143 solution and plotted on a graph to get the slope and R²-Value indicated in [Equation 5](#).

$$y = -3.7029x + 7.9886$$

$$R^2 = 0.9767$$

Equation 5: Slope & R²-value of co-packaged virus.

Using these values the Amplification Efficiency (E) could be calculated with the following formula (Equation 6)

$$E = 10^{\left(-\frac{1}{\text{slope}}\right)} = 1.8623$$

Equation 6: qPCR Efficiency of co-packaged virus.

Giving an amplification efficiency of 86.23% for this specific PCR.

The concentration of the individual AAV dilutions were then calculated by solving for x in Equation 5 as shown in Equation 7,

$$y = -3.7029x + 7.9886$$

$$y = c(q) \text{ value}$$

$$x = \log(\text{conc.}) = \frac{y - 7.9886}{-3.7029}$$

$$\text{Concentration} = 10^{(\log \text{conc.})}$$

Equation 7: Concentration of co-packaged virus.

and multiplied by the respective factor of dilution to get an averaged concentration for the according range of 1.67 µg/µl.

Using the calculation as seen in Table 22 the weight of the plasmid used for the standard curve was determined, to get the number of copies within 1ng of the plasmid.

Table 22: Calculation for Genome copy for the plasmid

Size of the plasmid	4,853.00	bps
1bp	660.00	Da
Weight of the plasmid in Daltons	3,202,980.00	Da
1 Da = (1/N _A)g (N _A is Avagadros Number)	1.6605E-24	g
Weight of the plasmid in grams	5.3187E-18	g
	5.3187E-09	ng
1ng of the plasmid	188,016,815.59	genome copies

Since our qPCR results for the virus were interpreted using this standard, those copy numbers were then further used to calculate the total copies of DNA within our end solution of concentrated virus, amounting to a total amount of 2.51×10^{14} copies in 800 μl of solution (Table 23).

Table 23: Calculation for Virus titer (Virus genome copy/ml)

1ng of the plasmid	188,016,815.59	genome copies
AAV Genome concentration (ng/ μl)	1,667.36	ng/ μl
Virus Genome copy/ μl	3.13×10^{11}	vg/ μl
Virus Genome copy/ml	3.13×10^{14}	vg/ml
Total Virus Genome copy (in 800 mL)	2.51×10^{14}	vg

Another 50 μl of viral DNA was extracted with the RNA/DNA Extraction Reagent ViralXpress (EMD Milipore) to check for the success of the self-complementation. For this the viral DNA was let run for 2 hours at 50V on a 0.8% alkaline agarose gel and dyed for 20 hours with a SYBR-GOLD staining solution (Material and Methods; Alkaline Gel Electrophoresis), to get the following results (Figure 11).

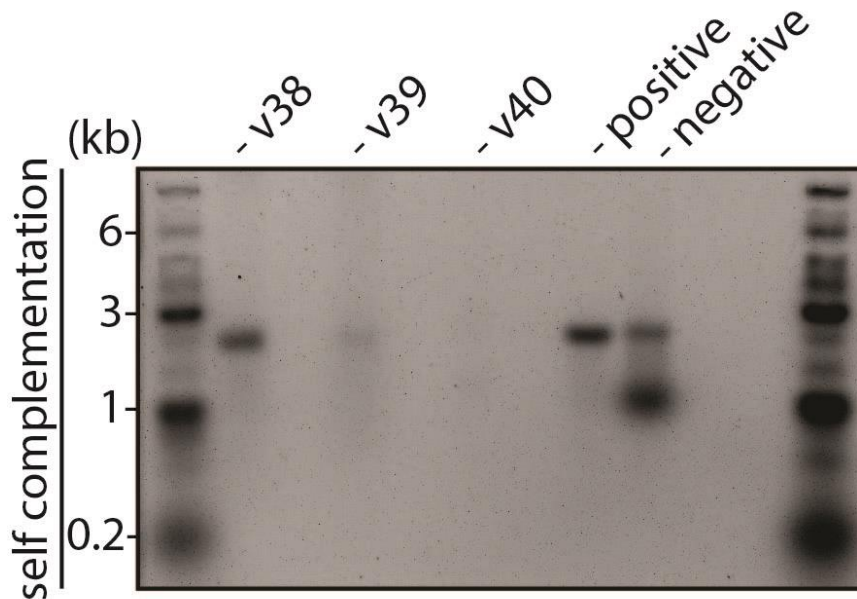


Figure 11: Control of AAV Self-Complementation. Comparison of viral genome sizes to check for correct self complementation during virus production. V38 represents the co-packaged AAV vector mix used during the experiments. V39 and v40 are different self complementing AAVs not related to this thesis. The positive control has double the size of the non self-complementary virus used as the negative control.

6.4 Test Injections

After the self-complementation of the virus was confirmed, I performed test-injections on the auditory cortex of 6-8 week old C57BL/6 mice using 50nl of Bromphenole blue dye to find out the correct stereotactic coordinates that would guarantee that the virus gets expressed in the intended region (Material and Methods; Injection).

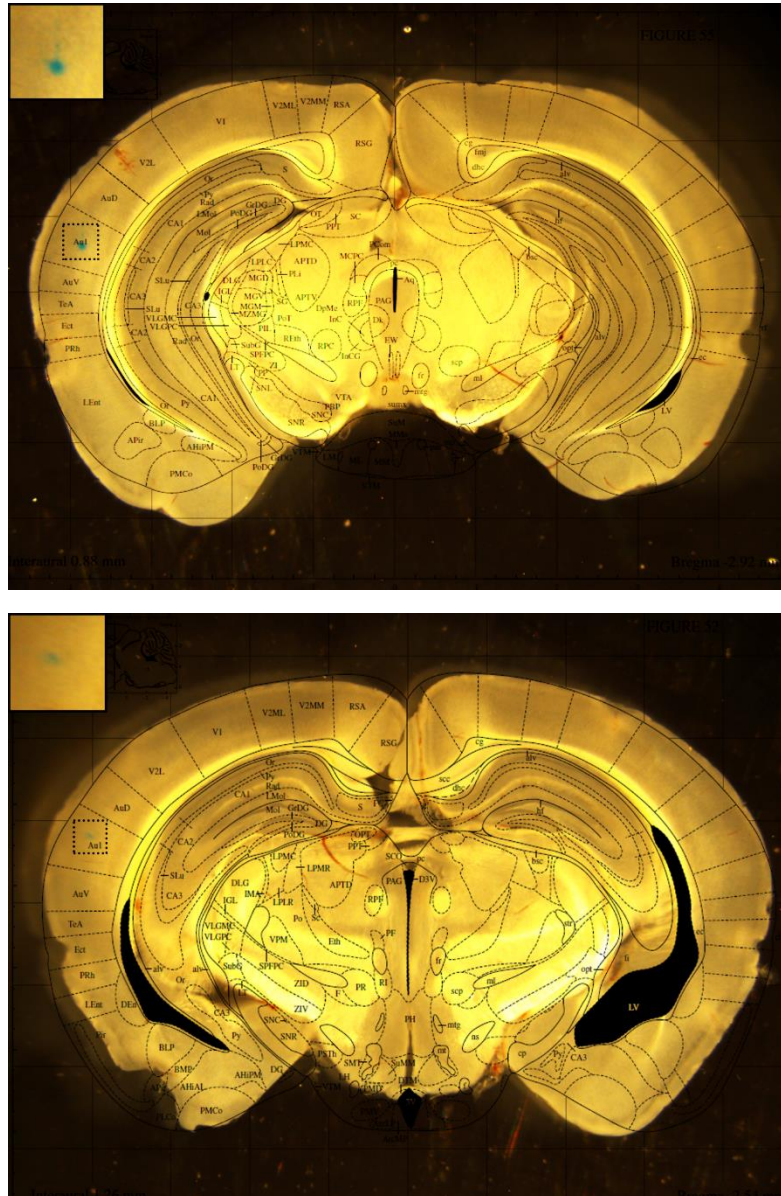


Figure 12: Test-Injections. Test-Injections were performed on 6-8 weeks old C57BL/6J mice targeting the Auditory Cortex (ACx) until the correct coordinates were found. Shown are two examples of successful targeting with 50nl of Bromphenol Blue.

Directly after the injections all mice were sacrificed without waking them from the anaesthesia and an encephalectomy was promptly performed. The brains were washed once in 1xPBS and immediately put into 15ml of a 4% paraformaldehyde (PFA) (Thermo Fisher Scientific Inc.) solution for a total duration of 24 hours at 4°C. After fixation, the brains were washed three times with PBS and stored in the same solution.

The fixed brains were then sliced into 100 µm thick slices using a Vibratome (Material and Methods; Slice Preparation) and stored in PBS. Those slices covering the region of the auditory cortex were analysed under a Leica MZApo-Stereomicroscope (Meyer Instruments Inc.) (Figure 12).

If necessary coordinates for injection were adjusted and the test-injections repeated until proper and consistent results were achieved.

6.5 Virus Injection

After establishing a routine at targeting the auditory cortex via stereotactic injections, the virus was injected into 13 C57BL/6 mice between the age of 10 and 12 weeks. To establish a time course for expression efficiency and to guarantee maximal mRNA transport. The mice were split into three groups and injection sessions were performed on different time points for each group. The first group consisting of 7 mice that were injected 16 days prior to sacrifice and the second and third group each comprised 3 mice that were injected 8 and 4 days before sacrifice respectively (Material and Methods; Injection). Each mouse received therein four consecutive injections with 200nl of a 3.1×10^{14} concentrated virus along the auditory cortex of the right hemisphere, to ensure an even and thorough spread of the virus.

6.5.1 mRNA Reporter Extraction

After the planned incubation period, mice were sacrificed and an encephalectomy was performed. From each brain three samples were taken as promptly as possible to avoid RNA degradation. Those samples were the auditory cortex on the ipsilateral site of injection in order to analyse the virus expression, the auditory cortex on the contralateral side of injection to analyse the degree of mRNA transport and finally the bulbus olfactorius, acting as a negative control where no transport of reporter mRNA should take place.

All samples were quickly handled and RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, #74804) and Dynabeads Oligo dT₂₅ (Thermo Fischer Scientific Inc., #61002) (Material and Methods; RNA extraction and cDNA conversion).

For the Reverse Transcription step three different gene specific primers (GSP) were tested for both their specificity and efficiency, eventually resulting in the use of GSP-3 (ATCGAGATCGCAGGTGAG) for further steps. A gene specific primer guarantees that only reporter mRNA gets reverse transcribed into cDNA before the subsequent RNase A treatment.

To further minimize a potential false negative signal from the viral input DNA a junction PCR was performed. For this purpose another set of forward and reverse primers were ordered, whereby the forward primer was specifically designed to span the betaglobin intron inside the iCre gene, causing the PCR to only amplify target sequences that have been converted into cDNA in contrast to the viral input DNA who's iCre genes still possess an intron and thereby preventing the successful binding of the forward primer. The individual combinations between forward and reverse primers ([Table 24](#)) were again tested for their efficiency, resulting in the use of the primers junction_1.1 (CAGATGCCAGGATATCAGGA*A*C*C*T*G) and junction_2.3 (ATCG-CAGGTGAGGCCT*A*G*C) for further experiments.

Table 24: Junction Primers

betglobl-spliced-5xPTO_fw	junction_1.1	CAGATGCCAGGATATCAGGA*A*C*C*T*G
	junction_1.2	GAACTCTGACAGATGCCAGG*A*T*A*T*C
	junction_1.3	CTCTGACAGATGCCAGGATA*T*C*A*G*G
	junction_1.4	GACAGATGCCAGGATATCAG*G*A*A*C*C
T7-M13-3'-01-3xPTO_rev	junction_2.1	GAGATCGCAGGTGAGGCC*T*A*G
	junction_2.2	CATCGAGATCGCAGGTGA*G*G*C
	junction_2.3	ATCGCAGGTGAGGCCT*A*G*C

The junction PCR was performed for 15 consecutive cycles and was afterwards purified using the QIAquick PCR purification kit (Material and Methods; Sequencing).

Finally another PCR had to be performed in order to amplify the reporter cDNA above threshold level and at the same time attach the necessary barcodes for the Illumina Sequencing. Therefore 3 Test PCRs were performed, to determine the threshold. 20 and 25 cycles were used for a KAPA 2xHiFi Mix PCR (Material and Methods; Sequencing) and the resulting reaction put onto a gel (Figure 13).

As expected there was a great discrepancy between the thresholds of the input sample taken from the ipsilateral side and the output sample from the contralateral side.

6.6 Next Generation Sequencing

Since we wanted to be above threshold for our Contralateral samples but didn't want to risk over-amplifying the Input samples we decided to go with 25 PCR cycles. As seen in Figure 13 the input bands were already quite pronounced while the output bands just barely started to come up.

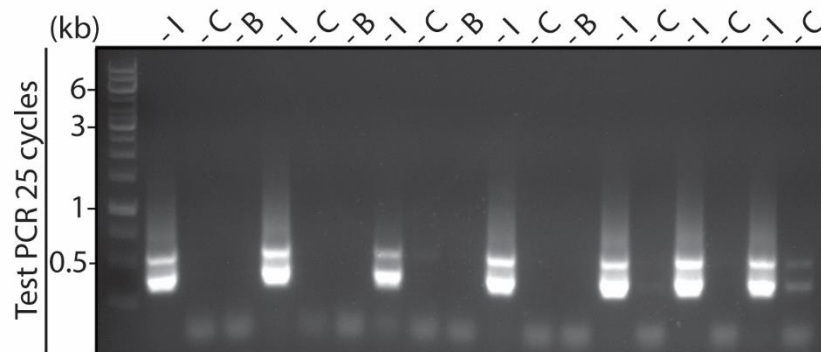


Figure 13: Threshold Determination for Target Enrichment. Gel Electrophoresis results after 25 cycles during enrichment PCR. While the signal of Ipsilateral samples are already quite pronounced, the signal of the Contralateral sample are just beginning to come up. Legend. I: Ipsilateral sample, C: Contralateral Sample, B: Bulbus Olfactorius sample

In addition to the 38 in vivo samples, 2 further samples were added to function as a control for the virus packaging efficiency. One of them comprised an equimolar mixture of all 10 construct plasmids and the other a DNA extract of the same rAAV mix that was used for the injections. The comparison of these two samples is supposed to give an idea about whether the AAV co-packaging process already results in a discrimination between individual samples.

For the adapter adherence, I used PE_1.0 as a forward- and PE RV 1-40 as reverse primers each of the latter only differing in a 6 bp long barcode sequence that allow you to differentiate between the various samples during sequencing, even after combining samples into a single reaction.

All the samples were now purified again using Agencourt AMPure XP – PCR purification beads (Beckman Coulter, Inc.) to remove primer dimers (Material and Methods; Sequencing) and were handed in to the CSF for Next Generation Sequencing.

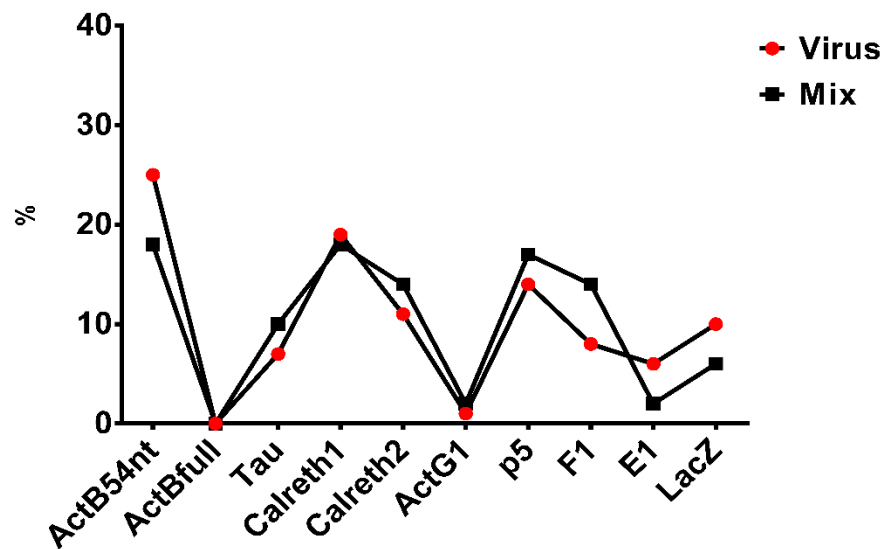


Figure 14: Equimolar Mix vs Viral Extract. Deep Sequencing results of an equimolar mixture of all 10 control plasmids plotted against viral DNA extract.

To our surprise the equimolar mixture did not show an even distribution, which should be around 10% for our 10 constructs (Figure 14), but the virus sample showed the same pattern in distribution and no significant difference was found after analysis with Two-way ANOVA and Multiple Comparisons.

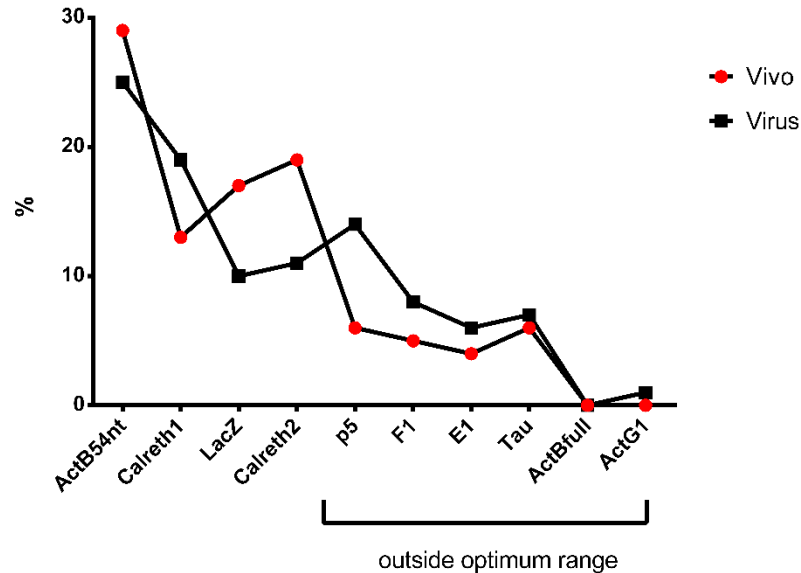


Figure 15: Viral Extract vs In-Vivo Sample. cDNA from Ipsilateral Extract plotted against the initial Viral Extract. Localization Sequences are ordered from smallest (left) to largest (right). While there is no significant difference between both samples, a correlation between relative amount of cDNA and Sequence Length can be observed.

Neither were any statistically significant differences present when comparing the Virus with the In-Vivo sample but after plotted the sequences in order of size (Figure 15) we found a negative correlation of the relative amount within the samples with their sequence length (Figure 17).

When looking at the translocation data we decided to use the mice with a 16 days incubation period (Figure S 1). After normalizing the Negative Control “Bulbus ol.” and the Contralateral Sample with the expression level on the ipsilateral side we plotted the results on Figure 16 and found no reliable sign for transsynaptic transport.

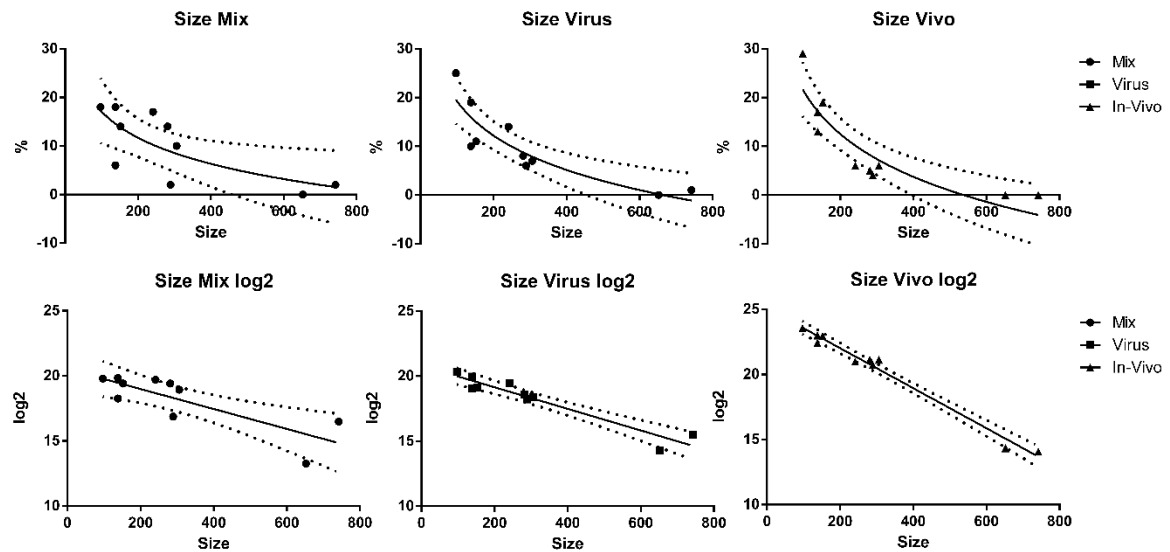


Figure 17: Correlation between Translocation Sequence Size and cDNA. Amount of cDNA plotted against Translocation Sequence Length. First Row: Interpolation with 95% Confidence Intervals, relative amount. Second Row: Interpolation with 95% Confidence Intervals, log2. Size Correlation. Mix: $r^2=0.5365$, $p=0.0160$, *; Virus: $r^2=0.9119$, $p=0.0004$, ***; In-Vivo: $r^2=0.9052$, $p=0.0005$, ***.

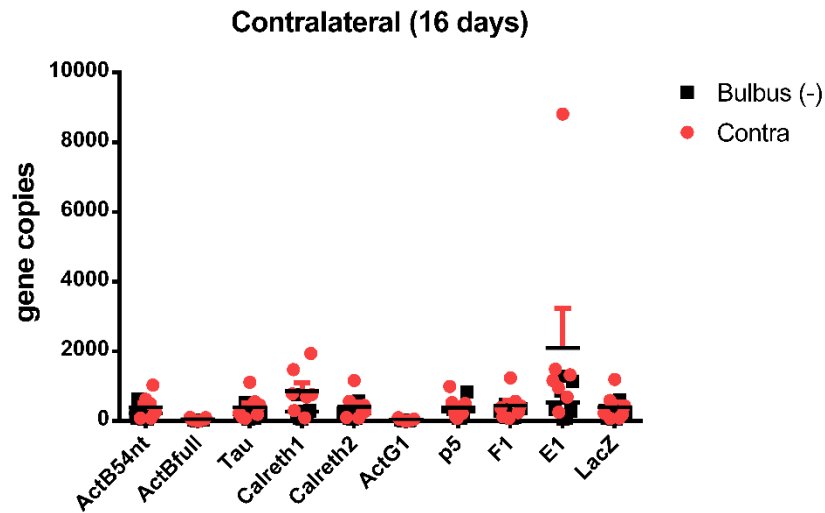


Figure 16: Expression at contralateral side of Injection. Gene copies detected via Next Generation Sequencing at the contralateral side of injection, plotted against expression levels of the negative control "Bulbus olfactorius". $n = 7$.

6.7 qPCR

To verify these results additionally a qPCR was performed on the samples of 2 mice with an incubation period of 16 days. As input the DNA product from the junction PCR was used with the primer “new_qPCR_fw_150bp” and their respective reverse primers, each binding specifically to a bar code sequence that prior was cloned right before the translocation sequence. The results of this qPCR can be seen in Figure 18. Despite the high variation between mice, results from the qPCR were consistent with the results from Deep Sequencing.

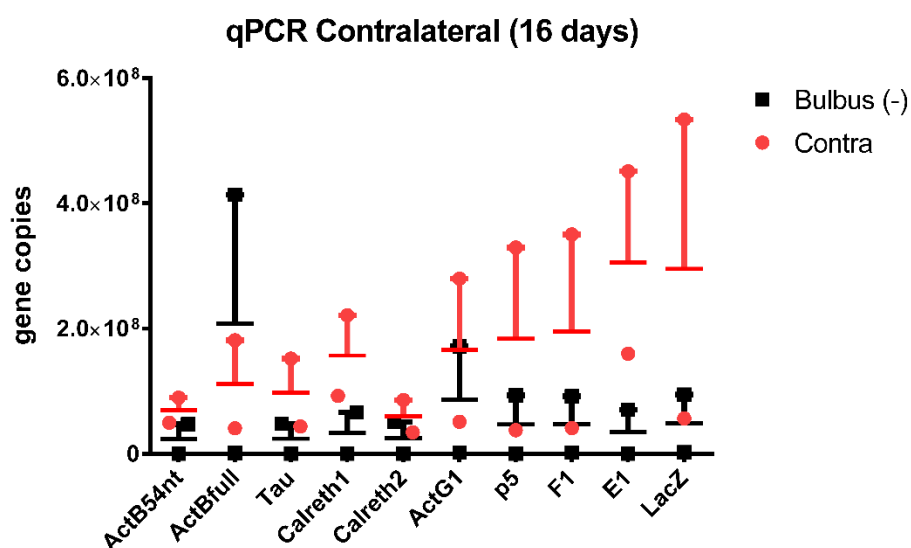


Figure 18: Expression at contralateral side of Injection. Gene copies detected via qPCR at the contralateral side of injection, plotted against expression levels of the negative control “Bulbus olfactorius”. n = 2.

7 Discussion

AAVs broad tropism and ability to stably express its transgenes in non-dividing cells while maintaining low immunogenicity, makes it one of the most efficient vehicles for in-vivo gene delivery in current research but its limitations in both packaging capacity

and manufacturing pose an obstacle for many of its users. The traditional AAV production method through the triple transfection of human embryonic kidney 293 (HEK293) cells (Grimm et al., 1998) and the subsequent purification of the virus using a CsCl₂ density gradient centrifugation (Zolotukhin et al., 1999) is very cost and time intensive, especially for methods that rely on gene delivery using multiple vectors at once. In some instances, the required expression cassette exceeds AAVs packaging capacity and the cassette has to be fragmented and delivered in individual vectors (Gosh, Yue & Duan, 2010; Zhang et al., 2013). In other instances, it might be beneficial to control transgene expression by using different regulatory elements like cell type specific promoters to either express different transgenes in each affected cell type or express the same transgene in various cell types but excluding expression in for example antigen presenting cells that would otherwise provoke an immune response (Zhang et al., 2012). In such cases researchers have at the moment only one option, and that is to produce and characterize each virus independently and subsequently co-administer them in the appropriate ratio, thereby multiplying the efforts involved in the already cost- and labour-intensive production.

One of the goals of this study was therefore to try and provide a new production method that lowers both cost and labour required for the manufacture of multiple vectors. Here I showed that after co-packaging a total of 10 distinct AAVs in a single step during production I could produce a virus batch comprising each individual virus in a ratio comparable to the ratio of the expression plasmids used during packaging.

In the following we are going to have a look at:

- The distribution pattern of our constructs
- The translocation of constructs to the contralateral side of injection

While one would expect an even distribution with each vector accounting for roughly 10% of the total titre, especially for the Equimolar Mixture, we see a stronger variation in our results with two stark outliers “ActBfull” and “ActG1”. But since the same pattern of variation is present in both the viral as well as the mixture sample, we can conclude that this error must have already occurred during the preparation of our library. Since the variation is too large for a simple pipetting error, especially for our outliers, the most likely source of the discrepancy is a wrongly assumed Plasmid concentration after performing the Maxi prep of our expression plasmids.

In fact, after analysing the data using a Two-way ANOVA and Holm-Šídák test for Multiple Comparisons, no statistically significant difference between the viral and in-vivo sample was found (Figure 14), meaning that despite packaging our library in the wrong ratio, we succeeded in packaging a library consisting of ten different expression constructs simultaneously and in the same ratio.

When comparing the relative prevalence of sequences between the virus and the in vivo sample, we didn't find statistically significant differences either (Figure 15), yet after plotting our results in order of Insert length we found a discriminatory trend toward larger Sequences.

This was in part expected. As we were using a self-complementary construct, which already packages its genome as a dimer during virus production, we only had about half the packaging capacity of a regular AAV. So, with translocation sequences ranging after all from 54bp to 685bp and doubling their variation in a scAAV vector, we knew that it would shift individual samples out of the optimal AAV packaging range between 4.1 kb to 4.9 kb, whereby especially larger samples have already been shown to lead to a strong reduction in packaging efficiency (Dong, Fan & Frizzell, 1996). Since our Plasmids ranged in size between 2300 and almost 2800 base pairs, which would be roughly equivalent to 4.6 and 5.6 kb for a regular AAV vector, I plotted all constructs in order of translocation

sequence length which as expected showed a discrimination for shorter sequences, especially for constructs outside of the optimal packaging range (Figure 15).

Even though a small trend in size distribution was already observable in the Mixture sample, a clear correlation can be observed in both the Virus ($r^2 = -0.9119$; $p = 0.0004$) and In-Vivo ($r^2 = 0.9052$; $p = 0.0005$) samples (Figure 17).

If applicable special attention should be placed to keep the expression cassette inside the optimal range of the AAV and even then try to keep individual plasmids as close in size as possible to reduce the need for normalisation.

In total though, the simultaneous packaging of a small library during AAV production using a self-complimentary Expression Plasmid, leads to a reliable virus batch without sacrifices in titre.

The second question I tried to answer using this translocation sequence library was whether the co-packaging of various vectors can be used as an easy and cheap method to characterize and compare multiple genes or regulatory elements in vivo concurrently. In the course of this project I therefore co-packaged a small synaptic targeting library into a recombinant adeno-associated virus, comprising the zipcode of the Actin Beta mRNA as well as its whole 3'UTR, both of which have already been shown to facilitate synaptic targeting of mRNA (Willis, 2007); the tau zipcode (Litman et al., 1993; Aronov et al., 2001) and both identified calreticulin zipcodes (Vuppalachchi et al, 2010) as well as five constructs incapable of axonal transport which would serve as a negative control. The library was injected into the right hemisphere of the auditory cortex in order to measure interhemispheric transport using deep sequencing and qPCR. At three different time points samples were taken ipsilateral of the injection site to measure input; contralateral to test for transport of our mRNAs and at the bulbus olfactorius which does

not have any connections to either of the other sites and thereby serves as a negative control.

Since the individual candidates did not show an even distribution in the ipsilateral sample where each of the ten candidates should have been expressed with an average incidence of 10% of the whole sample as previously discussed (Figure 14), we decided to normalize the values of both the Contralateral as well as the Bulbus sample using the distribution pattern found in the Ipsilateral sample to correct for this distribution anomaly. Compared to the Virus and Equimolar Mix sample the Ipsilateral sample underwent the exact same extraction procedure as the test and control sample with the same amount of intermediate PCR steps and was therefore favoured for normalization compared to the Mixture or Virus sample since this would inevitably lead to a correction of most of the possible biases introduced during extraction and sequencing. Normalized values of the Contralateral and Bulbus sample should therefore in the following be interpreted as deviations from an even baseline.

To achieve optimal expression levels while decreasing the waiting period during a potential assay we split our test subjects into three different groups, with an incubation period of 4, 8 and 16 days after virus injection, respectively. As seen in Figure S 1 the expression of mRNAs needed more than 4 days to reach its plateau but the extent of transport to the contralateral site still changed between 8 and 16 days. To measure the axonal transport of our mRNA's we therefore only included individuals with a 16 day incubation period.

When looking at the normalized mRNA values in our contralateral and bulbus olfactorius sample in Figure 16 there does not appear to be a clear difference between our region of interest and the negative control.

A slight trend can be observed for the sequence “Calreticulin 1” and “E1”, even though the latter is a negative control. And although an analysis with Two-way ANOVA and multiple comparison does indeed show a statistically significant increase of “E1” levels in the contralateral sample when compared to the negative control, it should be viewed with caution, as the levels are still far below the values of the Ipsilateral samples. The fact that four of the other sequences which have already been reported to initiate transport failed to show even a trend towards localization corroborates that a systematical error might be present in the experiment, most probably in either the design of the transport construct or the retrieval of the contralateral sample. The fact that the qPCR showed a similar result also suggests this.

8 Outlook

In order to actively use such a co-packaging mechanism for actual screening purposes, it would to be repeated with a whole genome library consisting of millions of different candidates, paying attention to keeping the size distribution of the inserted genome fragments at an even level and possibly already adding the adapters for deep sequencing beforehand to minimize amplification bias.

Should such a screening method get successfully implemented, it would not only shed a new light on compartmentalized transcription and the cells transport machinery as a whole, but one could also continue to try and find sequences that cause transsynaptic mRNA transport.

The fact that intercellular RNA exchange takes place between various cells types has already been shown whereby Exosomes (Batagov & Kurochkin, 2013; Valadi et al, 2007) or Tunneling Nanotubes (Gerdes & Carvalho, 2008; Hase et al., 2009, Gerdes et al., 2013) have been proposed as intercellular bridging devices.

Exosomes are intraluminal vesicles of multivesicular bodies (MVBs) and are released upon the exocytic fusion of the MVB with the plasma membrane, releasing the exosomes into the intracellular space, often toward a recipient cell and have already been discussed as a novel way of communication between cells (Lakkaraju & Rodriguez-Boulan, 2008). Additional roles that have been proposed are removal of unwanted or excess proteins (Pan et al., 1985) or transfer of pathogens like prions in diseases such as Creutzfeld-Jakob disease or bovine spongiform encephalopathy. They are released by virtually all types of mammalian cells and have so far been shown to contain messenger RNA (mRNA), micro RNA (miRNA), characteristic lipids and an evolutionary conserved set of proteins which generally varies with tissue and cell of origin (Bellingham et al., 2012).

While multivesicular bodies bud off directly from the plasma membrane and are heterogeneous in size (up to 1000 nm in diameter) (Frühbeis et al., 2013), does the biogenesis of exosomes occur within those very same multivesicular bodies in the endosomal system - which shuttles cargo between the plasma membrane, the trans-Golgi network and lysosomes - and are homogeneously shaped instead with a diameter between 50 and 100 nm (Bellingham et al., 2012; Frühbeis et al., 2013). From here the MVBs can either fuse with the lysosomal membrane and discard their content into the lysosome for degradation or fuse with the plasma membrane and release the exosomes into the extracellular environment. The existence of multiple mechanisms of protein sorting into exosomes has given rise to the possibility that there are different sorts of MVBs that each vary in content and/or destination (Bellingham et al., 2012; van Niel et al., 2006; Simons and Raposo, 2009). But independent of the actual means of transport, being able to identify the sequences responsible for transsynaptic localization would give rise to the ability to further modify the behavior of transgenes by selectively attaching those sequences and thereby controlling their eventual destination even outside the boundaries of the transfected cell. Combined with the correct reporter gene this would make it possible to create a transsynaptic marker assay allowing one to map not only anterograde

but also retrograde connections of the transfected ROI, providing an invaluable tool for connectomics.

9 Appendix

9.1 Supplemental Figures

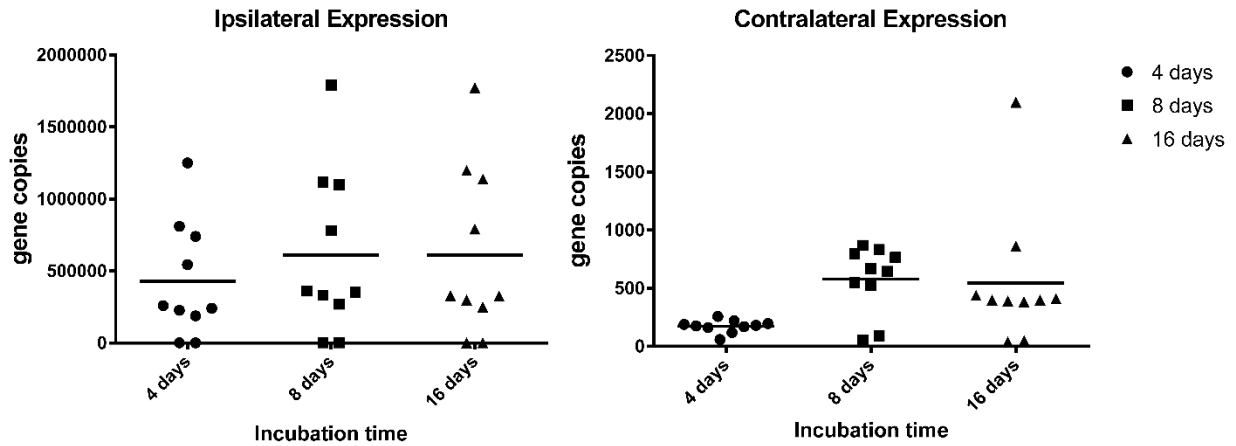


Figure S 1: Timeline of mRNA Expression. Gene copies after 4, 8 and 16 days respectively. Horizontal bars plot mean value.

9.2 Plasmid Maps

9.2.1 pscAAV_CMV-GFP

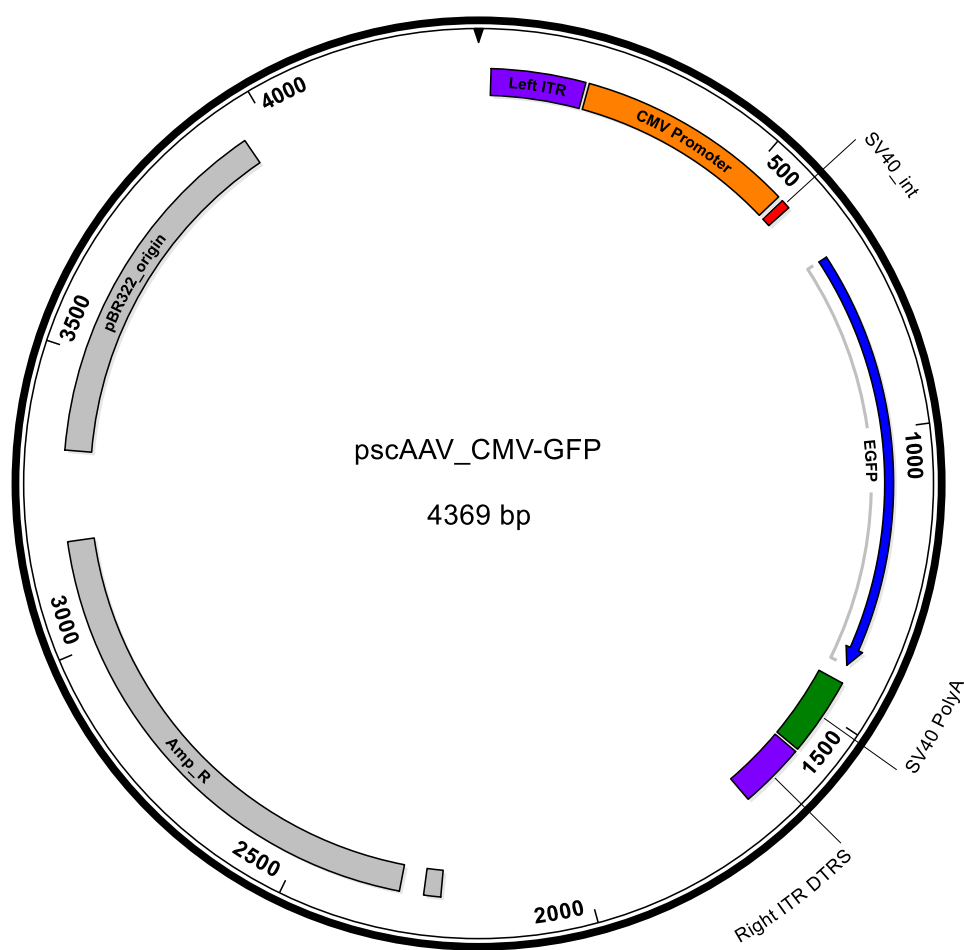


Figure S 2: Plasmid Map pscAAV_CMV-GFP. Map of Plasmid pscAAV_CMV-GFP [ITR, inverted terminal repeats; CMV, cytomegalovirus promoter; eGFP, enhanced GFP; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.2 46_pCMV-MCS-iCRE-mKO-beta globinintron

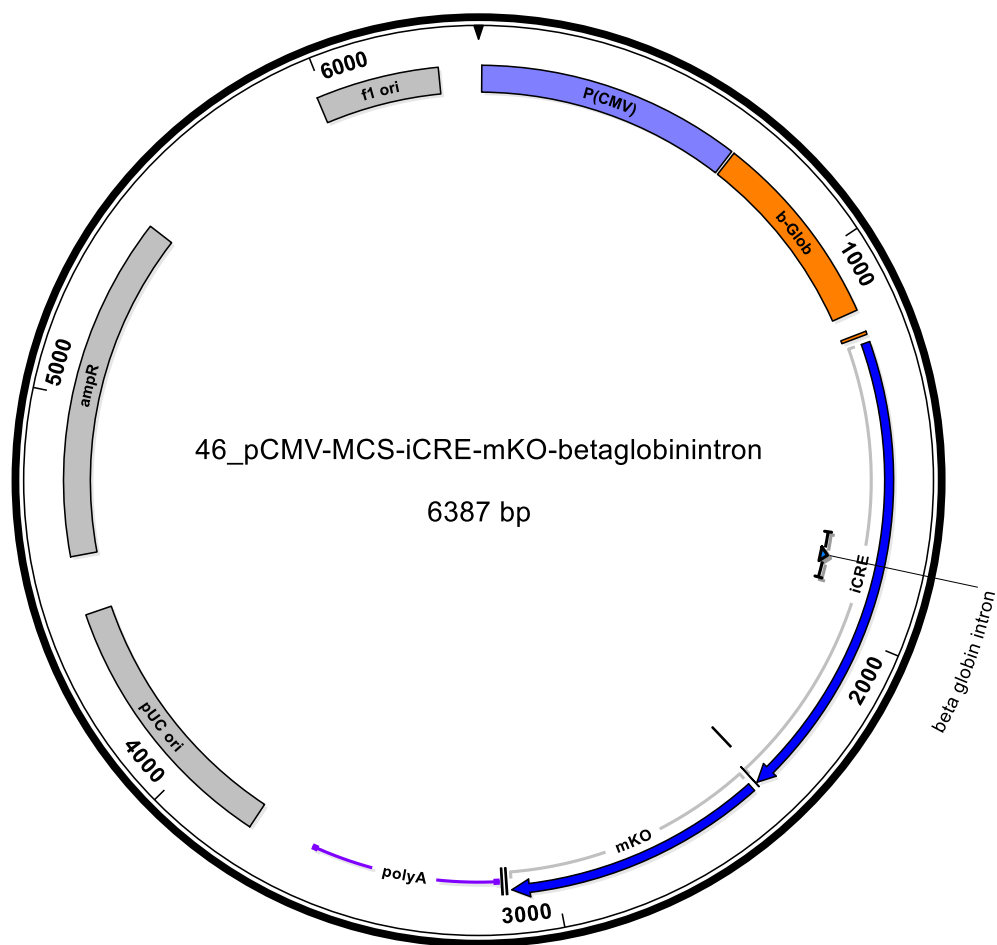


Figure S 3: Plasmid Map 46_pCMV-MCS-iCRE-mKO-beta globinintron. Map of Plasmid 46_pCMV-MCS-iCRE-mKO-beta globinintron [CMV, cytomegalovirus promoter; b-Glob, beta globin; iCRE, improved CRE; PolyA, polyadenylation site; pUC ori, origin of replication; Amp_R, Ampicillin Resistance; f1 ori, origin of replication]

9.2.3 131_pAAV_hSyn-3NLS-RGECO

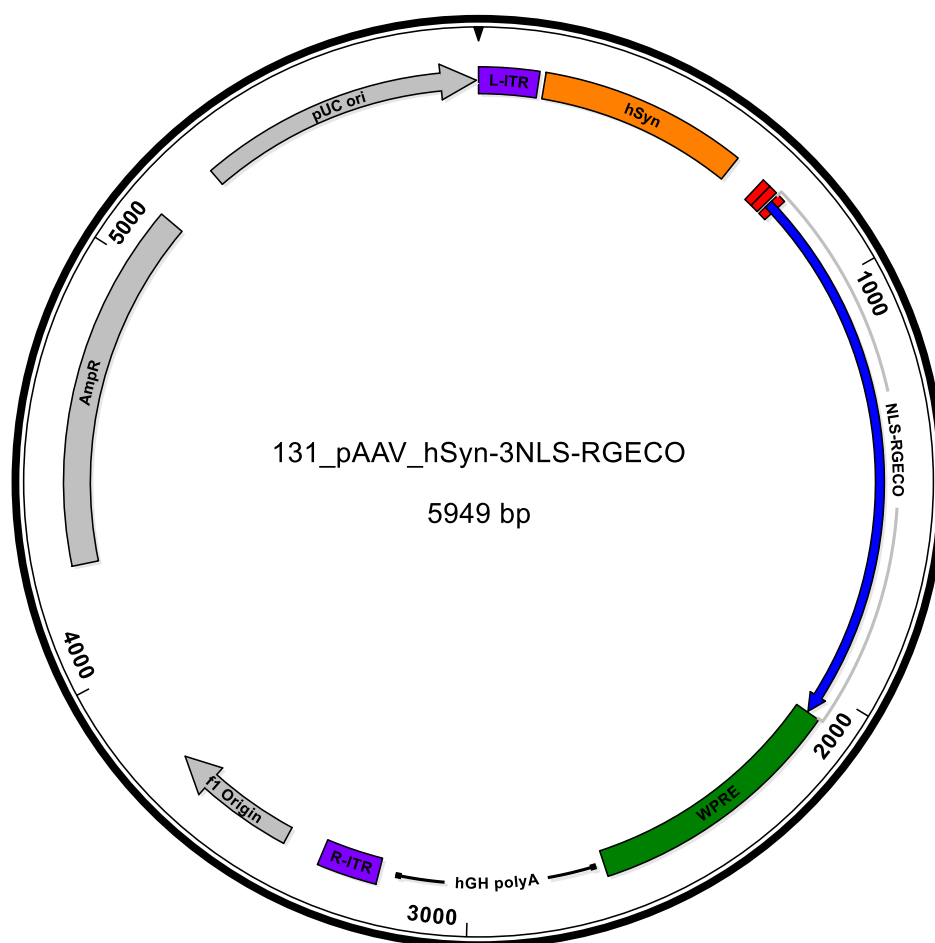


Figure S 4: Plasmid Map 131_pAAV_hSyn-3NLS-RGECO. Map of Plasmid 131_pAAV_hSyn-3NLS-RGECO [ITR, inverted terminal repeats; hSyn, human Synapsin Promoter; NLS-RGECO, Red fluorescent genetically encoded Ca²⁺ indicator for optical imaging; WPRE, Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element; hGH polyA, human growth hormone polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.4 pscAAV_pSyn_iCRE_intron_new

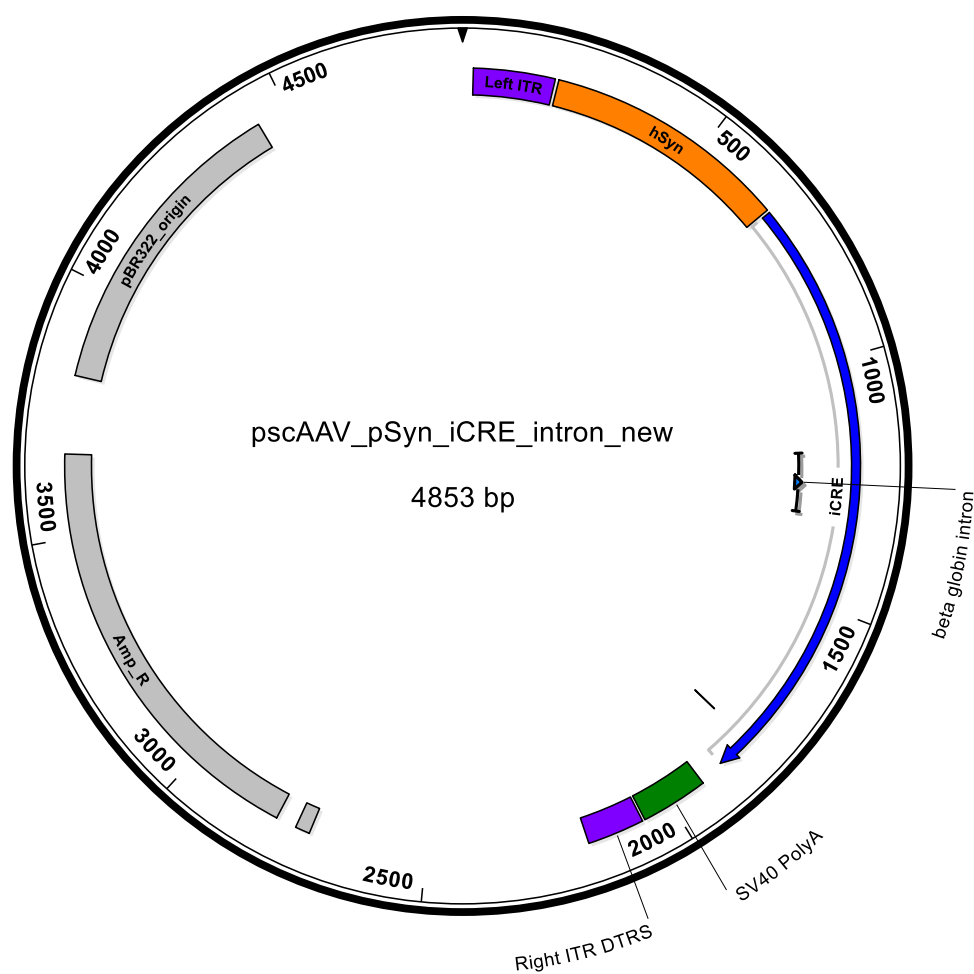


Figure S 5: Plasmid Map pscAAV_pSyn_iCRE_Intron. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.5 54_pAAVCamKII-PamCherry1-NLS

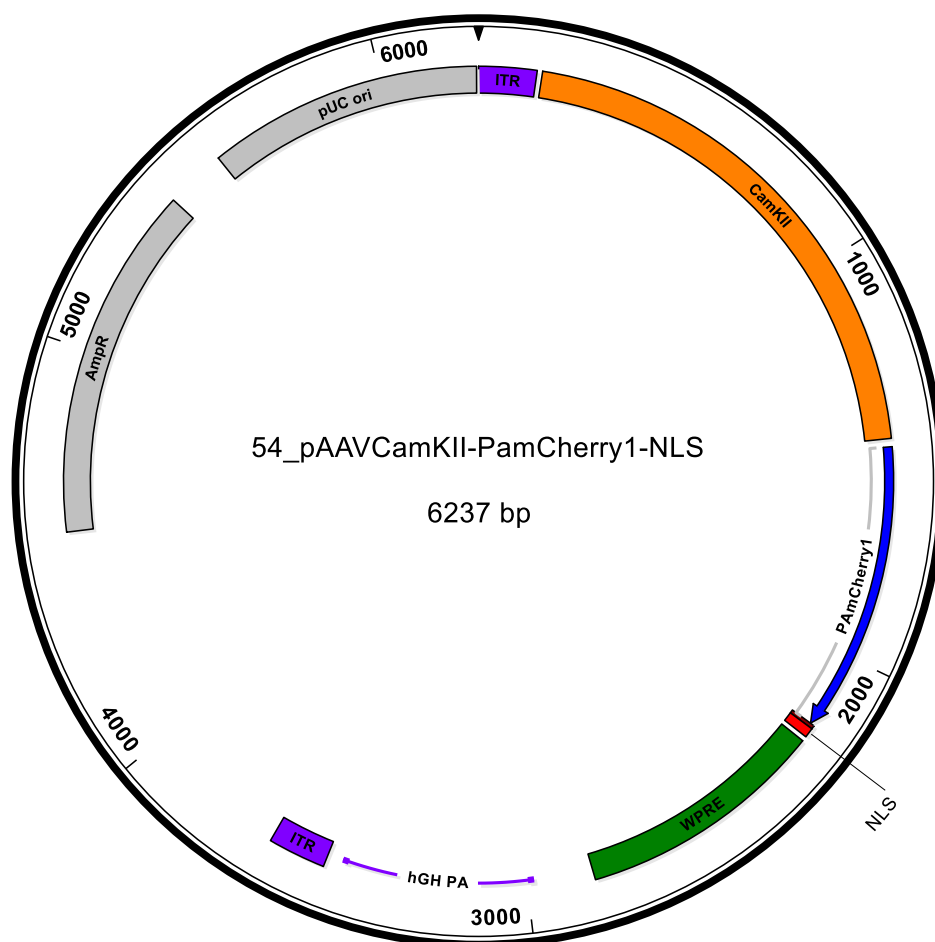


Figure S 6: Plasmid Map 54_pAAVCamKII-PamCherry1-NLS. Map of Plasmid p54_pAAVCamKII-PamCherry1-NLS [ITR, inverted terminal repeats; CamKII, Ca²⁺/calmodulin-dependent protein kinase II; PamCherry1, Photo Activateable mCherry 1; NLS, nuclear localization sequence; WPRE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; hGH polyA, human growth hormone polyadenylation site ; Amp_R, Ampicillin Resistance]

9.2.6 5_pHelper

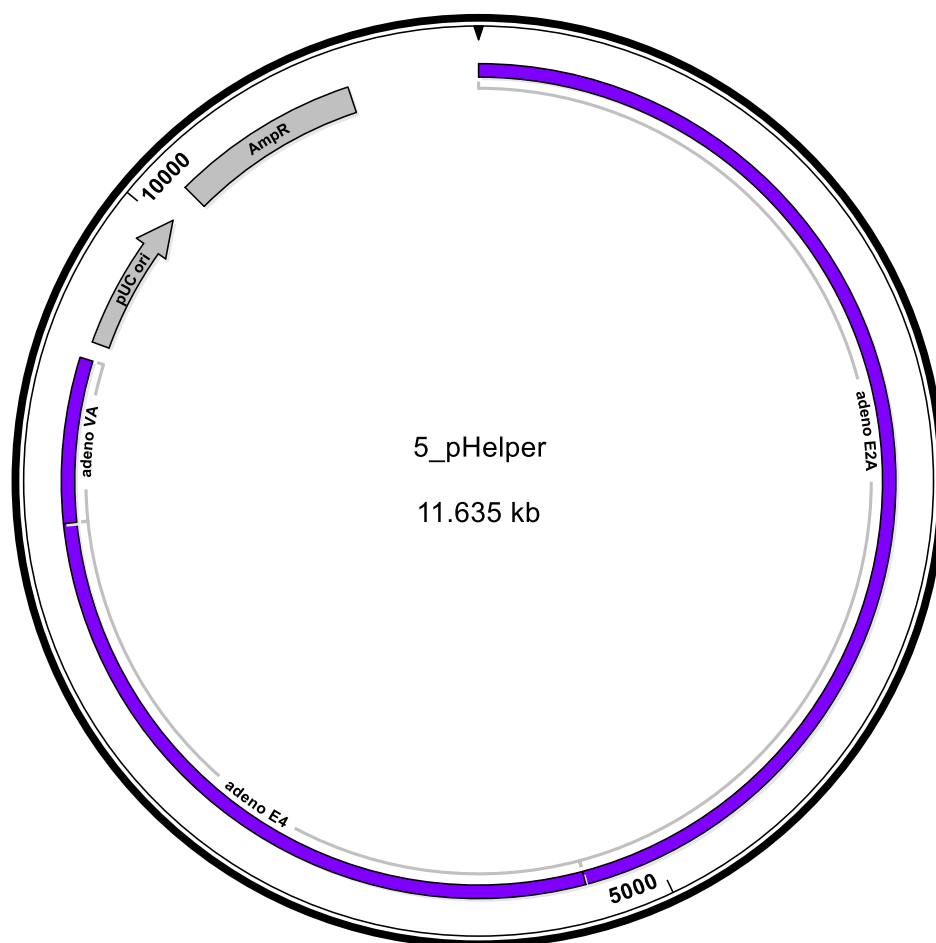


Figure S 7: Plasmid Map 5_pHelper. Map of Plasmid 5_pHelper [Amp_R, Ampicillin Resistance]

9.2.7 10_pLECYT

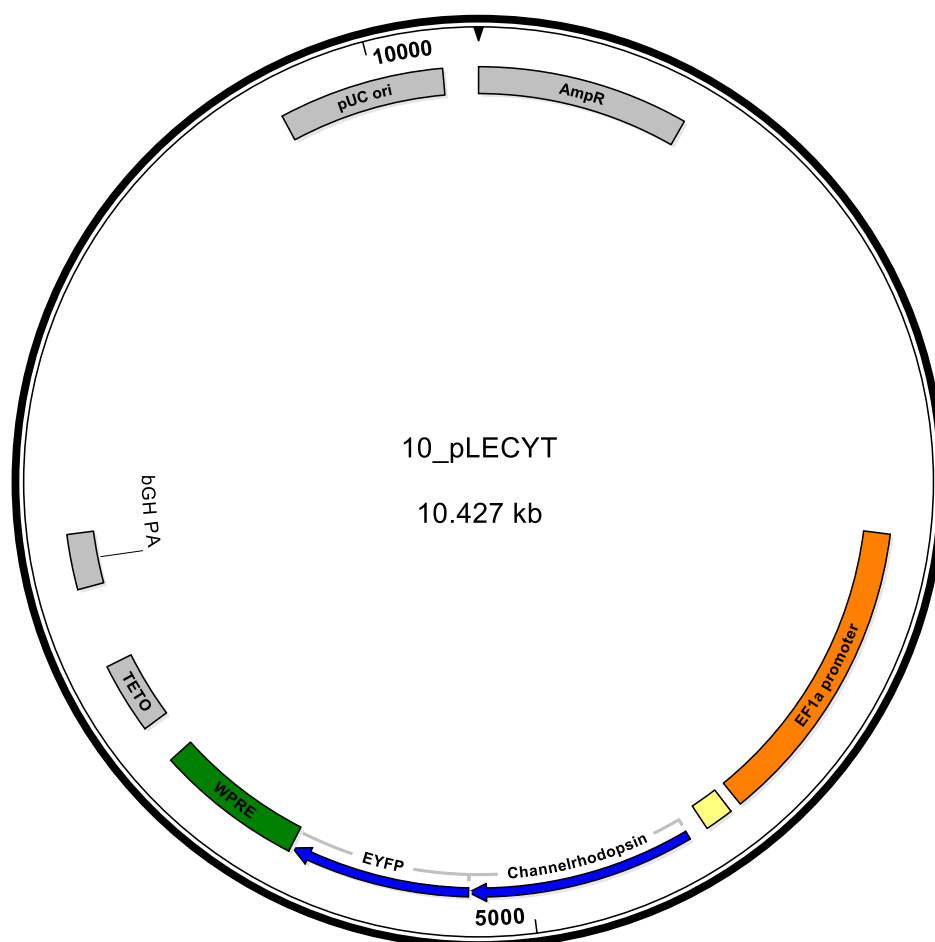


Figure S 8: Plasmid Map 10_pLECYT. Map of Plasmid 10_pLECYT [EF1a, translation elongation factor EF1a promoter; eYFP, enhanced Yellow Fluorescent Protein; WPRE, Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element; TETO, tetracycline operator; hGH PA, human growth hormone polyadenylation site ; Amp_R, Ampicillin Resistance]

9.2.8 26_pBluescript_II_SK(-)

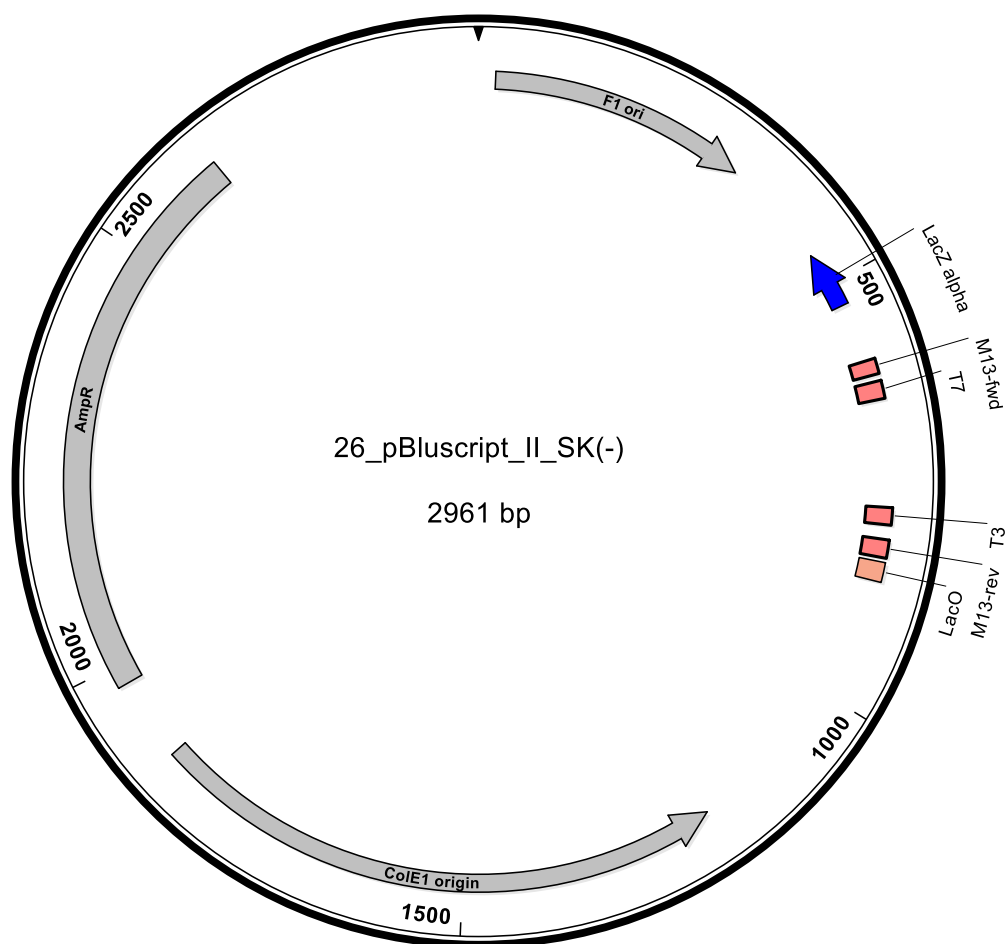


Figure S 9: Plasmid Map 26_pBluescript_II_SK(-). Map of Plasmid 26_pBluescript_II_SK(-) [Amp^R, Ampicillin Resistance]

9.2.9 pscAAV_pSyn_iCRE_intron_ActB54nt

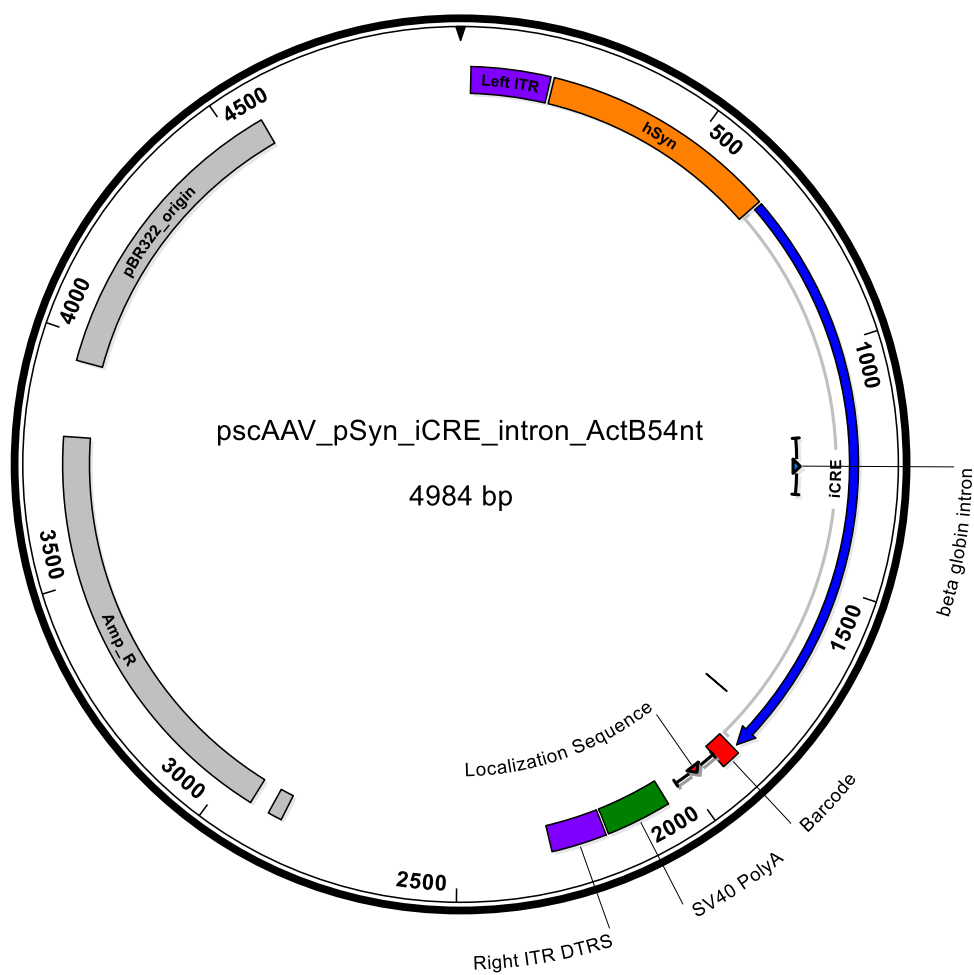


Figure S 10: Plasmid Map pscAAV_pSyn_iCRE_Intron_ActB54nt. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_ActB54nt [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.10 pscAAV_pSyn_iCRE_intron_ActBfull

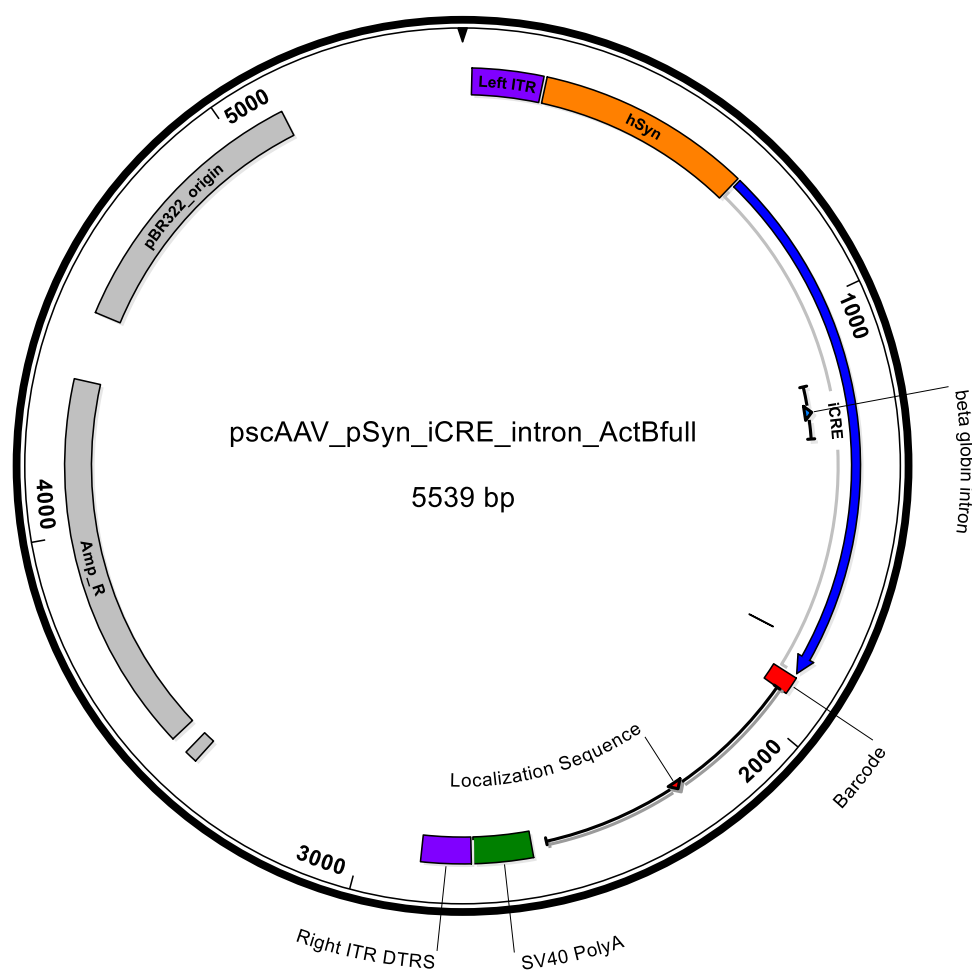


Figure S 11: Plasmid Map pscAAV_pSyn_iCRE_Intron_ActBfull. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_ActBfull [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.11 pscAAV_pSyn_iCRE_intron_Tau

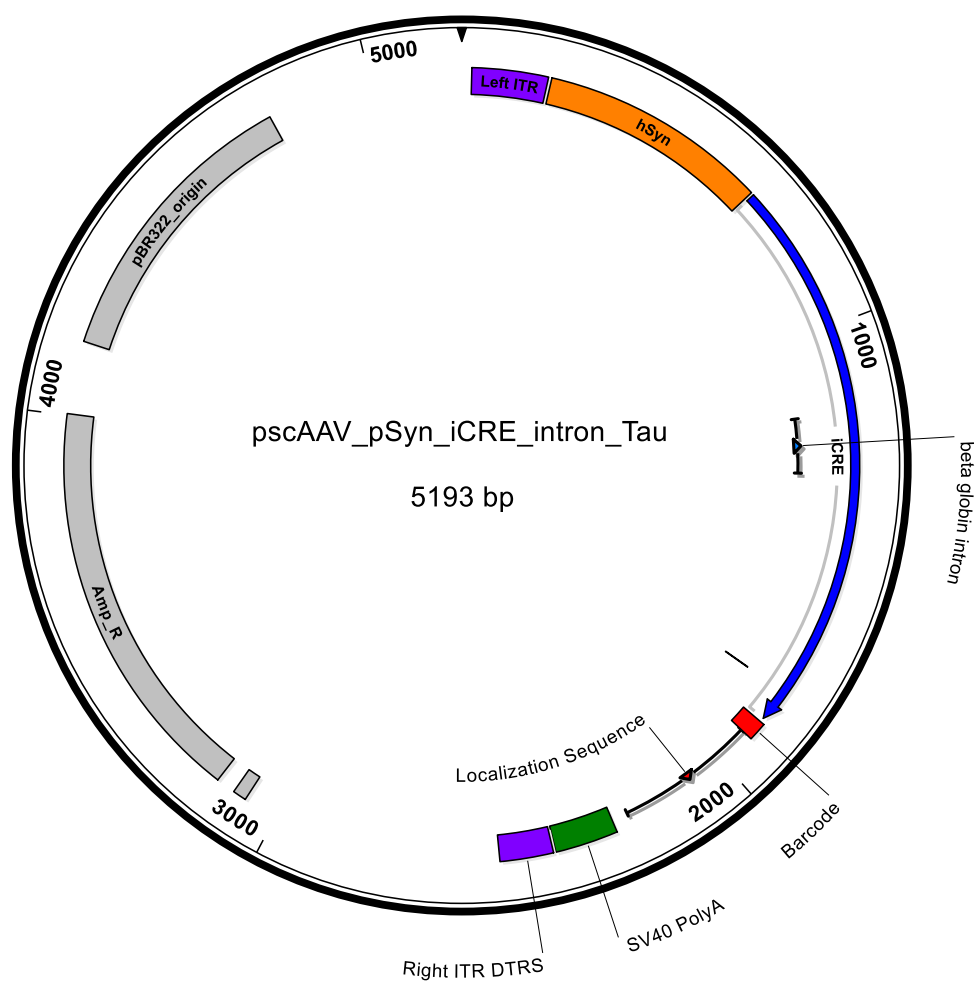


Figure S 12: Plasmid Map pscAAV_pSyn_iCRE_Intron_Tau. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_Tau [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.12 pscAAV_pSyn_iCRE_intron_Calreticulin_1

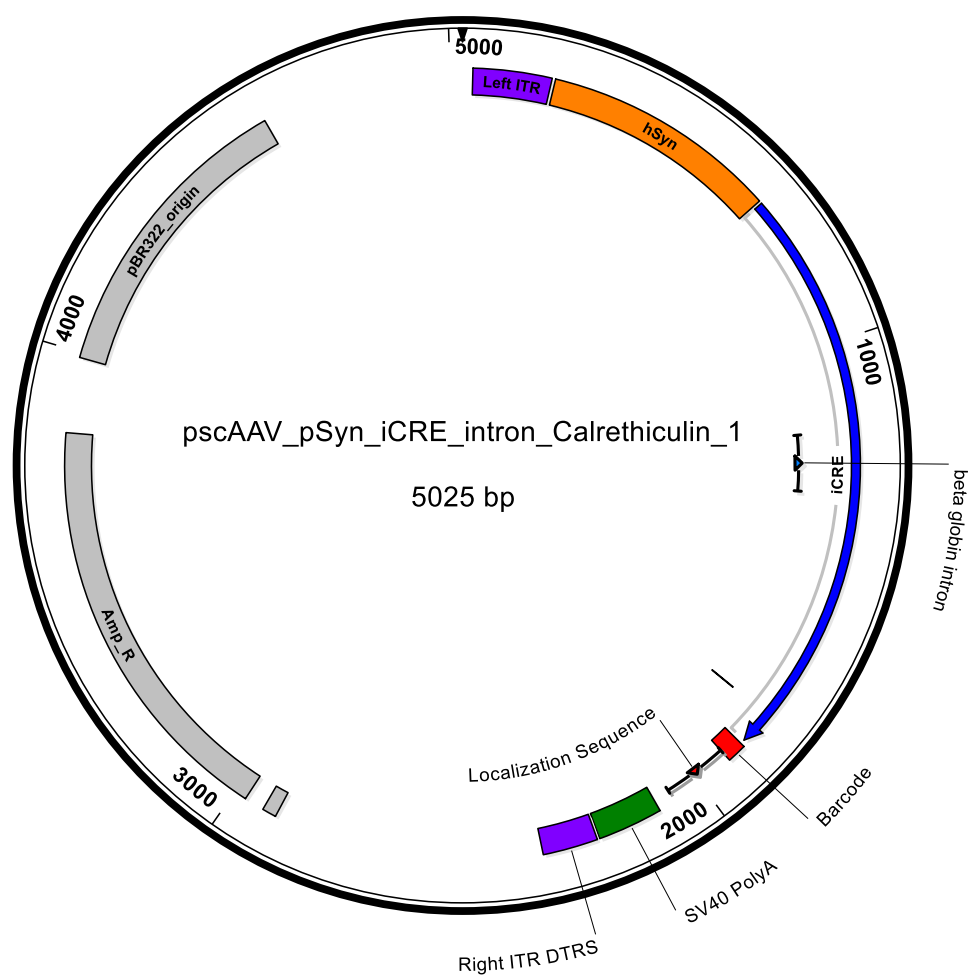


Figure S 13: Plasmid Map pscAAV_pSyn_iCRE_Intron_Calreticulin_1. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_Calreticulin_1 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.13 pscAAV_pSyn_iCRE_intron_Calreticulin_2

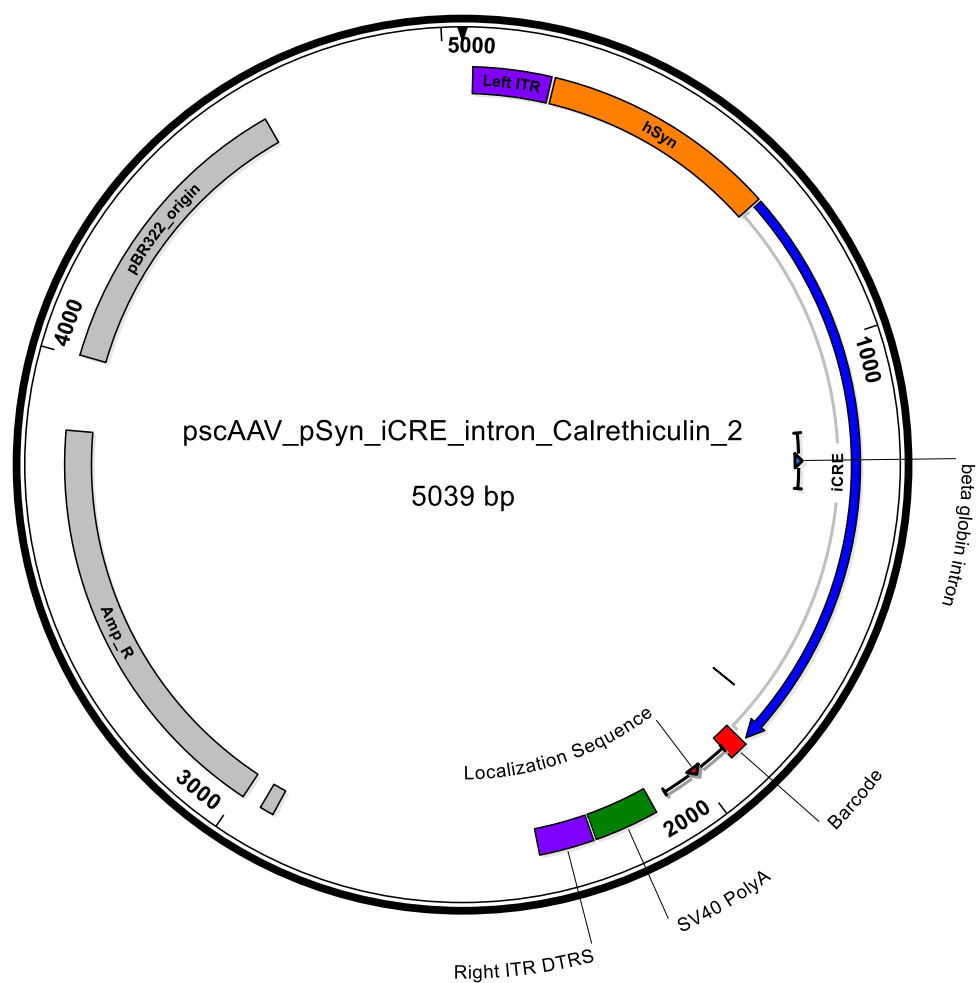


Figure S 14: Plasmid Map pscAAV_pSyn_iCRE_Intron_Calreticulin_2. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_Calreticulin_2 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.14 pscAAV_pSyn_iCRE_intron_ActG1

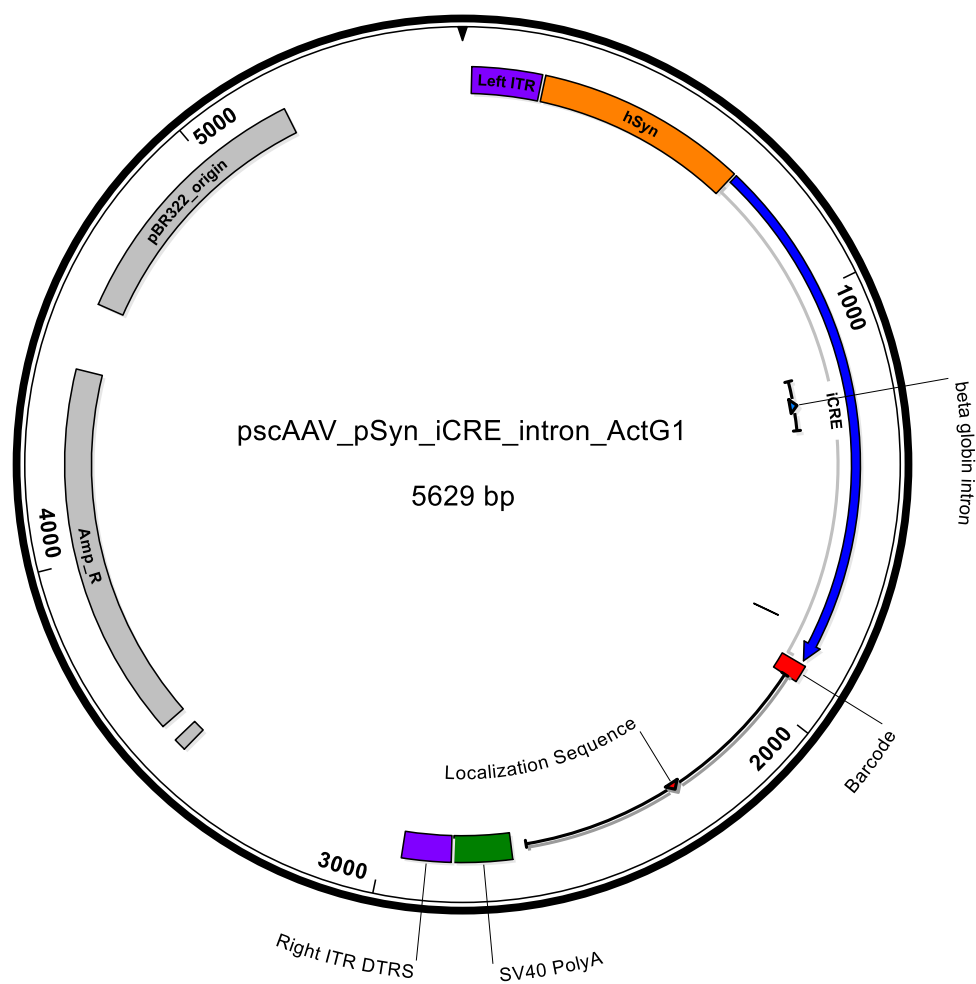


Figure S 15: Plasmid Map pscAAV_pSyn_iCRE_Intron_ActG1. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_ActG1 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.15 pscAAV_pSyn_iCRE_intron_p5

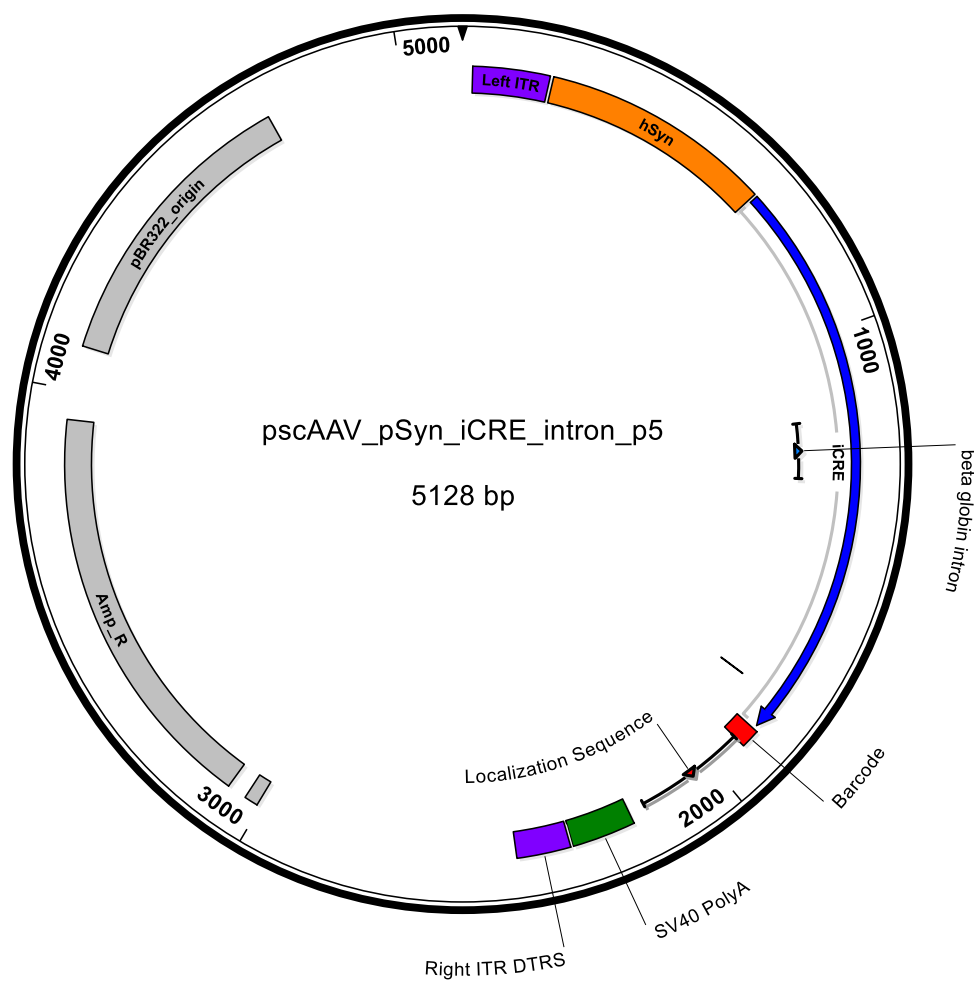


Figure S 16: Plasmid Map pscAAV_pSyn_iCRE_Intron_p5. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_p5 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.16 pscAAV_pSyn_iCRE_intron_p26_F1

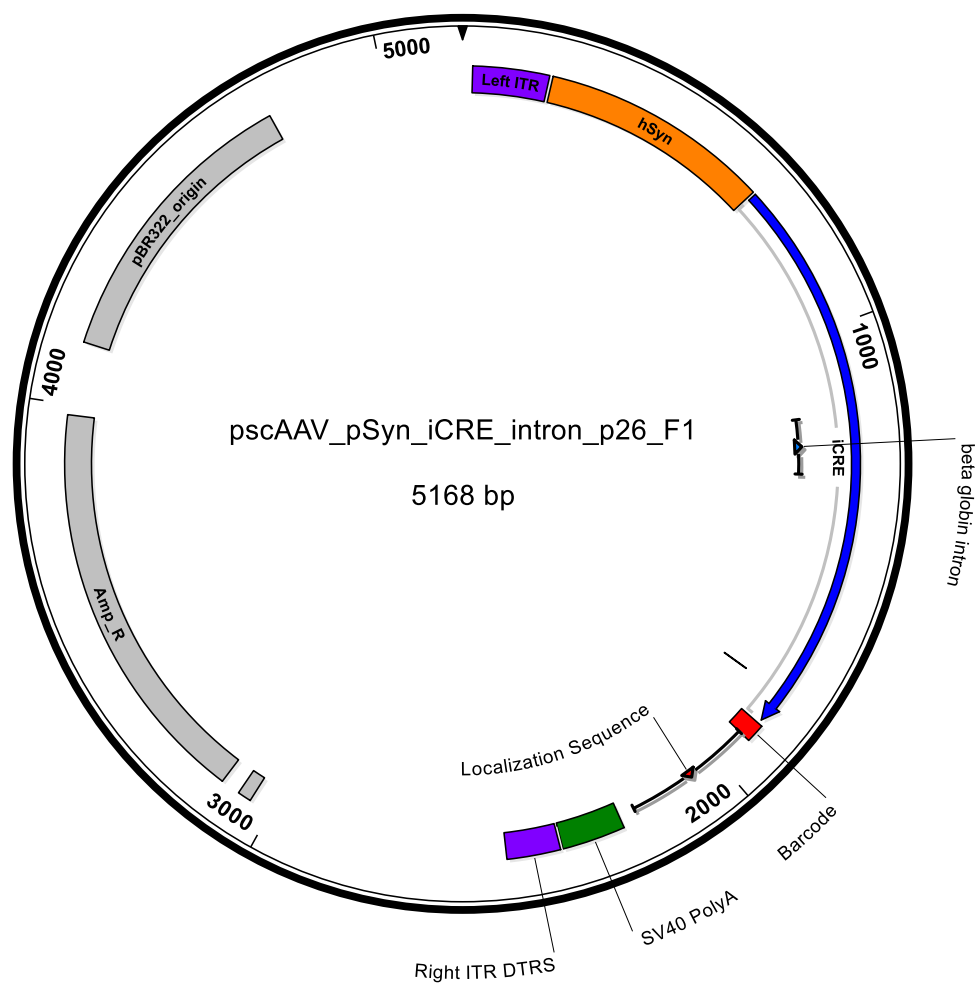


Figure S 17: Plasmid Map pscAAV_pSyn_iCRE_Intron_p26_F1. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_ActG1 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp^R, Ampicillin Resistance]

9.2.17 pscAAV_pSyn_iCRE_intron_P26_E1

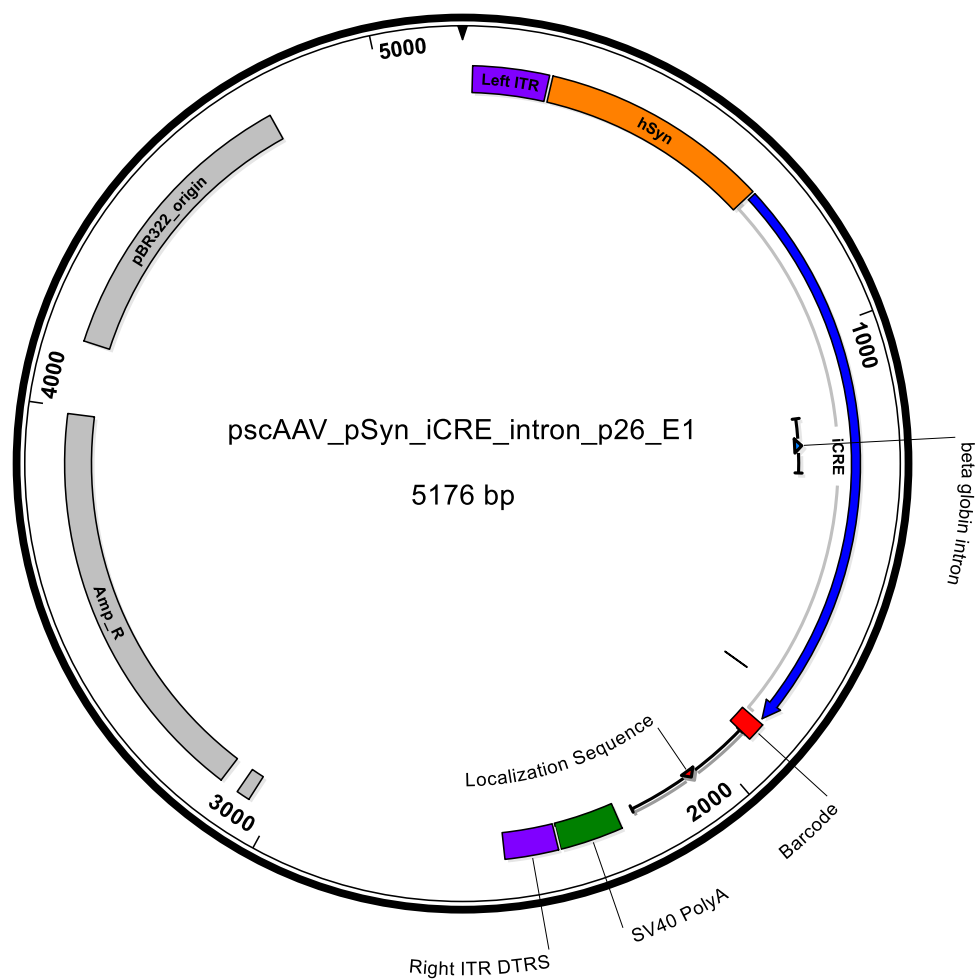


Figure S 18: Plasmid Map pscAAV_pSyn_iCRE_Intron_p26_E1. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_p26_E1 [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

9.2.18 pscAAV_pSyn_iCRE_intron_p26_LacZ

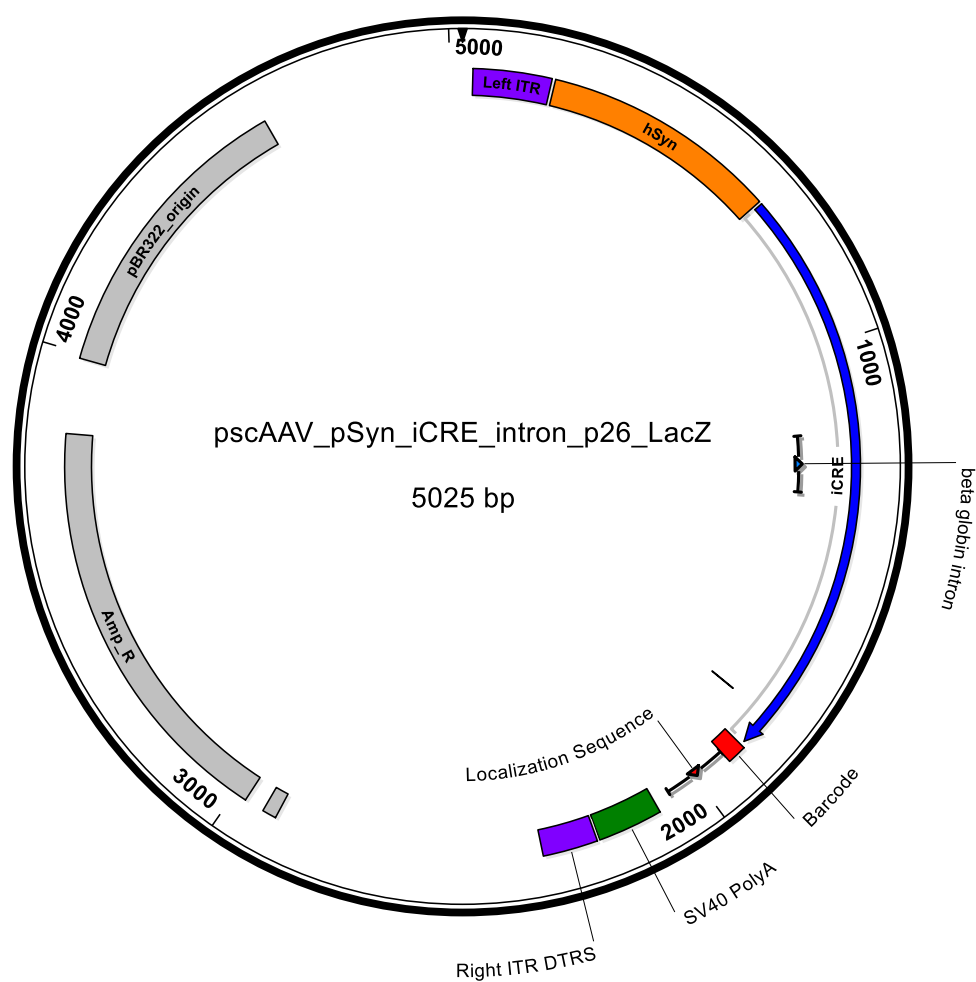


Figure S 19: Plasmid Map pscAAV_pSyn_iCRE_Intron_p26_LacZ. Map of Plasmid p143_pscAAV_pSyn_iCRE_Intron_p26_LacZ [ITR, inverted terminal repeats; hSyn, human synapsin promoter; iCRE, improved CRE recombinase; SV40 PolyA, SV40 polyadenylation site; Amp_R, Ampicillin Resistance]

10 Literature

10.1 References

- Pollock, J. D., Wu, D.-Y. & Satterlee, J. S. Molecular neuroanatomy: a generation of progress. *Trends Neurosci.* **37**, 106–23 (2014).
- Köbber, C. *et al.* Current concepts in neuroanatomical tracing. *Prog. Neurobiol.* **62**, 327–51 (2000).
- Huh, Y. & Oh, M. Gene transfer in the nervous system and implications for transsynaptic neuronal tracing. *Expert Opin. ...* **10**, 763–772 (2010).
- Schiavo, G., Matteoli, M. & Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* **80**, 717–66 (2000).
- Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R. Simultaneous amplification and detection of specific DNA sequences. *Biotechnology NY* **4**, 413-7 (1992).
- Azevedo, F. a C. *et al.* Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. *J. Comp. Neurol.* **513**, 532–41 (2009).
- Yoshihara, Y. Visualizing selective neural pathways with WGA transgene: combination of neuroanatomy with gene technology. *Neurosci. Res.* **44**, 133–40 (2002).
- Luo, L., Callaway, E. M. & Svoboda, K. Genetic dissection of neural circuits. *Neuron* **57**, 634–60 (2008).
- Kissa, K. In Vivo Neuronal Tracing with GFP-TTC Gene Delivery. *Mol. Cell. Neurosci.* **20**, 627–637 (2002).
- Aranda-Abreu, G. E., Hernández, M. E., Soto, A. & Manzo, J. Possible Cis-acting signal that could be involved in the localization of different mRNAs in neuronal axons. *Theor. Biol. Med. Model.* **2**, 33 (2005).
- Di Liegro, C. M., Schiera, G. & Di Liegro, I. Regulation of mRNA transport, localization and translation in the nervous system of mammals (Review). *Int. J. Mol. Med.* **33**, 747–62 (2014).
- Will, C. L. & Lu, R. Spliceosome Structure and Function. 1–23 (2011).

Willis, D. E. *et al.* Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J. Cell Biol.* **178**, 965–80 (2007).

Welshhans, K. & Bassell, G.J. Netrin-1-induced Local β -actin Synthesis and Growth Cone Guidance Requires Zipcode Binding Protein 1. *J Neuroscience.* **31**, 9800–9813 (2012).

Kislauskis, E. H., Zhu, X. & Singer, R. H. Sequences Responsible for Intracellular Localization of α -Actin Messenger RNA Also Affect Cell Phenotype. **127**, 441–451 (1994).

Sundell, C. L. & Singer, R. H. Actin mRNA localizes in the absence of protein synthesis. *J. Cell Biol.* **111**, 2397–403 (1990).

Eng, H., Lund, K. & Campenot, R. B. Synthesis of α -Tubulin, Actin, and Other Proteins in Axons of Sympathetic Neurons in Compartmented Cultures. **19**, 1–9 (1999).

Vuppalanchi, D. *et al.* Conserved 3'-untranslated region sequences direct subcellular localization of chaperone protein mRNAs in neurons. *J. Biol. Chem.* **285**, 18025–38 (2010).

Jirikowski, G. F., Sanna, P. P. & Bloom, F. E. mRNA coding for oxytocin is present in axons of the hypothalamo-neurohypophyseal tract. **87**, 7400–7404 (1990).

Bi, J., Tsai, N.-P., Lin, Y.-P., Loh, H. H. & Wei, L.-N. Axonal mRNA transport and localized translational regulation of kappa-opioid receptor in primary neurons of dorsal root ganglia. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 19919–24 (2006).

Litman, P., Barg, J., Rindzoonski, L. & Ginzburg, I. Subcellular localization of tau mRNA in differentiating neuronal cell culture: Implications for neuronal polarity. *Neuron* **10**, 627–638 (1993).

Aronov, S., Aranda, G., Behar, L. & Ginzburg, I. Axonal Tau mRNA Localization Coincides with Tau Protein in Living Neuronal Cells and Depends on Axonal Targeting Signal. **21**, 6577–6587 (2001).

Antar, L. N., Li, C., Zhang, H., Carroll, R. C. & Bassell, G. J. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. *Mol. Cell. Neurosci.* **32**, 37–48 (2006).

Willis, D. E. *et al.* Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J. Cell Biol.* **178**, 965–80 (2007).

- Aschauer, D. F., Kreuz, S. & Rumpel, S. Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PLoS One* **8**, e76310 (2013).
- Doerfler, P. a, Byrne, B. J. & Clément, N. Copackaging of Multiple Adeno-Associated Viral Vectors in a Single Production Step. *Hum. Gene Ther. Methods* **8**, 1–8 (2014).
- Doutin, S., Monteilhet V., Veron, P., Leborgne C., Benveniste, O., Montus, M.F., Masurier, C.. Prevalence of Serum IgG and Neutralizing Factors Against Adeno-Associated Virus (AAV) Types 1, 2, 5, 6, 8, and 9 in the Healthy Population: Implications for Gene Therapy Using AAV Vectors. *Hum. Gene Ther.* **6**, 704–712 (2010).
- Lakkaraju, A. & Rodriguez-Boulan, E. Itinerant exosomes: emerging roles in cell and tissue polarity. *Trends Cell Biol.* **18**, 199–209 (2008).
- Bellingham, S. A., Guo, B., Coleman, B. & Hill, A. F. Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? *Front. Physiol.* **3**, 1–12 (2012).
- Frühbeis, C., Fröhlich, D., Kuo, W. P. & Krämer-Albers, E.-M. Extracellular vesicles as mediators of neuron-glia communication. *Front. Cell. Neurosci.* **7**, 182 (2013).
- van Niel, G., Porto-Carreiro, I., Simoes, S. & Raposo, G. Exosomes: a common pathway for a specialized function. *J. Biochem.* **140**, 13–21 (2006).
- Simons, M. & Raposo, G. Exosomes--vesicular carriers for intercellular communication. *Curr. Opin. Cell Biol.* **21**, 575–81 (2009).
- Arnold, C. D. *et al.* Genome-wide quantitative enhancer activity maps identified by STARR-seq. *Science* **339**, 1074–7 (2013).
- Heintzman, N. D. *et al.* Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* **459**, 108–12 (2009).
- Dong, J., Fan, P., Frizzell, R. A. & Al, D. E. T. Quantitative Analysis of the Packaging Capacity Recombinant Adeno-Associated Virus of. **2112**, 2101–2112 (1996).
- Grieger, J. C., Samulski, R. J., Hill, C. & Carolina, N. Packaging Capacity of Adeno-Associated Virus Serotypes : Impact of Larger Genomes on Infectivity and Postentry Steps. **79**, 9933–9944 (2005).

- Yan, Z., Zhang, Y., Duan, D. & Engelhardt, J. F. Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc. Natl. Acad. Sci.* **97**, 6716–6721 (2000).
- Duan, D., Yue, Y. & Engelhardt, J. F. Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol. Ther.* **4**, 383–91 (2001).
- Ghosh, A., Yue, Y. & Duan, D. Efficient transgene reconstitution with hybrid dual AAV vectors carrying the minimized bridging sequences. *Hum. Gene Ther.* **22**, 77–83 (2011).
- Wisden, W. & Meier, J. C. Genetic techniques and circuit analysis. *Front. Mol. Neurosci.* **3**, 4 (2010).
- Alilain, W. J. & Silver, J. Shedding light on restoring respiratory function after spinal cord injury. *Front. Mol. Neurosci.* **2**, 18 (2009).
- Adamantidis, A., Carter, M. C. & de Lecea, L. Optogenetic deconstruction of sleep-wake circuitry in the brain. *Front. Mol. Neurosci.* **2**, 31 (2010).
- Han, X., Qian, X., Stern, P., Chuong, A. S. & Boyden, E. S. Informational lesions: optical perturbation of spike timing and neural synchrony via microbial opsin gene fusions. *Front. Mol. Neurosci.* **2**, 12 (2009).
- Nichols, C. D. & Roth, B. L. Engineered G-protein Coupled Receptors are Powerful Tools to Investigate Biological Processes and Behaviors. *Front. Mol. Neurosci.* **2**, 16 (2009).
- Wisden, W., Murray, A. J., McClure, C. & Wulff, P. Studying Cerebellar Circuits by Remote Control of Selected Neuronal Types with GABA(A) Receptors. *Front. Mol. Neurosci.* **2**, 29 (2009).
- Hodge, J. J. L. Ion channels to inactivate neurons in *Drosophila*. *Front. Mol. Neurosci.* **2**, 13 (2009).
- Holford, M., Auer, S., Laqua, M. & Ibáñez-Tallón, I. Manipulating neuronal circuits with endogenous and recombinant cell-surface tethered modulators. *Front. Mol. Neurosci.* **2**, 21 (2009).
- White, B. H. & Peabody, N. C. Neurotrapping: cellular screens to identify the neural substrates of behavior in *Drosophila*. *Front. Mol. Neurosci.* **2**, 20 (2009).

Kasuya, J., Ishimoto, H. & Kitamoto, T. Neuronal mechanisms of learning and memory revealed by spatial and temporal suppression of neurotransmission using shibire, a temperature-sensitive dynamin mutant gene in *Drosophila melanogaster*. *Front. Mol. Neurosci.* **2**, 11 (2009).

Tessier, C. R. & Broadie, K. Activity-dependent modulation of neural circuit synaptic connectivity. *Front. Mol. Neurosci.* **2**, 8 (2009).

Huang, S. & Kamihira, M. Development of hybrid viral vectors for gene therapy. *Biotechnol. Adv.* **31**, 208–23 (2013).

Torashima, T., Koyama, C., Higashida, H. & Hirai, H. Production of neuron-preferential lentiviral vectors. *Protocol Exchange* **89** (2007).

Heister, T., Heid, I., Ackermann, M. & Fraefel, C. Herpes Simplex Virus Type 1/Adeno-Associated Virus Hybrid Vectors Mediate Site-Specific Integration at the Adeno-Associated Virus Preintegration Site, AAVS1, on Human Chromosome 19. *J. Virol.* **76**, 7163–7173 (2002).

Mulligan, R. The basic science of gene therapy. *Science* (80-.). (1993).

Montini, E. *et al.* The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. *J. Clin. Invest.* **119**, 964–975, doi:10.1172/jci37630 (2009).

Kotin, R. & Siniscalco, M. Site-specific integration by adeno-associated virus. *Proc. ...* **87**, 2211–2215 (1990).

Smith, R. H. Adeno-associated virus integration: virus versus vector. *Gene Ther.* **15**, 817–22 (2008).

Duan, D., Sharma, P. & Dudas, L. Formation of adeno-associated virus circular genomes is differentially regulated by adenovirus E4 ORF6 and E2a gene expression. *J. ...* **73**, 161–169 (1999).

Al, G. E. T. N o v e l T o o l s f o r P r o d u c t i o n a n d P u r i f i c a t i o n R e c o m b i n a n t A d e n o - a s s o c i a t e d V i r u s V e c t o r s o f D I R K G R I M M , ! A N D R E A K E R N , ' K A R O L A R I T T N E R , ^ a n d J U R G E N A . K L E I N S C H M E D T ' 2746. **2760**, 2745–2760 (1998).

- Zolotukhin, S., Potter, M., Zolotukhin, I. & Sakai, Y. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* **28**, 158–167 (2002).
- Zhang, Y. *et al.* Dual AAV therapy ameliorates exercise-induced muscle injury and functional ischemia in murine models of Duchenne muscular dystrophy. *Hum. Mol. Genet.* **22**, 3720–9 (2013).
- Zhang, P. *et al.* Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. *Hum. Gene Ther.* **23**, 460–72 (2012).
- Day, T. P., Byrne, L. C. & Flannery, J. G. Retinal Degenerative Diseases. **801**, 687–693 (2014).
- McCarty, D. M., Monahan, P. E. & Samulski, R. J. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther.* **8**, 1248–54 (2001).
- Batagov, A. O. & Kurochkin, I. V. Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3[prime]-untranslated regions. *Biol. Direct* **8**, 12 (2013).
- Valadi, H. *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**, 654–9 (2007).
- Gerdes, H.-H. & Carvalho, R. N. Intercellular transfer mediated by tunneling nanotubes. *Curr. Opin. Cell Biol.* **20**, 470–5 (2008).
- Gerdes, H.-H., Rustom, A. & Wang, X. Tunneling nanotubes, an emerging intercellular communication route in development. *Mech. Dev.* **130**, 381–7 (2012).
- Pan, B., Teng, K., Wu, C., Adam, M. & Johnstone, R. M. Electron Microscopic Evidence for Externalization of the Transferrin Receptor in Vesicular Form in Sheep Reticulocytes. **101**, (1985).
- Pan, W. *et al.* DNA polymerase preference determines PCR priming efficiency. *BMC Biotechnol.* **14**, 10 (2014).

Loya, A. D. I., Pnueli, L., Yosefzon, Y., Wexler, Y. D. O. & Ziv-ukelson, M. The 39-UTR mediates the cellular localization of an mRNA encoding a short plasma membrane protein. *Spring* 1352–1365 (2008).

Chabanon, H., Mickleburgh, I., and Hesketh, J. Zipcodes and postage stamps: mRNA localization signals and their trans-acting binding proteins. *Brief Funct. Genomic. Proteomic.* **3**: 240–256 (2004).

Lopez de Heredia, M. and Jansen, R.P. mRNA localization and the cytoskeleton. *Curr. Opin. Cell Biol.* **16**: 80–85 (2004).

10.2 Books

Kandel, E.R., Schwartz J.H., Jessel, T.M., (1996) *Neurowissenschaften – Eine Einfuehrung*

Kopf, D. Instruments, Model 1900 Stereotaxic Alignment System Set-Up Procedure & Operation Manual

Purves, D., Augustine, G.J., Fitzpatrick, D., Hall, C.W., LaMantia, A-S., White, L.E., (2012) *Neuroscience Fifth Edition*

Dorak, M.T. (2006) *Real-time PCR*

Logan, J., Edwards, K., Saunders, N. (2009) *Real-time PCR – Current Technology and Applications*

Kaplitt, M.G., Loewy, A.D., (1995) *Viral Vectors – Gene Therapy and Neuroscience Applications*

10.3 Internet sources

<http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/>

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