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**Long-term, stable, functional and non-invasive labeling across the brain of
a preclinical animal model**

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Abstract

Brain disorders remain difficult targets for pharmacological compounds that rarely can provide a therapeutic effect without considerable off-target effects. To provide more specific therapeutic effect, adeno-associated viral (AAV) vectors are increasingly used to target an exogene to the central nervous system. In mice, the most widely adopted model species, AAV-based gene delivery is used extensively. In stark contrast, in large-animal preclinical models, we still largely lack efficient and long-term safe AAV-based gene delivery methods. Synthetic capsid variants derived from the wild-type AAV9 have been shown to cross the blood-brain barrier and achieve widespread and homogeneous labeling of the brain upon minimally-invasive administration in mice. In my work, I ask whether new synthetic AAV variants can achieve efficiency and safety of delivery in large-animal preclinical species that is similar to that observed in mice. I define as “successful and functional delivery” in the context of optogenetic activation of visual cortex. I leveraged recent breakthroughs of optogenetic protein optimization and demonstrate that a minimally-invasive gene delivery combined with minimally-invasive optogenetic brain stimulation is ready to serve as the core technology foundation for a completely new mode of brain stimulation. I target the therapeutic optogenetic protein to specific cell types in the brain of a large-animal preclinical species at unprecedented efficiency. The development of safe and efficient gene delivery combined with high-density optogenetic stimulation may open a new era of brain therapy.

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1. Introduction

Understanding big brains requires big science. The Human Genome Project that ran around the turn of millennia led to the explosion of new biotechnology methods making nucleotide sequence engineering more efficient, reliable and accessible. Initially, engineered DNA sequences have been injected or electroporated into cells to study or modify their function. However, these methods are low-throughput and cannot be easily reconciled with the need of long-term and safe survival of the targeted cells. During the first decade of the 21st century, adeno-associated viruses (AAVs) were picked up as an efficient and reliable vector system for getting engineered DNA into target cells. Today, AAVs are a standard tool used to deliver sequences encoding proteins that report, or modify cell function. In parallel to the increasing adoption of AAV-based gene delivery, translational medicine also picked up the new tool for the purpose of gene therapy. Gene therapy can target a wide range of medical conditions such as metabolic, cardiovascular, haematological, neurological, ophthalmological diseases, as well as infectious diseases and cancer. High proportion of gene therapy targets are rare diseases caused by defects occurring on a single-gene. Additionally, gene therapy holds promise as a viable approach for individuals not only with rare single gene defects but for the ones who presently lack any means of treatment. More than 2000 clinical trials [1] have focused on AAV-based gene therapy over the last decades. Already three out of five FDA-approved gene therapies successfully implemented the use of AAVs as a vector system.

transgene, that is delivered into the host. When we look at basic science applications, the exogenous protein expressed upon transduction can implement a gain-of-function in the target cells, in the form of e.g. making the cells fluorescent. In translational medicine applications – gene therapy –, the exogenous sequence often encodes the correct version of a mutated endogenous gene. AAVs possess distinct advantages that set it apart from other gene delivery vectors. AAV demonstrates remarkable selectivity in transducing various tissue types. The introduction of novel capsids not only enhances this specificity in tropism but also mitigates the neutralizing impact of antibodies generated in humans against wild-type AAV capsids. Additionally, AAV enables long-term transgene expression without the need for integration into the host genome avoiding any insertion caused malfunction, and it is characterized by its non-pathogenic nature. Thanks to the simple genomic structure its DNA can be easily modified and designed in accordance with our objectives. Distinct cell or tissue specific promoters can be inserted into the viral DNA to further specify the transgene expression in the injected individual, creating a platform for precise genetic targeting. Capsid and promoter engineering

can minimize off-target effects, reduce the occurrence of side effects, lower the required viral load for clinical effectiveness, thus mitigating the magnitude of the immune response and minimizing treatment costs. Hence, AAV with its outstanding performance and safety profile, along with modern biotechnological tools, holds a great deal of additional potential as a therapeutic gene delivery vector.

Ongoing clinical trials are thoroughly examining another crucial aspect alongside the AAV viral construct design: the route of administration. The viral vector can be introduced directly into the affected area, organ, or tissue or another approach is to deliver the therapeutic vehicle into circulating body fluids such as blood or cerebrospinal fluid, from which the targeted area can uptake the particles ensuring minimal invasiveness of the therapeutic delivery. The results from small-scale human tests highlighted that the number of clinical trials that met primary efficacy endpoints based on whether they followed the exact method of vector administration used in the final large animal studies or deviated from it has a significant difference. A higher translational success rate was observed when the administration route and dosage of AAV in human applications remained consistent with those used in the final large animal studies [1]. Efficacy of AAV-based gene therapy by route of administration shows that the intrathecal and intravenous (IV) delivery routes results in the highest success rate, 100% and 80% respectively [1]. Although, the safety profiles show that besides their non or minimal invasiveness regarding the delivery the ratio of unexpected side effects is high. The solution is the application of selective and efficient AAV vectors, achieved through specific capsid and promoter designs. In summary, adeno-associated viral-based therapeutic advancements administered via intrathecal or intravenous delivery routes holds the potential to revolutionize gene therapy by enabling non-invasive or minimally invasive procedures with precise genetic targeting capabilities.

Our research team aims to develop a minimally invasive AAV based gene therapy combined with high density optical brain interface for optogenetic visual restoration. We would like to apply our technology in blind patients who have lost the eye-to-brain connection caused by optic nerve atrophy or due to conditions such as glaucoma, diabetic retinopathies or nerve trauma where the first stages of the visual pathway (eye, optic nerve) are affected with the condition but the rest of the sensory system remains largely intact [2]. As the pre-dominant structure of the sensory system is spared it is thought that a device-based prosthetic approach to restoration of visual function will be effective and will enjoy similar success as cochlear implants have for restoration of auditory function, the optimal technology is yet to be developed [2]. Our goal is to achieve this development of technology that would be of great significance

as there is yet no treatment for such blind patients. The aim is to apply novel adeno associated virus based genetic targeting of the thalamic *Dorsal Lateral Geniculate Nucleus* (LGN) that receives primary input from the retina. To maximize efficiency and safety, targeted labeling is accomplished through minimally or non-invasive delivery methods avoiding brain damage, while target specificity is achieved using LGN specific promoters and novel AAV capsids. The therapeutic gene is responsible to express light sensitive opsins that functions as cation channel upon light activation enabling optical stimulation of the nerve endings of neurons projecting from the LGN to the primary visual cortex. Application of red light-sensitive opsins facilitate deeper brain area stimulation from the brain surface by taking advantage of the fact that longer-wavelength light is less scattered in living tissue. By modulating the activity of the axon terminals from above the visual cortex by a high-density red LED array, high resolution restored function can be achieved with minimal invasiveness.

This research mainly focuses on the development of long-term, stable and functional labeling of the whole brain with red-shifted opsin expressing recombinant adeno-associated viral vectors delivered via intrathecal or intravenous administration routes in preclinical animal model. The selected model animals for this research are cats, which possess a visual system that closely resembles that of humans in both structure and function. This choice enhances the translational potential of the results, as findings from cat models can provide valuable insights directly applicable to human visual therapies. The research addresses to find the optimal dose depending on the delivery route and the best performing viral construct to achieve safe, effective and functional labeling of the whole brain. The results would serve as a fundamental basis for further development to realize LGN specific expression with engineered promoters. In addition, I pursue to find the optimal stimulation parameters for the applied red-shifted opsin to be able to efficiently activate the targeted brain area or cells while the brain function is monitored chronically via fluorescent widefield imaging. The applied viral vectors and methods described in this study represent a significant step forward in the development of gene therapy platform integrated with optogenetics, aiming to revolutionize the treatment of individuals with visual impairments.

2. Literature background

2.1 Recombinant adeno-associated viruses are good candidates for targeting the central nervous system

Understanding how the neuronal networks and local circuits work would give an insight into our most complex organ, the brain and also it would reveal the underlying conditions of

neurodevelopmental or neurodegenerative diseases. By having a region or cell type specific targeting the role of different functional units of the circuits can be precisely described by modulating their activity and examine the caused effect. There is a wide variety of modulatory tools that are based on chemogenetics or optogenetics, for activity indicators different voltage sensors or fluorescent calcium indicators can be applied combined with optical imaging. There is no other way to gain explicit information on neurons or local circuits as all other functional measurements such as the electrophysiological characterization with electrodes, electroencephalograms or electrocardiograms are a summation of the activity of a larger brain area or only give results about a single cell action potentials and not about its function in a network. Upon understanding a disease-causing malfunction, disorder or developmental problem, genetic targeting could be suitable to deliver neuromodulators as therapeutic tools to decrease, reverse or repair the condition.

Adeno-associated viruses (AAV) belong to the family of single-stranded DNA parvoviruses. As either pathogenic behaviour or toxicity can be connected to this virus type it is a very attractive tool for gene therapy. The simple structure enables the facile and effective production of the AAVs as the virus consists of a single-stranded DNA and a packaging capsid. The single-stranded DNA of the wild-type AAV contains two open reading frames that are dragged along by two inverted terminal repeats (ITR). One of the coding regions contains the genes that are required for the replication (*rep*) of the viral DNA, the other one codes the genes of the capsid (*cap*) peptides. In recombinant AAVs (rAAV) the DNA sequence between the ITRs is replaced by the desired promoter and the transgenic gene or genes with a limited 4,7 kbp total length. During the rAAV production the necessary *rep* and *cap* genes and the required helper genes from adenoviruses are provided as plasmids (Fig. 1). In the final recombinant AAV only the single-stranded DNA flanked by the ITR sequences is packed into the capsid and lack the genes that helps in virus production after viral infection therefore the resulted rAAV is not able to replicate and only delivers the desired transgene(s) [3] [4].

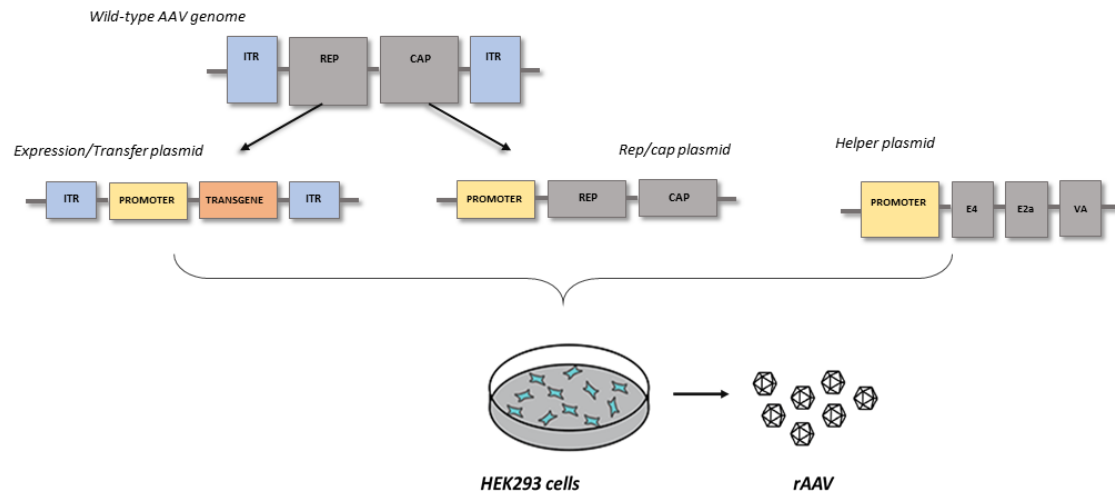


Figure 1: Recombinant adeno-associated virus production. The wild-type genome is modified as between the ITR sequences the desired promoter and transgene(s) are coded and the genes that are essential for the viral production are provided in the form of additional circular plasmids (rep/cap and helper plasmids). All of the three plasmids are transfected into HEK293 cells that serve as a host for rAAV production. After the viral purification the final rAAV only contains the DNA sequence with the ITR regions.

After delivering the rAAV by injection into the host organism the first step in the life cycle (Fig. 2) of the rAAV is that it enters into the cells via receptor and co-receptor binding although the infection mechanism of AAV is still poorly understood. Upon cellular recognition the particles go through endocytosis and the viral particles are transported in the cell through endosomal trafficking. The AAV transported into the nucleus and after the nuclear entry, it goes under uncoating and the single-stranded DNA is released although the mechanisms involved in uncoating are entirely unknown [5]. While the wild-type AAV is tend to integrate at non-homologous sites in the genome the rAAV DNA is typically remain extrachromosomally avoiding the risk of causing any damage or mutation in the host genome. The rAAV's single-stranded DNA is processed into a double-stranded episomes through double stranded synthesis that is facilitated by the secondary structure of ITRs that allow for self-priming. It worth to mention that self-complementary vectors (scAAV) that contain double-stranded DNA are overcoming this synthesis step where the payoff is that the length of the delivered gene is halved. The episomes concatemerize to produce high molecular weight structures that persist long-term in non-dividing cells such as neurons [3]. The formed

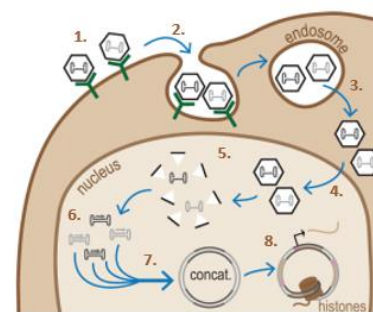


Figure 2: The life cycle of recombinant AAVs [11]. 1.) Cellular recognition through receptors and co-receptors. 2.) Endocytosis. 3.) Endosomal trafficking in the infected cell. 4.) Nuclear entry. 5.) Viral uncoating. 6.) Release of single-stranded viral DNAs and complementary strand synthesis. 7.) Extrachromosomal concatemer formation. 8.) Transcription of the delivered transgene(s).

circular double-stranded concatemers localize to the periphery of the nuclei where active transcription and DNA repair are known to occur. Probably the concatemers are also packed on histones that could result in epigenetic influence on the transcription although there are no clear data that describes the mechanism and the temporal progression. In summary, the advantage of replication-defective, non-pathogenic rAAV over wild-type AAV or other viral vectors is its relatively simple structure, the capability of entry into cells and its ability to deliver foreign genetic material into the nucleus, which can be stably expressed over the lifetime of the cell without DNA integration [6].

2.2 Tropism of naturally occurring and engineered AAV capsids

Naturally occurring AAVs have the ability to infect most tissues and cell types because the capsid responsible for packaging the DNA and forming the particle's surface is built up with different viral protein (VP) compositions [3]. To date, at least 12 natural serotypes and hundreds of variants of AAVs have been described with different tissue tropism and transduction efficiency making AAV a very useful system for preferentially infect specific cell types [7]. There are several capsids that show tropism towards the central nervous system (CNS) however, most of the commonly used natural serotypes are inefficient in crossing the blood brain barrier (BBB) therefore these must be delivered via injections given directly into the target brain area resulting in tissue damage and limited and uneven spatial coverage [8]. AAV9 due to its unique serological profile has an exclusive feature as it can cross the BBB and it has the ability to transduce neuronal and non-neuronal tissue while it also outperforms other capsids in cell transduction efficiency [9], [10]. To achieve non-invasive gene therapy in the brain by targeting the CNS with intravenously delivered viral vehicle,

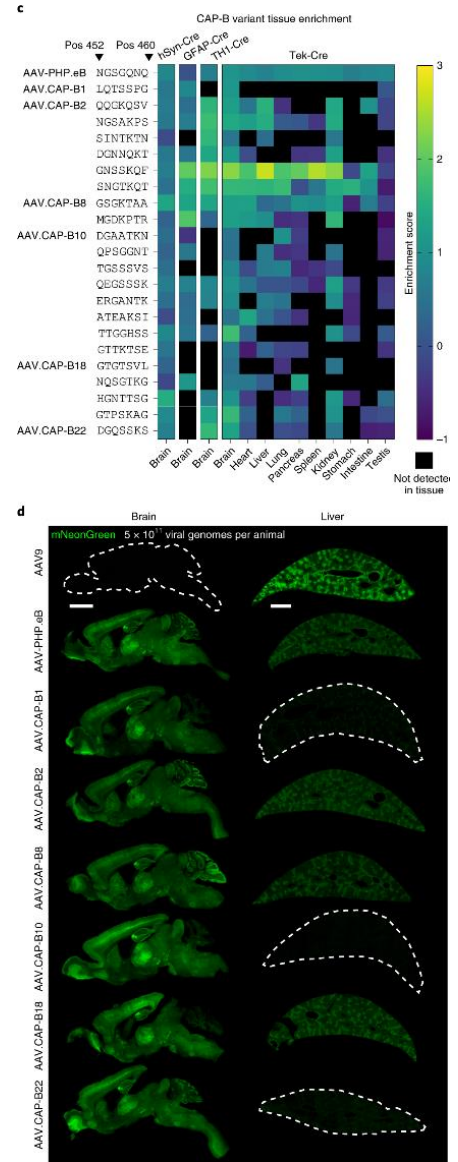


Figure 3: Comparison of AAV capsid variants regarding their expression efficiency, specificity and off-target transduction level [19]. c) The heat map shows the enrichment score across the different transgenic lines after sequencing, that is a calculated value based on the relative abundance of the variant's sequence in different tissues. d) Anatomical sections of the mouse brain and liver shows the fluorescent intensity and labeling density of each variant. Based on these data AAV.CAP-B10 and B22 are the best choice to target the CNS with systemic injections and avoiding liver transduction.

penetrating the blood-brain barrier is an essential property therefore AAV9 is a very prominent viral vector candidate in brain research and therapy. However, AAV9 transduces tissues other than the brain as well such as lung, cardiac muscles, skeletal muscles, pancreatic cells and hepatocytes [10]. By infecting other cell types upon intravenous injection may induce a strong systemic immune response, creating a confounding effect especially in the case of the liver that is a highly immunogenic organ. To overcome this issue genetic modifications were done on AAV9 capsid to optimize the delivery of a transgene achieving a transduction level similar to that of direct administration in affected cells while minimizing off-target transduction [11]. The first variant AAV-PHP.B transduced adult mouse brains with significantly higher efficiency than AAV9 although it requires a substantial dose of virus for effective labeling and it also failed to translate to non-human primates (NHP) [8], [12]. To enhance potency in the central nervous system new capsids were engineered such as AAV-PHP.eB later AAV.CAP-B10, AAV.CAP-B22, AAV-PHP.C1, AAV-CAP.Mac the latest ones shows more neuron biased tropism than AAV-PHP.eB [12], [13] [14], [15]. This represent notable progress as these capsids are capable of efficiently, specifically and safely delivering genes to the brain minimizing off-target labeling and in mouse lines and NHPs with moderate load of viral vectors (Fig. 3).

Reportedly, the promising effective labeling within the CNS through the BBB is not universally valid across species in the case of AAV-PHP.eB capsid [16]. Although, mechanism by which AAV crosses the BBB has not been fully understood experiments on human *in vitro* blood brain barrier model suggests that the crossing is tightly regulated by receptor-mediated transcytosis [17]. AAV-PHP.eB has been shown to rely on improved binding to Ly6a receptor for its increased CNS tropism [18] and researchers showed that probably due to the specific binding the cell transduction is mouse strain dependent [16]. These findings suggest that other AAV9 derivatives could also show expression differences across species even between rodents. The solution is to develop newly engineered capsid variants and also validate the translatability of capsids into human therapy on species that are genetically closer to humans [19].

2.3 Genetically encoded calcium indicators for optical neuronal activity readout

The central goal of neuroscience is to elucidate how information is encoded and processed at the circuit, neuronal ensemble or single neuron resolution in the intact brain chronically. Following the neural activity in real time requires well-developed activity indicators and recording methodologies. There are plenty of possibilities in both fields, researchers have to choose wisely which suits the most to their aims. Directly recording of electrical signal are like

single action potentials or a sum of fluctuating potentials of a large group of neurons are enabled by whole cell patch clamp or multi electrode measurements that are really advantageous from the time resolution point of view although they lack high throughput. They are also highly invasive methods so tracing neural activity change in chronic experiments cannot be performed in addition to the fact that multiunit recordings have poor spatial resolution. On the other pole of the scale there are the methods that enables large-scale recordings in a cost of temporal and spatial resolution such as the electroencephalogram (EEG) or functional magnetic resonance imaging (fMRI). One and two photon optical imaging techniques combined with viral delivery of sensors create the bridge between large-scale recordings and good spatial and temporal resolution over a long time.

Optical imaging creates the opportunity to follow neuronal activity indirectly with fluorescence change in a single cell or in a given brain area *in vivo*. As neurons communicate with each other through action potentials that is a result of a membrane potential change following different cellular events, activity can be recorded based on distinct indicative steps or substances. The most commonly used indicators detect neuronal activities via voltage or ion dynamics [23]. Voltage sensitive dyes are used to detect action potentials and subthreshold responses in neurons. Dyes can be delivered to the brain via different routes although chemical substances cannot be localized within the tissue with cell type specific manner therefore voltage indicator proteins are needed which expression can be under the control of specific promoters. Since there are no known naturally-occurring fluorescent proteins with acceptable characteristics for optical detection of membrane voltage transients it has been a challenge to use them due to a combination of small signal-to-noise ratio, slow kinetics, and poor membrane expression [24]. Additionally, the membrane potential is quite significant in amplitude, but it only exists in a confined fraction of space that further hardens the use of voltage based methods and prioritize the use of ion dynamic detection. The question is which ion should be monitored in neurons? Potassium ions are already abundant in intracellular spaces and are released after sodium influx during the repolarization phase, enabling the recording of the slow signal dissolution within the cells. Better to detect the ions, the sodium or the calcium that are responsible for membrane depolarization within neurons. Sodium ion has higher basal intracellular concentration and a smaller activity-driven concentration gap between extra- and intra-cellular concentrations compared to calcium ions therefore calcium detection is the most advantageous activity indicator in neurons. The low basal calcium concentration state enables minimizing nonspecific background signal, and the concentration gap between the basal and activated states has 7-20

fold difference that is 5.5 for sodium [23]. The calcium ion (Ca^{2+}) regulate activity-dependent signaling, it is the main second messenger that helps to transmit depolarization status and synaptic activity of a neuron thereby relaying information on neuronal activity status both locally (in a dendritic spine, for example) and globally (to initiate widespread changes in energy metabolism, for example) [25]. There are Ca^{2+} sensitive dyes that are widely used in neuroscience because they are relatively easy to implement and provide large signal-to-noise ratios [26] although the same limitation applied as with the voltage sensitive dyes, they are not capable of genetically defined cell type specific labeling. As a result, intracellular calcium sensing proteins, the so called genetically encoded calcium indicators (GECIs) are the superior activity tracers in neuroscience research.

The term “genetically encoded” refers to the fact that the sensors are solely composed of amino acids with no addition of any synthetic compound or cofactor that would make it difficult to apply in the living brain [27]. The most widely used, well-characterized and developed GECI is the G-CaMP that is based on single green fluorescent protein (GFP) with high Ca^{2+} binding affinity that enables visualizing intracellular Ca^{2+} in living cells by the emission of green fluorescence upon neural activity. GFP has been modified on sites that determines fluorescence characteristics this is how circularly permuted enhanced green fluorescent protein (cpEGFP) was created that has no or very low fluorescence in a certain conformation and bright green light emission in another. GCaMP is composed of three domains and the connecting linker regions. The N terminus of cpEGFP is connected to the M13 fragment (a mutated version of RS20) of myosin light chain kinase is a target sequence of calmodulin (CaM), whereas the C terminus of cpEGFP is connected to CaM. When Ca^{2+} binds to CaM, cpEGFP conformational changes due to the Ca^{2+} –CaM–M13 interaction that induce the fluorescence intensity changes [28].

As earlier versions of GCaMP reported neural activity at timescales much slower than electrical signalling and are limited by trade-offs between sensitivity and kinetics further developed version of the probe was engineered. There are three main targets of modifications: the fluorophore to increase fluorescence, the calcium binding domain that influences probe sensitivity and the calcium-dependent interactions between CaM and the CaM-binding peptide that help to determine sensor kinetics. With structure-guided mutagenesis at the hotspots and with CaM binding peptide replacement (RS20 to ENOSP or CaMKK in the

latest versions respectively) four newer generations of GCaMPs were developed in the last 10 years, GCaMP6, jGCaMP7, XCaMP-G and jGCaMP8, in time order respectively, for optimal sensitivity and kinetics [29] [30] [31]. There are fast (f) variants that were optimized for kinetics, they have faster rise and decay times, but with reduced sensitivity; slow (s) variants that are more sensitive in a cost of slower kinetics response, GCaMP6 and 8 has medium (m) variants that has medium rise and medium decay time [30] (worth to mention that GCaMP7 has several other more specific variants as well). The latest engineered GCaMP7f, XCaMP-G and GCaMP8f are able to resolve real time single action potentials from a firing sequence up to 50 Hz without waiting for the sensor rise time in between spikes that is required with GCaMP6, 8f achieve this fast kinetics with excellent sensitivity. Comparing their induced fluorescence change and kinetics (Fig. 5) the latest GCaMP8 series are the most advantageous sensors as they have the fastest kinetics and highest sensitivity.

There is only one limitation of GCaMP indicators as they could cause side-effects in neurons especially for enhanced or prolonged expressions or for transgenic. Side-effects include the damage or growth impairments in neurites accompanied by aberrant Ca^{2+} oscillations or even results in cell death [32]. These side-effects seem to be closely associated with abnormal accumulation of GCaMP filling in the nuclei which mechanism is not described yet [33]. Probably this is due to the fact that GCaMP containing calmodulin acts as an impaired apoCaM that interferes with both gating and signaling of L-type calcium channels which disrupts Ca^{2+} dynamics and gene expression [33]. Documented imaging problems occur with GCaMP

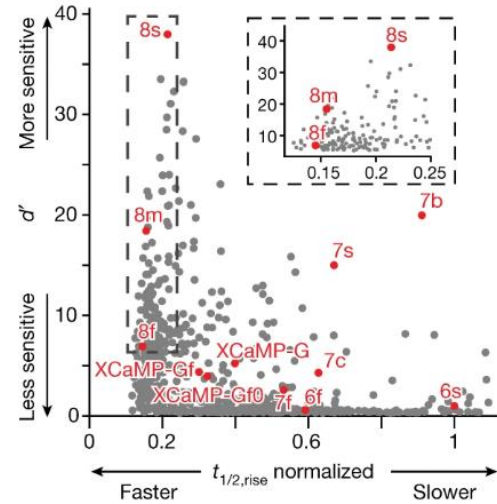


Figure 5: Comparison of different GCaMP variant regarding their sensitivity and kinetics [39]. There is always a trade-off between the indicators sensitivity and its kinetics. This plot shows that the latest versions of GCaMPs achieved fast kinetics and high sensitivity.

during long expression of the protein, such as weak or diminished readout of Ca^{2+} signal that are likely due to excitation-transcription perturbations and calcium channel dysregulations in excitable cells. To overcome this problem special (weaker) promoters, optimal viral dilutions, conditional expression, and other tactics are utilized to reduce the expression level/time of GCaMP but keep the required level for *in vivo* imaging. On top of that researchers developed GCaMP-X by engineering an additional apoCaM-binding protective motif and an extra tag ensuring subcellular localization into conventional GCaMP [33]. There is no significant performance difference compared to GCaMP6m so GCaMP-X inherits the excellent properties of Ca^{2+} -sensing evolved from generations of GCaMPs, while avoiding Ca^{2+} distortion and other problems.

For combinatorial use with other activity indicators or optogenetic proteins for light-based control of neuronal activity further color variants were developed beyond green fluorescence indicators such as jRGECO1a [34] that emit red light upon activity or other multicolor variants of XCaMPs. Pairing these activity tracers with modern light-based modulatory elements within a rAAVs DNA as a bicistronic cargo lot of possibilities arise, researchers only have to choose the combination and variant(s) that suits best their aims.

2.5 Precise neuronal activity modulation by light-sensitive proteins

To understand or to repair the function of individual cells in a neuronal circuit or to reveal the functional connection between brain areas precise activity modulators are required. To date, there is a wide variety of neuronal modulators that are used for these purposes such as electrical stimulators, pharmacons, chemogenetic tools and light-sensitive opsins. The difference between these methods is in their invasiveness, specificity or spatial resolution and temporal precision. Electrodes cannot be used to precisely target defined cells and they are also highly invasive so their long-term usage in therapy is questionable and drugs and drug-related mechanisms act much slower compared to neuronal firing rate [35]. Only the light-sensitive opsins used in joint with genetic targeting are capable of real-time optical neuronal activity modulation with high spatial (cell type-specific) and temporal (millisecond-scale) resolution [35], [36].

Opsins are light-sensitive proteins that directly regulate the flow of ions across the plasma membrane or initializing distinct signaling pathways or interactions. Different subtypes of it are found in some form in all living organisms and used for energy capture and storage, to regulate developmental processes, to mediate orientation and in higher order species it serves for visual

signaling [37]. Generally retinal is the chromophore inside many types of opsins, that is responsible for photon absorption and for the initialization of conformational switch in the protein causing a cellular event. As the chromophore, the all-*trans* retinal is universally available in all kinds of tissues only the opsin's gene is required to be delivered into the target cell or area used as a single-component actuator system [38]. Different protein interactions cause the spectral tuning of the chromophore absorption which results in that different type of opsins can be activated by different wavelengths of light [39].

Three major classes of microbial ion conducting opsins are used as activity modulators in the central nervous system such as the halorhodopsins that pumps anions into the cell, bacteriorhodopsins that translocates cations outside of the cell, these serve as inhibitory or hyperpolarizing components and channelrhodopsins that are non-selective cation channels, causing cation influx resulted in neuronal excitation [38]. The most widely used actuator for neuronal excitation is the

naturally occurring channelrhodopsin 2 (ChR2) that functions as a non-selective cation channel upon blue light absorption. This has a limitation when large-animal research or human therapy is the goal as blue light is not capable to penetrate deeply in the brain tissue (Fig. 7). Therefore, the cells expressing this opsin must be located in the apical layers of the cortex if the light comes externally or the activating light must be presented invasively through optical fibers if deeper cortical layers or brain areas are investigated. Red-shifted opsins have the properties to overcome this issue to enable non-invasive deep brain stimulation and large-animal or human cortical stimulation in any layer of it. As the human cortex is 1-4,5 mm [40] thick the red-shifted opsins seems to be very promising in terms of therapy for neurodevelopmental or neurodegenerative diseases.

ChRmine, the most efficient red-shifted opsin was discovered via *in silico* opsin screening and its sequence was optimized for mammalian expression [41]. It is homologous in its function to cation-conducting channelrhodopsins but, by primary sequence, more closely resemble to ion pump bacteriorhodopsins with its short trimeric assembly [39]. ChRmine yields high photocurrents upon activation, it has high light-sensitivity, and spectral compatibility with fluorescent activity reporters such as with the GCaMP family as the peak activation wavelength

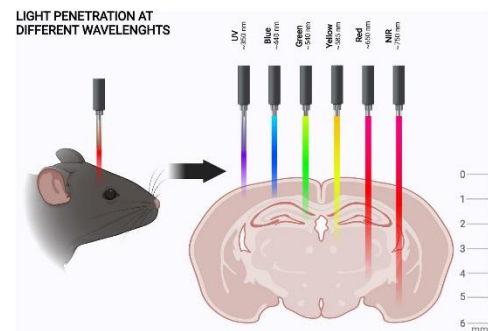


Figure 4: Representative image of how deep the different lights can penetrate [37]. Red light enables deeper brain area stimulation.

is 585 nm [41] enabling all-optical access to neurons. Additionally, it has very fast kinetics for example desensitization in darkness was found to be an order of magnitude faster than for other red-shifted opsins additionally ChRmine reliably induced spiking even with short red-shifted light pulses and at low irradiance values (100% spike success rate at 1 ms, 0.08 mW/mm² on cultured neurons) [41]. These properties of this opsin make it a promising therapeutic tool by which neuronal excitation can be performed with high temporal precision and with lower required irradiance. The only drawback of ChRmine in research is that the spiking probability when 488 nm light is applied is. This limits the usable excitation power in the case of one-photon imaging, control experiments are needed to test whether red light or blue light induced activation was recorded. There are further developments in this regard as more red-shifted opsins are developed called rsChRmine or ChRmine 2. [39].

ChRmine delivery as a transgenic opsin into higher order organisms realized as its gene is engineered into the rAAV's DNA with a codon optimized format as a monocistronic or bicistronic manner with different genetically encoded calcium indicators. The incorporation of the soma localization signal gene (Kv2.1) downstream of the opsin holds immense potential for optimizing membrane trafficking efficiency and facilitating highly specific targeting. Through this strategic localization of ChRmine to the soma, the stimulation can be exclusively directed towards the targeted cell, without inadvertently activating neighbouring axons or dendrites. In conclusion ChRmine is a very efficient platform to activate deeper brain areas with high specificity.

3. Plans

Reaching long-term, stable functional and non-invasive labeling of the whole brain with recombinant adeno-associated viral vectors requires a vast amount of testing beforehand we apply the viral vectors in cats. Not only the expenses of animal keeping are very high especially after surgeries but the necessary amount of virus injected through intravenous or intrathecal delivery routes cost a lot as well. Therefore, we aimed to test a custom conditional promoter system first in rats as the constructs without this system showed inefficient expression in both species via less invasive delivery routes. As this conditional system consists of two components we pop up the question that what is the most optimal ratio for these components. To approximate the optimal ratio, the different variations are injected intracortically and evaluated based on the labeled cell density and fluorescence intensity. In light of the findings the most promising ratio is used in cats through non- or minimally invasive routes.

Functionality of rAAV based expression is assessed from two approaches, from the read-out and from the modularity point of view. By that not only the successful protein expression can be evaluated but the functionality of the produced proteins as well. GCaMP indicated activity of the neurons is followed by widefield fluorescent imaging. Modulation of the activity is realized with the light sensitive opsin, the ChRmine that is activated with red light via optical fiber from the brain surface.

In this reasearch I aim to describe the differences between rAAV labeling with and without the custom conditional promoter system through distinct delivery routes in the two model animals, in rats and cats. I also aim to thoroughly assess the delivered viral vector's long-term safety and efficacy. My goal is also to test the functionality of the produced proteins with *in vivo* fluorescent imaging and optogenetic stimulation on rats and cats. In addition, I pursue to successfully validate a new optogenetics based activity modulation method. Specifically, my aim is to evoke targeted brain activity from the brain surface realizing a minimally invasive approach. The possible results looking ahead to therapeutic application possibilities regarding viral delivery and neural activity modulation.

4. Methods

4.1 Animals

Procedures involving animals have met the regulations of the local institutional ethical committees (Ethical Committee of Research Centre for Natural Sciences). All the experiments were designed by following the 3R principles (Replacement, Reduction and Refinement). Female adult (~270g) Hannover Wistar (n=3) and Long Evans (n=2) rats participated in the 2-week-long experiment for testing the optimal viral construct ratio of the custom conditional system. Additional Hannover Wistar (n=1) and Long Evans (n=2) animal were used for chronic *in vivo* fluorescent imaging and optogenetics experiments. Intravenous injection was given to young Hannover Wistar (n=3) and Long Evans (n=4) rats, the virus was injected 30 days after their born when their weight was approximately 100 g. These animal were participated in chronic *in vivo* experiments as well. The Hannover Wistar animals were purchased from Toxicoop Kft., the Long Evans rats were breed at the laboratory's own animal house facility. In total 15 animals were used for the viral tests in rats.

In the following experiments 8 cats were used that were breed at the laboratory's own animal house facility that took place in a well-isolated manner from rats. For the intracortical injections 2, for the intrathecal injection 4 and for the intravenous injections 1 cat was used. Not only

because of the 3R principle but also considering the high costs of animal care, one cat participated in several other experiments unrelated to this research.

4.2 Surgery procedures

In this section, I will provide detailed descriptions of the rat surgery protocols, as I personally performed these procedures. Cat surgeries, intrathecal and IV injections were done under the supervision of our professional vet. Briefly, the way intracortical injections were performed and how the chamber was implanted in cats have a very similar fundamental methodology to the following processes except the anaesthesia protocol, the complexity of the interventions and that individual cats have much more advanced implant systems for different imaging techniques.

The different types of surgeries on rats were done under isoflurane or fentanyl-medetomidine-midazolam (FMM) anaesthesia. After the induction of the anaesthesia with isoflurane the animal was placed to the stereotaxic frame fixed with the ear bars. In the case of the inhaled isoflurane anaesthesia an inhalation mask was applied, the isoflurane flow was kept at 2 or 2,5% mixed with generated oxygen at a flow rate of 1,2 mL/min. Preoperative medications were given to the animal, medetomidine subcutaneously in a dose of 0,1 mL/100 g and lidocaine was applied for local analgesia. In the form of liquid lidocaine was injected subcutaneously to the operated area and in the form of cream it was put into the ears for less painful stereotaxic head fixation. 10 minutes after the drug administration the animal head was fixed with the help of the ear bars. The breathing rate and the temperature was regularly monitored by the surgeon. During the surgery the animal's body heat was kept within a normal range with the help of a heating pad. Postop the animals got paracetamol based painkiller and clavulanate/amoxicillin antibiotics for 10 days portioned and solved into the animal's daily water after each intervention.

4.2.1 Intracortical injections

The skin was removed from the skull after the shaving of the animal's head. Injections were given locally into the left hemisphere. 3 holes were drilled each with a 0,5mm diameter, the injection sites were equally spaced apart for expression check but for chronic *in vivo* experiments the 3 holes were located only in the visual cortex within a circle of 1,5mm diameter. All the 3 injections in a given animal contained the same viral composition. Pulled glass micropipette was filled with mineral oil and with NANOLITER2010 Microprocessor controlled injector the virus was sucked into the capillary and then 400 nL virus per hole was

injected with 2 nL/s speed into ~500 μ m depth. When the virus cleared out of the capillary the micropipette was left in the injection depth for 2,5 mins then at half depth for another 2,5 mins to avoid the leaking of the virus. After the injections the skin was sewn and the animal was placed back into a new cage after the animal was totally awake.

4.2.2 Intravenous injection

For this procedure only an inhalation mask was applied to the animal, no stereotaxic holder was needed. AAV viruses were delivered through the tail vein of the animals. Before inserting the small cannula into the vein, the tail was placed into warm water to apply its vasodilator function as well as the tail was sprayed with alcohol that also helped a lot to visualize the main tail vein. After the successful insertion of the cannula the virus was injected then washed in with ringer lactate. After the injections the animal was placed back to its cage.

4.2.3 Craniotomy

Longitudinal *in vivo* brain studies require a stable and life-compatible cranial window. To meet with the criteria a 3D-printed chronic implant was fixed to the skull with bone cement (PALACOS, Heraeus Medical). It was used as a skin retainer to avoid skin regrowth and decrease the chance of any wound formation or infection development around the craniotomy. 2 weeks after the injections the rat was anaesthetized with the above-mentioned method. After the skin reopening the skull was cleaned with peroxide, dried with phosphate acid and grooved adhesion helping layers were applied (Universal Kit, Super Bond and Adper, 51202, 3M) and a 3 mm diameter craniotomy was drilled above the visual cortex where the virus had been injected. After the skull removal the dura was carefully eliminated. Bleeding was decreased by continuous washing with ice-cold physiological saline solution and by gelita application. 3 cover glasses (2 pieces of 3mm diameter and 1 piece of 4mm) were glued together with an optical glue (146, Norland) to form a cylindrical structure that enables fixing the glass with cement (RelyX Luting, 3505P, 3M) and superglue on the top of the skull while the brain is pushed softly to decrease brain motion-caused artefacts. No bone area was left without a supporting glue on its surface by the end of the intervention. The skin was glued with a biocompatible glue (Vetbond, 1469C, 3M) to the plastic to prevent the entry of any foreign materials. After the operation the animal was placed into a new cage.

4.3 Virus constructs

The viruses were stored at -80°C for long-term storage and after the thawing, the viruses were placed at a 4°C refrigerator. 10 recombinant AAV batches were used. Viral constructs

expressed either the hSyn neuron-specific promoter or a custom conditional construct encoding a genetically encoded indicator (GCaMP6m or GCaMP7s) but some of them also expressed the applied red-shifted opsin with a soma targeted motif (ChRmine-Kv.2.1) as well. I performed intracortical injections and intravenous injections with AAV.PHP.eB or AAV.B22 capsid. The custom conditional promoter system was either delivered with AAV.B22 or AAV.C1 capsid. Viruses were produced at IOB Basel within a cooperation framework or within our laboratory.

4.4 Widefield imaging

The GCaMP caused fluorescence change can be monitored by widefield microscope to capture the activity of hundreds of neurons across large fields of view. The microscopy setup was designed and built in our laboratory with Teledyne BSI Express camera for high quality image recording. For the GCaMP excitation a 470 nm Thorlabs LED source (0.035 mW/mm^2) was used. During the in vivo measurement the animal was anesthetized with minimal isoflurane. The rat was placed into the head holder system designed in our laboratory for stable imaging without pain. A head plate can be screwed to the implanted chamber then this head plate is fix to the built Thorlabs holder system by that minimizing the pain of the animal. We placed shadings around the space between the objective and the animal's head to exclude the light coming from the visual stimulation. Then the first step in the imaging workflow is to habituate the animal to the dark environment to increase the evoked response. Then to record 30s of spontaneous activity before run the stimulation and measure the fluorescence change in the brain. The used full screen white flash stimulation was presented to the animal by a Dell monitor that was placed 17 cm from the animal's eye, situated to cover most of its visual field. In one 5 s-long stimulation cycle 5 flashes were shown to the animal with a temporal frequency of 2 Hz in 2.5 s, the rest of the recording measures the basal fluorescence for baseline correction. Generally, we applied 10 repetitions to increase the signal to noise ratio. Upon somatosensory stimulation we applied 50 ms airpuff with 2 Hz with 5 s period. Analysis for both recordings were done in the software developed in our laboratory.

4.5 Optogenetic stimulation

Minimally invasive light stimulation using the red-shifted ChRmine opsin was achieved through the utilization of a fixed and externally positioned mirror 45° angled, 200 μm or 400 μm diameter, 20 mm long fiber (Doric Lenses, Silica/Silica, 0.48, 20 mm, M45 end). Precise laser manipulation was carried out by connecting the fiber to the Neurophotometrics FP3002 via a patch cord. The laser (635 nm) stimulation parameters were systematically adjusted, and the most successful settings were observed with a stimulation cycle of 5 seconds, a 0.2 second

laser stimulation duration at 30 Hz frequency, using a 25 ms pulse width, and delivering a total of 5 laser pulses with 60% laser intensity (321 or 119 mW/mm²). The optogenetically evoked activity was monitored by employing the GCaMP activity followed by wide-field imaging setup.

4.6 Immunohistochemistry

The rats used for the optimization of the custom conditional promoter were perfused 2 weeks after the injections, the other rodents and cats lived longer. The animals were perfused transcranially, first with physiological salt solution then with 4% paraformaldehyde (PFA). The whole rat brain or in the case of the cats the sectioned 3 mm thick block were post fixed overnight at 4 °C in PFA. Then 60 µm thick sections were cut by Vibratome (Leica, VT1002S) for further anatomical analysis. After the sectioning 3 times washing with 0,1 M phosphate buffer (PB) was done. If the sections were not used immediately they were placed into 30 % sucrose solution for overnight to process them to long-term -80°C storage.

We applied immunohistochemical staining on the selected sections with diaminobenzidine (DAB). First, the samples were immersed in 0.1M PB three times, each time for 10 minutes. Next, a 1% peroxide solution was applied for 10 minutes. The samples underwent another three rinses in 0.1 M PB for 10 minutes each to ensure proper preparation. For blocking, the samples were incubated for 45 minutes in a blocking solution composed of 5% Normal Goat Serum (Vector Laboratories, S-1000-20), 4% Bovine Serum Albumin (Sigma-Aldrich, 232-936-2), and 0.05% Triton. This step aimed to reduce non-specific binding and enhance specific antibody interactions. Following the blocking step, the samples were incubated at 4°C for two days with a rat anti-GFP (Weber Consulting, 04404-84, 1:2000), rabbit anti-GFP (Sigma-Aldrich, SAB4301138, 1:5000) or mouse anti-NeuN (EMD Millipore, MAB377, 1:2000) primary antibody. After primary antibody incubation, the samples underwent another three rinses in 0.1M PB for 10 minutes each. Subsequently, goat anti-rat (Vector Laboratories, BA-4000, 1:250), goat anti-rabbit (Vector Laboratories, BA-1000, 1:250) or goat anti-mouse (Vector Laboratories, BA9200, 1:250) biotinylated secondary antibody was applied and allowed to incubate for 2 hours. This secondary antibody binds to the primary antibody, facilitating further detection and visualization. The samples underwent three additional rinses in 0.1 M PB for 10 minutes each. To amplify the signal and enable visualization, an Avidin Biotin Complex (ABC, Vector Laboratories, PK-4000, 1:250) containing hydrogen peroxidase activity was applied and allowed to incubate for 1.5 hours. For staining, the samples were treated with DAB-Ni solution for 20 minutes. This solution consisted of 8 ml 0.1 M PB, 2.5 mg

DAB, 3.33 mg NH₄Cl, and 0.4 ml 0.05 M Ni(NH₄)SO₄. Lastly, to complete the protocol, a 30 µl solution of 0.03% peroxide was applied for at least 5 minutes. This step aimed to ensure optimal staining and signal development. The samples underwent a final three rinses in 0.1 M PB for 10 minutes each to remove any residual reagents or staining components.

The native sections were mounted with Vectashield with DAPI (Vector Laboratories, H-1200-10) to the coverglass, the stained samples were fixed on slides with chrome gelatine and then covered with DePex. The images were obtained with confocal microscope (Zeiss LSM710), epifluorescent microscope or bright field microscope depending on the sample's nature.

4.7 Quantification of cell properties

Mean fluorescence of a given brain region or cell was measured with ImageJ. Cell quantification was done manually with the Cell Counter plugin in ImageJ on the immunohistochemically stained sections. By clicking on the labeled cells the program automatically labels and counts them. The cell counting was performed in an area with a given size to be able to compare the samples. Labeled cell percentages was calculated by the ratio of labeled cell in a layer/all cell in a layer. The latter information was counted on NeuN stained samples (data not shown).

When the two components' ratio was tested in each case one injection site was selected which was not overlapped with the neighbouring injection. 3 sections were used, one at the injection site and the 4th and 8th from it to be able to detect the spherical spreading of one injection and quantify the labeled cells as a result cell number/mm² (apart from the 60 µm thickness) can be calculated.

5. Results

5.1 Comparison of the expression pattern and efficiency of viral labeling via different delivery routes in cats

In this research, I examined the impact of three different routes for delivering viral vectors in cats. The objective was to analyse and describe how each delivery route affected the spatial distribution pattern of labeled cells, their contribution to whole-brain expression, their suitability for functional measurements in large animals, and a comparison of their invasiveness. We administered recombinant adeno-associated viral vectors through local injections into the visual cortex, intrathecally, and intravenously into the systemic circulation. Intracortical injections are commonly used to target expression in a desired brain region for *in vivo* functional measurements or tracing studies, regardless of whether the target area is cortical

or deeper within the brain. Although this method is highly invasive as it requires penetration into the brain, it offers advantages in terms of required viral particle quantity and the selected recombinant adeno-associated viral vector. The injected volume of a single injection ranged from 100 nL to 1.2 μ L depending on the specific experiment. In addition to AAV9 and its synthetic derivatives, AAV1, AAV2, AAV5, and AAV8 are also capable of yielding high neuronal expression in the central nervous system delivered this way [42]. In our experiment, we delivered 300 nL of the virus intracortically at thirteen locations in a cat. All viruses were AAV-PHP.eB, AAV-CAP-B10, or AAV-CAP-B22 capsids, and the expression of GCaMP6m was regulated by a neuron-specific hSyn promoter. The results (Fig. 6/a) indicate that a small area within the visual cortex was infected with a single injection, encompassing only 12% of the Area17 and Area18 in cats (calculated based on the Catlas [43]) on a 60 μ m thick coronal section. In comparison, applying 260 nL from the same virus in the rat somatosensory cortex results in 80% labeling of the cells in the targeted area (data not presented) showing the applicability differences between species of local injection. Considering that the medial-lateral and anterior-posterior number of labeled cells decrease further from the injection site a larger volume, more viral genome, or multiple injections may be required to cover for example the entire visual cortex in large animals. We also examined the effect of the injections on the brain's immune defense by staining the samples with the IBA1 marker, which results in positive staining of activated microglial cells. The results (Fig. 6/b) suggest that cortical injections induce a repair mechanism that could lead to less effective labeling or, in the worst case, a decrease in the number of cells. Based on our findings, it can be concluded that achieving viral expression over a larger area or throughout the entire brain would require a significant amount of virus, in addition to resulting in unequal distribution of expression around the injection sites. In the context of whole-brain functional intracortical injections, alternative approaches are required as local injections are not considered optimal.

Intrathecal injections offer a less invasive approach compared to intracortical delivery; however, their administration still demands the expertise of skilled professional. Intrathecal delivery requires a significantly higher amount of injected virus, as it aims to target the entire brain with one injection. One advantage of intrathecally delivered AAVs is that the viral particles come into direct contact with the brain after the injection, bypassing the need for transport through the blood-brain barrier. In an adult cat we injected the virus (AAV-CAP.B22-hSyn-GCaMP7s-ChRmine) aiming to achieve highly neuronal biased expression. The results obtained (Fig. 6/d) reveal certain limitations in the distribution of the virus achieved through

this delivery method. The labeled cells were predominantly observed on the top of the cortical gyri, while very few or no labeled cells were visible within the sulci. Additionally, the subcortical regions exhibited poor labeling, with only a few cells observed in the *Superior Colliculus* (Fig. 6/e). Notably, no labeled cells were found in the *Periaqueductal Grey Area* and *Lateral Geniculate Nucleus* (Fig. 6/e). Furthermore, when examining the distribution of labeled cells across cortical layers, it was predominantly layer 3 neurons (maximum 9.4 %) that were affected across regions, with lower percentages of labeled cells found in layers 2 (maximum 4.9 %) and 5 (maximum 1.1 %) (Fig. 6/c). Moreover, an uneven distribution was observed in the number of labeled cells per layer when different gyral areas were compared, indicating that the labeling is highly dependent on the specific brain region and cortical layer. It is likely that neurons in direct contact with the cerebrospinal fluid (CSF) are labeled. This delivery route represents a significant advancement towards achieving minimally invasive whole-brain labeling. However, it is important to acknowledge and consider its inherent limitations.

The intravenous injection is considered the least invasive method for viral delivery. Its efficiency and expression level primarily depend on the chosen capsid, especially on its ability to cross the blood-brain barrier. Synthetic derivatives of AAV9 are the most prominent capsids for this purpose, as they can efficiently overcome neutralizing effects developed against wild-type AAV capsids while crossing the barrier; however, intravenous injections require a higher amount of viral vectors. One advantageous approach to overcome this issue is to inject young animals, as the required viral amount is determined based on viral genome to body weight ratio. In our experiment, we injected two 4-day-old cats with the AAV-PHP.eB-hSyn-GCaMP6m-ChRmine viral construct. We observed sparse labeling in one animal (Fig. 6/f), but no labeling at all in the other. This difference could be attributed to individual physiological variations in neutralizing effects or the influence on the blood-brain barrier and how the capsid interacts with it but also problems in technical implementations can be the cause. Based on these results, we cannot definitively support or reject the idea that intravenous injection leads to a homogeneous labeled cell distribution in large animal models. The sparse labeling could be due to the use of a less effective capsid (AAV-PHP.eB) than the reportedly most effective ones (AAV-CAP.B10, AAV-CAP.B22) or the lower dosage that others reported to be effective.

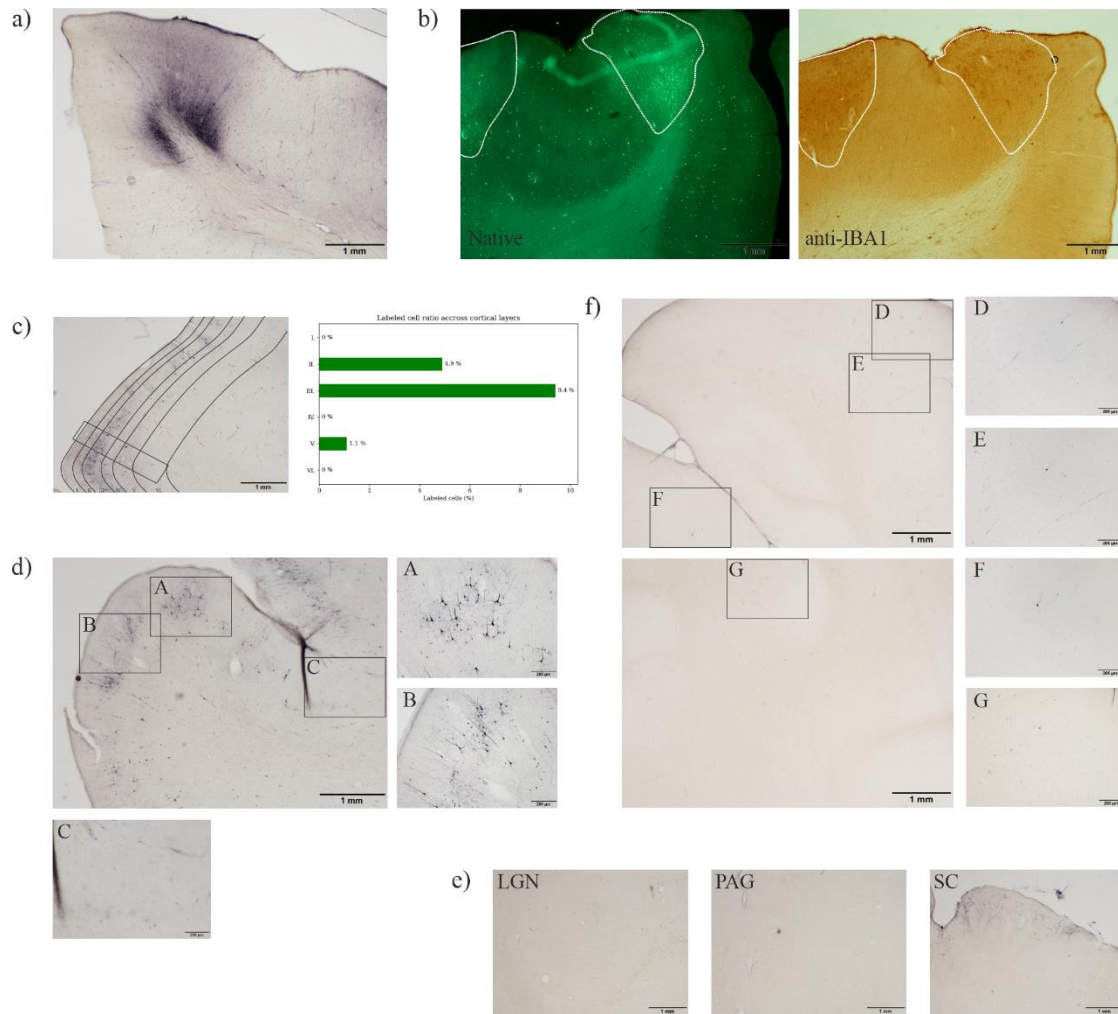


Figure 5: Comparison of the expression patterns resulting from three distinct viral administration routes. a) Intracortical injection. b) Intracortical injection induced microglia activation. Injection sites are indicated with their outline. c) Calculating labeled cell number density in each layer and determine their ratio to the number of all cells in the given layer. On the left image, cortical layers and the densest area is shown. d) Labeled cells via intrathecal injection. e) LGN-Lateral Geniculate Nucleus, PAG – Periaqueductal Grey Area, SC-Superior Colliculus f) Sparse labeling after intravenous injection.

5.2 Analysis of factors influencing minimally and non-invasive delivery routes

We conducted an experiment to investigate the sensitivity of the intrathecal delivery route to different injected viral particles. Typically, when using intravenous injection, there is a lower dose limit that is commonly used in literature due to the non-linear nature of AAV. Our objective was to determine a similar limit for intrathecal delivery. To test this, we administered two different amounts of viral genome (vg) from the same viral construct: a lower amount and a higher amount of vg. The difference between the quantities was one logarithmic unit, equivalent to a tenfold difference. Our findings revealed that the lower number of particles only labeled a minimal number of cells. Specifically, in a 60 μm thick coronal section of a cat brain measuring 2.5 mm*3.5 mm, we detected only two cells in layer 5 on distinct samples across

multiple sections from various brain regions when using the lower viral genome number (Fig. 7/a). In contrast, when using the higher amount, we observed a total of maximum 48 labeled cells across different layers in a 350 μm wide cortical area (Fig. 7/b). Based on this comparison, we concluded that optimal expression or improved results require the higher amount of virus particles to be injected into the cerebrospinal fluid (CSF) of an adult cat from the applied construct.

We performed intravenous delivery experiments in rats to assess the efficiency of viral labeling. With the use of AAV-PHP.eB capsid, we achieved a maximum density of 31 cells/ mm^2 in a rat (Fig. 7/c). Although the labeling remained sparse regarding the whole section, we observed the presence of labeled cells in both the cortical and subcortical regions. However, when we administered the same dose and construct, except for the capsid which was AAV-CAP.B22, to a Long Evans rat, the viral labeling was lower, with only 3 cells observed rat section that is 1.4 $\text{cm} \times 1 \text{ cm}$ (Fig. 7/d). We expected AAV-CAP.B22, being a derivative of AAV-PHP.eB with enhanced properties, to yield more effective labeling. The strain specificity of AAV-PHP.eB has been proven by other laboratories so its derivatives, like AAV-CAP.B22, could potentially account for such differences. Additionally, variations in physiological factors between animals cannot be disregarded. Moreover, we encountered considerable technical challenges in implementing tail vein injections in young rodents, that could also count for such differences. We noted that achieving more than sparse labeling with intravenous injection requires higher dosages with the applied construct.

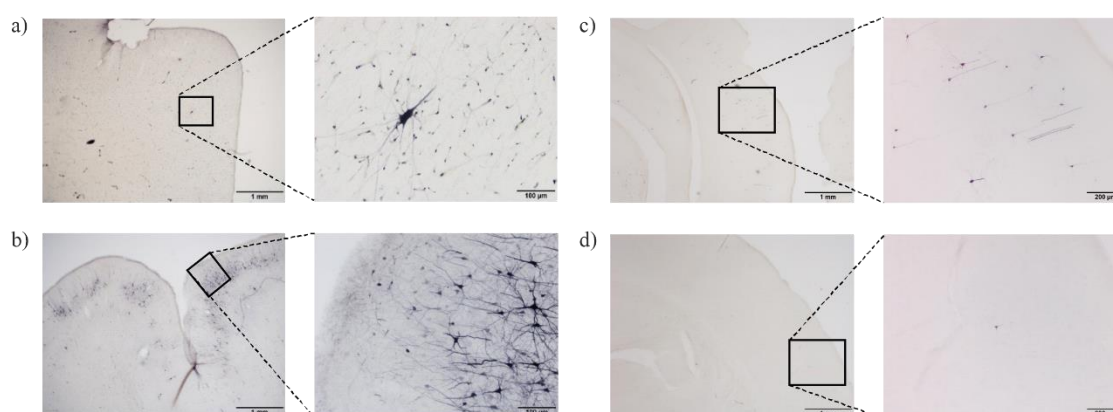


Figure 6: Testing how viral genome number and species strain affecting the expression. a) Low viral genome number intrathecal. b) High viral genome intrathecal. c) Wistar rat IV injected with AAV-PHP.eB. d) Long Evans rat IV injected with AAV-CAP.B22.

5.3 Optimizing gene expression with the custom conditional promoter system

Achieving high levels of transgene expression is a crucial criterion for effective *in vivo* brain function monitoring using fluorescence-based imaging techniques and for modulating neuron activity. Although we have already achieved considerable success with intrathecal injection, reaching up to 9.4% labeling in layer 3 cells, we encountered limitations when detecting infected cells solely through native fluorescent sections. Only faint or barely visible cells were observed (Fig. 8/a) while we were able to detect the presence of infected cells using immunohistochemical DAB staining. To address this limitation, we recognized the need to implement a viral-based custom conditional promoter system to enhance viral expression.

As the conditional system comprises two constructs, the question arises regarding the optimal ratio required to achieve maximal transgene expression while ensuring long-term stability. In our rat experiment, we aimed to determine the optimal ratio of the two components used in viral injections. Four rats were involved in the study, and each rat received injections at three locations on the left hemisphere, each rat were injected with a given ratio of the viral components. To ensure comparability, the total injected volume and the applied volume from each component were kept constant, while the ratio of the components depended solely on their titer. We evaluated the labeled cell density and fluorescence intensity within an area of a given size to assess the efficiency of transduction and the resulting fluorescence at the injection sites. Our findings indicated that, with the viral construct used in our study, the optimal ratio of the components was 1 (Fig. 8/b). Additionally, the results revealed a lower limit for the injected component A that is responsible for transgene delivery, which was within our expectations as by decreasing it the labeled cell density reduced (Fig. 8/b). Furthermore, increasing the component B beyond a certain threshold did not yield optimal results in terms of cell density or fluorescence intensity. While there is no existing literature on this specific topic, we speculate that excessive expression of component B may over activate the other component, potentially leading to harmful protein overexpression within the cells. From the obtained data, we are unable to determine whether this resulted in cell death or if the cells silenced the delivered viral DNA but lower fluorescence was measured when component B was elevated and also lower cell density was calculated that suggest the worse scenario (Fig. 8/b). Based on our experimental observations, we propose the following: component A determines the number of labeled cells, while component B influences the fluorescence intensity of the labeled cells. With the applied viral construct, the optimal ratio appears to be 1:1.

We conducted intrathecal injections of the custom conditional construct in an adult cat, utilizing the AAV-CAP.B22 capsid that was well-performed in earlier experiments via intrathecal delivery and we applied the cell type-specific custom construct to regulate component B expression, which labels projection neurons in the central nervous system. From the transgene coding component the lower amount of viral genome was delivered, while the component B was delivered at a same amount keeping their ratio 1. This virus injection resulted in highly intense native fluorescence (Fig. 8/c). Comparing it to the hSyn regulated normal construct with 10-fold higher viral genome injection than this injection, the fluorescence of the labeled cell soma was 3,9 times higher, 30 AU for the normal construct and 117 AU for the conditional construct (Fig. 8/d). Remarkably, individual axons were also clearly visible in their native state without requiring additional signal amplification (Fig. 8/e). In terms of labeled cell distribution, we observed the following percentages among the most densely labeled cells layer by layer: 27% in layer 1, 9.3% in layer 2, 9.4% in layer 3, and 3.7% in layer 5 (Fig. 8/g, h). Overall, this resulted in 71 labeled cells compared to the 48 cells from the hSyn normal construct. This outcome was unexpected considering that we anticipated a lower cell count due to the more specific promoter. In comparison to the basic construct, the custom conditional system proved to be significantly more effective in cell transduction and in resulted fluorescence as well. However, limitations of the delivery route were also evident, as the expression was still localized to gyral regions but with fewer density differences compared to the earlier experiments and here also only the superficial layers of the subcortex was expressing the transgene (Fig. 8/f). Surprisingly, we detected two labeled cells in the lateral geniculate nucleus, which is not in direct connection with the cerebrospinal fluid. One study suggests that some cells may be labeled through retrograde transport from intrathecal delivery [44], which could be the case here.

Although it is a significant success, concerns regarding long-term safety arise. Upon examining the stained samples, we observed a substantial background that is unusual (Fig. 8/i). We propose that the current delivery route, in conjunction with the custom system at the given viral genome quantity, led to the overexpression of GCaMP, which may negatively impact cellular machinery and calcium signalling within the cells. Confocal images revealed that the nuclei of many cells were completely filled with GCaMP, indicating its overexpression (Fig. 8/i). To address this issue, we propose using a more sophisticated version of GCaMP that incorporates additional defensive domains, or alternatively, reducing the overall delivered viral genome quantity.

Additionally, we cannot exclude the possibility that the optimal ratio of the two components may be dependent on the delivery route.

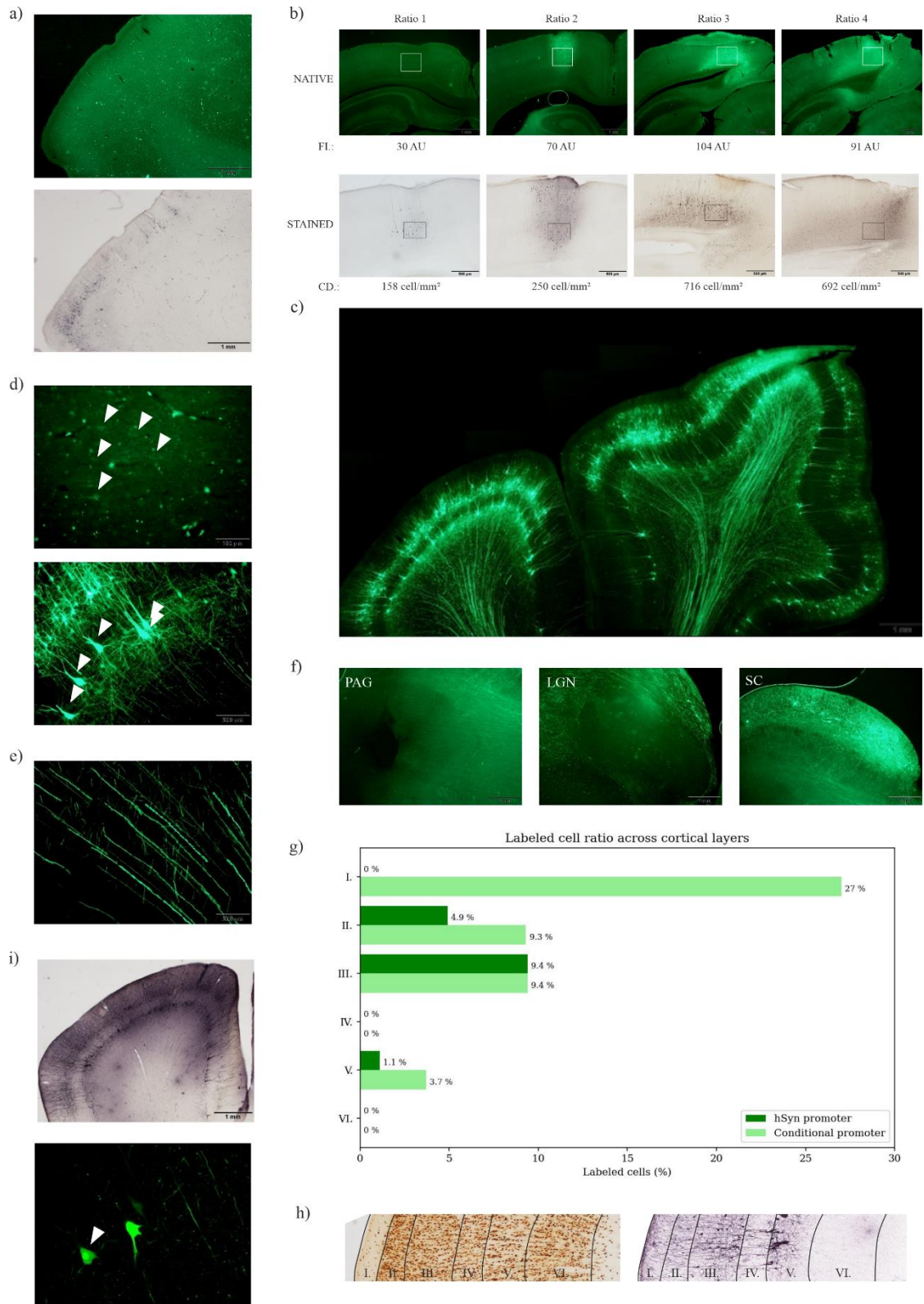


Figure 7: Application of the custom conditional promoter system in cats. a) The labeled cells are not visible on the native sections when no conditional promoter was used. b) Testing different ratios. Ratio 1, 2 and 3 contained the same amount from the component B and an increasing number of the component A, Ratio 4 contained the same component A than Ratio 3 but elevated amount from component B. FL: fluorescence intensity in arbitrary units (AU). CD: cell density in cell/mm². c) Bright fluorescence on the native section. d) Upper image: no custom conditional promoter in the viral construct, Lower image: custom viral construct. e) Individual axons in the native sample. f) Subcortical areas. g) Comparison of labeled cell density across cortical layers. h) anti-NeuN DAB staining on the left, anti-GFP DAB-Ni staining on the right, adjacent sections were used. i) GCaMP overexpression on DAB-Ni stained and native images.

5.4 Testing transgene expression functionality on the read-out and on the modularity level on rats

The effectiveness and success of viral delivery can be determined by assessing the expression pattern and stability. However, true success lies in our ability to express functional proteins in the targeted cells or area. We approached this assessment from two aspects: the readout and the modularity. To evaluate functionality, we employed the genetically encoded calcium indicator GCaMP for *in vivo* fluorescence-based calcium imaging, enabling us to monitor neural activity. Additionally, we aimed to modulate neural activity using ChRmine, a red-shifted opsin, and tested its functionality through red light stimulation from the brain surface, with GCaMP activity serving as an indicator of the induced activity. Our experimental subjects were rats injected intracortically with the viral constructs. We aimed to evaluate functional protein expression in both normal and conditional viral constructs.

During head-fixed, anesthetized measurements, two rats were subjected to external stimulation. A Long Evans animal, known for good vision among rats, was used for the normal construct. Consequently, the local injections and craniotomy were performed in the visual area. To evoke GCaMP activity in the visual cortex, we applied full-screen flash visual stimulation, taking advantage of the rats' sensitivity to contrast changes. For the conditional construct, we used a Wistar rat with relatively poorer vision. Thus, we chose to perform the local injections and craniotomy above the somatosensory area. Somatosensory-related brain activity was elicited by OpenSpritzer air puff stimulation. In both cases, we successfully recorded GCaMP activity in response to external stimulation (Fig. 9/a), validating the functionality of the expressed protein.

Next, we aimed to induce activity using the red-shifted opsin, ChRmine at the target area where we previously observed GCaMP activation. To ensure precise activation of specific cells and avoid passing axon activation, we applied soma localized ChRmine. As we see that the GCaMP is functional we supposed that the opsin is functional as well at the same area. Our objective was to determine the optimal stimulation parameters and find the most effective laser intensity. Throughout the experiments we used the 45° mirrored fiber as it is the most compatible with the widefield setup due to the imaging setup short, 1-inch working distance (Fig. 9/b). Precisely placing the fiber above the targeted area for a point-like stimulation presented a challenge, our holder system still requires a development. Different stimulation lengths and laser intensities were tested, and successful opsin-evoked activity was observed under specific parameters in

the normal construct injected animal (Fig. 9/c). However, the same protocol did not yield satisfactory results in the rat with the conditional system. This may be attributed to unequally distributed labeling within the field of view due to local injections, resulting in a lack or a very low amount of opsin-expressing cells in the intended activation area. Also of opsin-expressing cells are located at different depths those may require other laser intensities or pulse widths for proper stimulation. To address this, we plan to continue the experiments using intravenously injected rats if optimal expression can be achieved with the new construct. Alternatively, we will focus on refining the stimulation protocol for intrathecal or IV injected cats, where issues related to expression localization are less likely to affect the experiment.

In addition, this configuration was first done by our laboratory, namely the activation of ChRmine from the brain surface and the monitoring of activity with widefield GCaMP imaging that provides minimally invasive, all-optical access to the brain. It is a great success as it can be easily implemented in large animals as well due to the large field of view provided by the widefield setup developed by our laboratory.

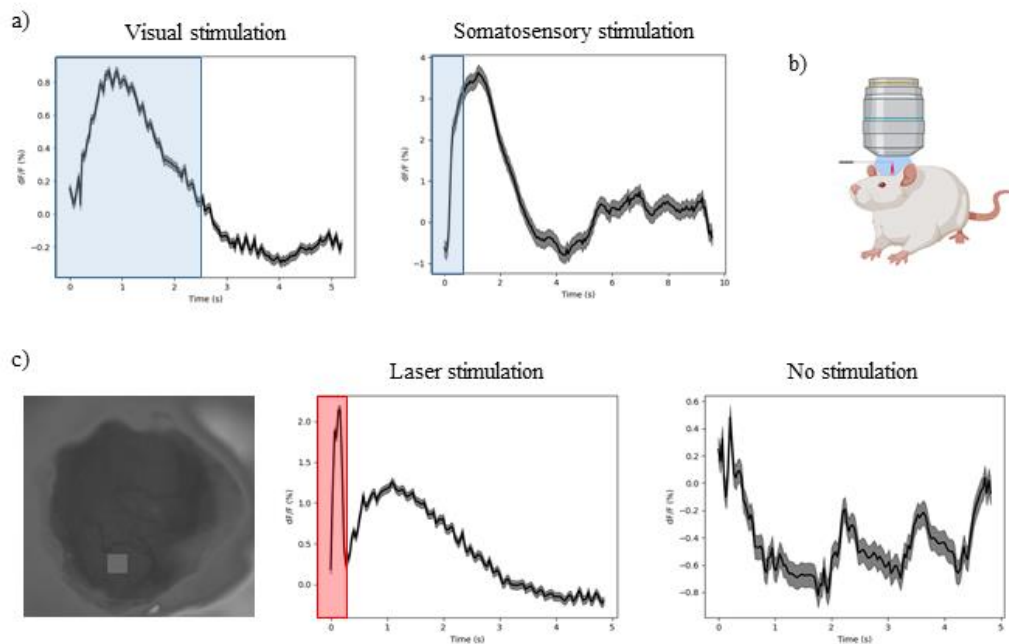


Figure 8: Widefield imaging in intracortically injected rat. Black curve shows the mean of the repetitions while grey area represents the standard error of mean. grey colored repetitions' mean. Colored box shows when the stimulation was on. a) External stimulation applied on two rats. Visual stimulation on the normal construct injected Long Evans, somatosensory stimulation on the custom conditional AAV-injected Wistar animal. b) Representation of the all optical access with the mirror fiber and widefield imaging setup. c) Region of interest shown with grey rectangle. Optogenetically evoked response from the brain surface is visible on the left side, control experiment on the right. Laser stimulation: one cycle is 5 seconds, a 0.2 second laser stimulation at 30 Hz frequency, using a 25 ms pulse width.

6. Discussion

We aimed to develop a long-term, stable, and functional labeling of the whole brain using adeno-associated viral vectors. The exceptional properties of this viral vector make it a promising tool not only in research but also in therapy. Its engineered capsid and DNA allow for precise targeting, including crossing the blood-brain barrier and specific neuron tropism. However, we have observed probably a strain-specific effectiveness, such as AAV-CAP.B22 not performed well in Long Evans rats through intravenous delivery but being highly effective when applied through intrathecal injection in cats.

In this research, my goal was to compare expression patterns depending on the delivery route. Local injection provided precise control over the delivered viral amount into the brain, but it resulted in unequal and limited labeling in large animals, covering only a small area (12.5%) of the targeted region with a single injection. This limitation hinders whole brain labeling and homogenous viral distribution. Intrathecal injection showed the greatest success, especially when the custom conditional promoter system was used. However, it led to overexpression of the GCaMP protein. To address this issue, we propose exploring the use of a safer alternative, such as GCaMP-X, which has also great properties regarding its sensitivity and brightness upon calcium binding or we suggest that the applied two-component conditional system could have delivery route dependent optimal ratio that should be investigated. Additionally, reducing the viral load could be considered with the applied constructs via intrathecal delivery in cats. Despite these limitations, the conditional system allowed us to achieve increased cell numbers and native fluorescence with a 10-fold reduction in viral dose. The resulting bright fluorescence enabled the visualization of individual cells and their projections without the need for staining, marking a significant advancement. However, it is important to note that there are differences in expression levels between gyral and sulcal areas in the cortex. In the cortical regions, the expression was predominantly observed in layers 1, 2, 3 and 5. Layer 3 and layer 5 labeling tendencies were observed without a specific promoter as well, suggesting that gyral layers and the superficial layers of the subcortex are favourable for viral delivery through intrathecal injection.

Regarding intravenous delivery, we only achieved very sparse labeling with normal constructs therefore we could not make a statement regarding the expression distribution. Exploring higher doses and investigating age-related factors in viral expression patterns could be potential avenues for improvement. As we have the efficient conditional system at hand, we have already injected young animals with a higher dose intravenously in a 1:1 ratio of this construct. We are

eagerly awaiting the cats to reach an operable age for fluorescence imaging-based *in vivo* measurements.

Furthermore, we successfully demonstrated the parallel application of the red-shifted opsin, ChRmine, and the calcium sensor, GCaMP, for *in vivo* monitoring using widefield imaging. Brain surface stimulation offers a minimally invasive approach to modulate neuronal activity, without the need for electrodes or optical fibers implanted into the brain. Although we achieved this in locally injected rats, further stimulation refinement experiments would be better performed on intrathecally or intravenously injected rats or cats, considering the 3D distribution limitations of intracortical injection especially in large animals. Our future steps involve the electronic and engineering tasks of designing a high-density optical stimulator for patterned optogenetic stimulation in the visual cortex.

In summary, this research represents a significant advancement in achieving high native fluorescence throughout the brain of large animals, bringing us closer to achieving homogeneous whole brain labeling. Intrathecal injection is a minimally invasive delivery route that enables dense and bright labeling, despite its specific area and layer dependent distribution. The observed expression levels provide a great opportunity for applying fluorescence-based, *in vivo* neuronal activity measurements in combination with brain surface optogenetic stimulation in cats. The applied initial parameter set provides a starting point for further investigations. Additionally, we are currently conducting intravenous injections using the custom conditional promoter system in large animals, and based on our preliminary data, we hold high hopes that this approach will result in long-term, stable, and functional labeling of the whole brain in a more homogeneous manner. Overall, the findings open up new possibilities for studying neuronal activity *in vivo* and manipulating brain function using optical techniques. Further research and refinement of our methods will contribute to a deeper understanding of brain circuitry and potential therapeutic applications.

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8. References

- [1] T. Burdett and S. Nuseibeh, “Changing trends in the development of AAV-based gene therapies: a meta-analysis of past and present therapies,” *Gene Ther.*, vol. 30, no. 3, Art. no. 3, Apr. 2023, doi: 10.1038/s41434-022-00363-0.
- [2] J. S. Pezaris and E. N. Eskandar, “Getting signals into the brain: visual prosthetics through thalamic microstimulation,” *Neurosurg. Focus*, vol. 27, no. 1, p. E6, Jul. 2009, doi: 10.3171/2009.4.FOCUS0986.
- [3] “Addgene: Adeno-associated virus (AAV) Guide.” <https://www.addgene.org/guides/aav/> (accessed Apr. 01, 2023).
- [4] R. C. Challis *et al.*, “Adeno-Associated Virus Toolkit to Target Diverse Brain Cells,” *Annu. Rev. Neurosci.*, vol. 45, no. 1, pp. 447–469, 2022, doi: 10.1146/annurev-neuro-111020-100834.
- [5] S. Bijlani, K. M. Pang, V. Sivanandam, A. Singh, and S. Chatterjee, “The Role of Recombinant AAV in Precise Genome Editing,” *Front. Genome Ed.*, vol. 3, 2022,

Accessed: Apr. 01, 2023. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fgene.2021.799722>

- [6] A. C. Maurer and M. D. Weitzman, “Adeno-Associated Virus Genome Interactions Important for Vector Production and Transduction,” *Hum. Gene Ther.*, vol. 31, no. 9–10, pp. 499–511, May 2020, doi: 10.1089/hum.2020.069.
- [7] C. Li and R. J. Samulski, “Engineering adeno-associated virus vectors for gene therapy,” *Nat. Rev. Genet.*, vol. 21, no. 4, Art. no. 4, Apr. 2020, doi: 10.1038/s41576-019-0205-4.
- [8] K. Y. Chan *et al.*, “Engineered AAVs for efficient noninvasive gene delivery to the central and peripheral nervous systems,” *Nat. Neurosci.*, vol. 20, no. 8, pp. 1172–1179, 2017, doi: 10.1038/nn.4593.
- [9] “Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes | Nature Biotechnology.” <https://www.nature.com/articles/nbt.1515> (accessed Apr. 07, 2023).
- [10] S. S. Issa, A. A. Shaimardanova, V. V. Solovyeva, and A. A. Rizvanov, “Various AAV Serotypes and Their Applications in Gene Therapy: An Overview,” *Cells*, vol. 12, no. 5, p. 785, Mar. 2023, doi: 10.3390/cells12050785.
- [11] A. R. Batista *et al.*, “Ly6a Differential Expression in Blood–Brain Barrier Is Responsible for Strain Specific Central Nervous System Transduction Profile of AAV-PHP.B,” *Hum. Gene Ther.*, vol. 31, no. 1–2, pp. 90–102, Jan. 2020, doi: 10.1089/hum.2019.186.
- [12] D. Goertsen *et al.*, “AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset,” *Nat. Neurosci.*, vol. 25, no. 1, Art. no. 1, Jan. 2022, doi: 10.1038/s41593-021-00969-4.
- [13] S. Jang, H. K. Shen, X. Ding, T. F. Miles, and V. Gradinaru, “Structural basis of receptor usage by the engineered capsid AAV-PHP.eB,” *Mol. Ther. - Methods Clin. Dev.*, vol. 26, pp. 343–354, Sep. 2022, doi: 10.1016/j.omtm.2022.07.011.
- [14] M. R. Chuapoco *et al.*, “Intravenous functional gene transfer throughout the brain of non-human primates using AAV,” *Res. Sq.*, p. rs.3.rs-1370972, Jan. 2023, doi: 10.21203/rs.3.rs-1370972/v1.
- [15] S. R. Kumar *et al.*, “Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types,” *Nat. Methods*, vol. 17, no. 5, p. 541, May 2020, doi: 10.1038/s41592-020-0799-7.
- [16] S. N. Mathiesen, J. L. Lock, L. Schoderboeck, W. C. Abraham, and S. M. Hughes, “CNS Transduction Benefits of AAV-PHP.eB over AAV9 Are Dependent on Administration Route and Mouse Strain,” *Mol. Ther. Methods Clin. Dev.*, vol. 19, pp. 447–458, Oct. 2020, doi: 10.1016/j.omtm.2020.10.011.

- [17] R. Song, K. Pekrun, T. A. Khan, F. Zhang, S. P. Pasca, and M. A. Kay, “Selection of rAAV Vectors that Cross the Human Blood-Brain Barrier and Target the Central Nervous System Using a Transwell Model.” *bioRxiv*, p. 2022.07.06.498990, Jul. 06, 2022. doi: 10.1101/2022.07.06.498990.
- [18] X. Chen *et al.*, “Functional gene delivery to and across brain vasculature of systemic AAVs with endothelial-specific tropism in rodents and broad tropism in primates.” *bioRxiv*, p. 2023.01.12.523844, Jan. 13, 2023. doi: 10.1101/2023.01.12.523844.
- [19] A. C. Stanton *et al.*, “Systemic administration of novel engineered AAV capsids facilitates enhanced transgene expression in the macaque CNS,” *Med*, vol. 4, no. 1, pp. 31-50.e8, Jan. 2023, doi: 10.1016/j.medj.2022.11.002.
- [20] B. Zingg, B. Peng, J. Huang, H. W. Tao, and L. I. Zhang, “Synaptic Specificity and Application of Anterograde Trans-Synaptic AAV for Probing Neural Circuitry,” *J. Neurosci.*, Mar. 2020, doi: 10.1523/JNEUROSCI.2158-19.2020.
- [21] “Addgene: Tetracycline Inducible Expression.” <https://www.addgene.org/collections/tetracycline/> (accessed Dec. 10, 2022).
- [22] J. Y. Qin *et al.*, “Systematic Comparison of Constitutive Promoters and the Doxycycline-Inducible Promoter,” *PLOS ONE*, vol. 5, no. 5, p. e10611, May 2010, doi: 10.1371/journal.pone.0010611.
- [23] J. Oh, C. Lee, and B.-K. Kaang, “Imaging and analysis of genetically encoded calcium indicators linking neural circuits and behaviors,” *Korean J. Physiol. Pharmacol. Off. J. Korean Physiol. Soc. Korean Soc. Pharmacol.*, vol. 23, no. 4, pp. 237–249, Jul. 2019, doi: 10.4196/kjpp.2019.23.4.237.
- [24] J. Platisa and V. A. Pieribone, “Genetically encoded fluorescent voltage indicators: are we there yet?,” *Curr. Opin. Neurobiol.*, vol. 50, pp. 146–153, Jun. 2018, doi: 10.1016/j.conb.2018.02.006.
- [25] M. Gleichmann and M. P. Mattson, “Neuronal Calcium Homeostasis and Dysregulation,” *Antioxid. Redox Signal.*, vol. 14, no. 7, pp. 1261–1273, Apr. 2011, doi: 10.1089/ars.2010.3386.
- [26] C. Grienberger and A. Konnerth, “Imaging Calcium in Neurons,” *Neuron*, vol. 73, no. 5, pp. 862–885, Mar. 2012, doi: 10.1016/j.neuron.2012.02.011.
- [27] M. Mank and O. Griesbeck, “Genetically Encoded Calcium Indicators,” *Chem. Rev.*, vol. 108, no. 5, pp. 1550–1564, May 2008, doi: 10.1021/cr078213v.

- [28] J. Nakai, M. Ohkura, and K. Imoto, “A high signal-to-noise Ca²⁺ probe composed of a single green fluorescent protein,” *Nat. Biotechnol.*, vol. 19, no. 2, Art. no. 2, Feb. 2001, doi: 10.1038/84397.
- [29] Y. Zhang *et al.*, “Fast and sensitive GCaMP calcium indicators for imaging neural populations,” *Nature*, vol. 615, no. 7954, Art. no. 7954, Mar. 2023, doi: 10.1038/s41586-023-05828-9.
- [30] T.-W. Chen *et al.*, “Ultra-sensitive fluorescent proteins for imaging neuronal activity,” *Nature*, vol. 499, no. 7458, pp. 295–300, Jul. 2013, doi: 10.1038/nature12354.
- [31] H. Dana *et al.*, “High-performance calcium sensors for imaging activity in neuronal populations and microcompartments,” *Nat. Methods*, vol. 16, no. 7, pp. 649–657, Jul. 2019, doi: 10.1038/s41592-019-0435-6.
- [32] J. Geng *et al.*, “Chronic Ca²⁺ imaging of cortical neurons with long-term expression of GCaMP-X,” *eLife*, vol. 11, p. e76691, Oct. 2022, doi: 10.7554/eLife.76691.
- [33] Y. Yang *et al.*, “Improved calcium sensor GCaMP-X overcomes the calcium channel perturbations induced by the calmodulin in GCaMP,” *Nat. Commun.*, vol. 9, no. 1, Art. no. 1, Apr. 2018, doi: 10.1038/s41467-018-03719-6.
- [34] H. Dana *et al.*, “Sensitive red protein calcium indicators for imaging neural activity,” *eLife*, vol. 5, p. e12727, Mar. 2016, doi: 10.7554/eLife.12727.
- [35] K. Deisseroth, “Optogenetics,” *Nat. Methods*, vol. 8, no. 1, Art. no. 1, Jan. 2011, doi: 10.1038/nmeth.f.324.
- [36] E. D. Melonakos, O. A. Moody, K. Nikolaeva, R. Kato, C. J. Nehs, and K. Solt, “Manipulating Neural Circuits in Anesthesia Research,” *Anesthesiology*, vol. 133, no. 1, pp. 19–30, Jul. 2020, doi: 10.1097/ALN.0000000000003279.
- [37] V. Emiliani *et al.*, “Optogenetics for light control of biological systems,” *Nat. Rev. Methods Primer*, vol. 2, no. 1, Art. no. 1, Jul. 2022, doi: 10.1038/s43586-022-00136-4.
- [38] K. Deisseroth, “Optogenetics: 10 years of microbial opsins in neuroscience,” *Nat. Neurosci.*, vol. 18, no. 9, Art. no. 9, Sep. 2015, doi: 10.1038/nn.4091.
- [39] K. E. Kishi *et al.*, “Structural basis for channel conduction in the pump-like channelrhodopsin ChRmine,” *Cell*, vol. 185, no. 4, pp. 672–689.e23, Feb. 2022, doi: 10.1016/j.cell.2022.01.007.
- [40] B. Fischl and A. M. Dale, “Measuring the thickness of the human cerebral cortex from magnetic resonance images,” *Proc. Natl. Acad. Sci.*, vol. 97, no. 20, pp. 11050–11055, Sep. 2000, doi: 10.1073/pnas.200033797.

- [41] J. H. Marshel *et al.*, “Cortical layer-specific critical dynamics triggering perception,” *Science*, vol. 365, no. 6453, p. eaaw5202, Aug. 2019, doi: 10.1126/science.aaw5202.
- [42] A. Watakabe *et al.*, “Comparative analyses of adeno-associated viral vector serotypes 1, 2, 5, 8 and 9 in marmoset, mouse and macaque cerebral cortex,” *Neurosci. Res.*, vol. 93, pp. 144–157, Apr. 2015, doi: 10.1016/j.neures.2014.09.002.
- [43] D. Stolzberg, C. Wong, B. E. Butler, and S. G. Lomber, “Catlas: An magnetic resonance imaging-based three-dimensional cortical atlas and tissue probability maps for the domestic cat (*Felis catus*),” *J. Comp. Neurol.*, vol. 525, no. 15, pp. 3190–3206, Oct. 2017, doi: 10.1002/cne.24271.
- [44] S.-Y. Song *et al.*, “The CSF-Contacting Nucleus Receives Anatomical Inputs From the Cerebral Cortex: A Combination of Retrograde Tracing and 3D Reconstruction Study in Rat,” *Front. Neuroanat.*, vol. 14, 2020, Accessed: May 29, 2023. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fnana.2020.600555>