

Final report

Neuro-Optogenetics

Leibniz-Institute: Deutsches Primatenzentrum GmbH, Leibniz-Institut für Primatenforschung

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Executive summary

Cognitive skills are based on complex networks of functionally specialized brain areas. Novel optogenetic methods expand the scope of neuroscientific testing, allowing the analysis of neuronal network functions by manipulating the activity of genetically modified but otherwise functionally intact neurons through light stimulation of the nerve cell tissue. In this project, different areas of the cerebral cortex in rhesus monkeys were made light-sensitive by transducing neurons with viral vectors. To test for the relevance of specific neural projections between brain areas, these were then silenced optogenetically in spatially and temporally precise fashion while animals conducted cognitive tasks.

As first important step, we provided a proof of the functionality, safety and effectiveness of the viral constructs. Production and quality assessments for adeno-associated virus (AAV) and a quick-cloning procedure to rapidly exchange the functional genetic elements for optogenetics approaches were established. Transduction efficiencies and expression of different opsin genes and fluorescent reporters were monitored *in vitro*, *ex vivo* and *in vivo* first in rodents. Also, alternative vector systems were explored and developed for future applications, including a highly efficient 4th generation of lentiviral vectors with superior transduction efficiency, exosome-associated AAVs with improved infection rates and immune-tolerance, and Polyoma JC virus derived virus like particles (VLPs) enabling highly specific and targeted delivery of genetic cargos.

Second, we established procedures for homogeneously transfecting larger volumes of cortical tissue. We provide evidence for a successful injection protocol, functionality of the viral constructs, and, based on quantitative immunohistochemical studies, neuroanatomical detail which is needed for the validation of the injection protocol and for proper interpretation of the physiological experiments.

Third, we developed hard- and software for laser stimulation at calibrated and selectable power in precise sync with the animals' behavior, via minimal-invasive optical fibers positioned stereotactically with computer-controlled micromanipulators. We can now record from individual neurons while animals work on their previously trained cognitive tasks and laser-stimulate the neuropil immediately surrounding the recorded neuron. In two experiment series, we applied light-driven local inactivation of long-range cortical communication between the frontal and the parietal and temporal lobe, respectively. We demonstrate changes in the local neural dynamics as a result of the laser stimulation, providing important evidence for a functional relevance of the respective neuronal projections.

Fourth, we tested larger-scale light-driven neural activation of premotor cortex while measuring whole-brain activation patterns with functional magnetic resonance imaging. This opto-fMRI approach allows us to investigate complex whole-brain responses to specific cortical network activation patterns in NHPs.

In summary, the Neuro-Optogenetic project allowed us to establish a set of highly advanced neuroscientific tools in a very fruitful collaboration between multiple labs of the German Primate Center, including the University Medical Center Göttingen. The new tools (will) benefit our ongoing (and future) research significantly.

1. Background and aims

1.1. Background and starting point

Neural processing in the cerebral cortex enables humans and other primates to achieve extraordinary sensory, motor and cognitive performance. These skills are based on complex networks of functionally specialized brain areas and cell types, particularly neurons. The functional properties of individual neurons and their contribution to these primate faculties are being investigated very successfully using methods from electrophysiology, neuropharmacology, and neuroanatomy. The dominating conceptual approach in this context so far was to work out correlations between neuronal signals and perceptual and/or motor performance.

Brain research was less successful so far in its contribution to the following central questions: How do different neuron types within a brain area and the neurons between different brain areas interact to produce complex cognitive skills? What is the causal relationship between the activity of nerve cells and their interaction on the one hand and the sensory, motor and cognitive performance of primates on the other? These questions represent the next level of understanding of the primate brain, the basis of which is an understanding of complex brain functions at the level of neuronal networks, i.e. beyond understanding of local functional nodes. Answering these questions will benefit the development of long-term diagnostics and therapies for neurological and neuropsychiatric diseases.

The technique of optogenetics allows the analysis of neuronal network functions by manipulating the activity of genetically modified but otherwise functionally intact neurons by light stimulation of the nerve cell tissue. With this, one is no longer dependent solely on the observation of neuronal activity patterns and their correlation with the behavior of the organism, but can analyze causal relationships in a more targeted way than electrical, electromagnetic or pharmacological manipulation techniques.

1.2. Aims of the project

The aim of the Neuro-Optogenetics project was to establish the pioneering methodology of optogenetic manipulation of neurons for system neuroscientific research in non-human primates (NHP) at the DPZ. In rodent research, optogenetic methods were well established within a few years after their development. In primates, on the other hand, there were only few but very promising approaches public at the time of the project start. Until today, published work using optogenetic tools in the context of cognitive research in NHPs is relatively scarce. The idea of the project was to establish corresponding methods by three working groups of the DPZ and apply them in three highly topical research fields: sensory attention, decision making and neuroprosthetics. Technical support was provided by an institute-funded new technology platform with extensive knowledge in the fields of genetics, virology and other molecular-biological approaches. Researchers in all four subdivisions of the project were financed from the Neuro-Optogenetics grant.

The expectation towards this method was and is a fundamental advance in our understanding of elementary brain functions, which are the basis for the cognitive performance of primates, including humans. Parallel to the work in cognitive neuroscience, the idea of the technology platform was to contribute to the rapid development of optogenetic methods, among other things

by integrating the VLP (virus-like particles) technology invented and patented at the DPZ. This unique technique could provide an alternative to the use of viruses in optogenetics in the medium term and could be an important advantage for a later application of the technology in human patients.

In summary, the project combines the establishment of this technique in non-human primates, supported by an institute-funded technology platform, with its application in three subprojects in central areas of systemic cognitive neuroscience: sensory attention, decision making and neuroprosthetics.

2. Project development

2.1. Approach and overview of experiments

In the Neuro-Optogenetic project, different areas of the cerebral cortex in rhesus monkeys were made light-sensitive by introducing genetic constructs (composed of cell-type specific promoter, opsin and fluorescence reporter genes) into neurons by means of viral transduction (viral vectors). Virally infected neurons then expressed and incorporated the light-sensitive channel proteins (opsins or ion-pumps) into their cell membranes. By illumination with laser light of a certain wavelength, such modified neurons could be activated or inactivated very quickly (within a few milliseconds) and reversibly. This approach of light-controlled activation and inactivation of genetically modified neurons was made accessible for application in NHPs in three working groups of the DPZ, with the intense help of a fourth group, and then applied for the first time in several specific scientific research questions in NHPs. The activation or inactivation of certain neuronal groups within a brain area, or of neuronal/axonal projections connecting different brain areas, was used to investigate the contribution of these neurons to higher cognitive performance, such as visual sensory attention, decision-making or the planning of coordinated movements, as laid out in more detail below (Results). Furthermore, as a yet to be implemented outcome of the project, the optical activation of neurons in somatosensory areas will be used to test the possibility of generating artificial body perceptions. This is a biomedical application for the improvement of motor neuroprostheses.

First, before testing the viral vectors in rhesus monkeys, a number of bench-side procedures and tests had to be established, for example, to proof the functionality, safety and effectiveness of the viral constructs. On the one hand, AAV production and quality assessments in terms of functionality, titers and purity of recombinant AAV were established, as well as a quick-cloning procedure to rapidly exchange the functional genetic elements for optogenetics approaches. Transduction efficiencies and expression of different opsin genes and fluorescent reporters were monitored on various cell lines *in vitro*, on organotypic CNS cultures *ex vivo* and *in vivo* in rodents and also compared to commercially available, validated AAVs for neuro-optogenetics. On the other hand, a number of alternative vector systems have been explored and developed to provide a solid base for future applications, including a highly efficient 4th generation of lentiviral vectors with superior transduction efficiency for accelerated *in vitro* testing of new opsin-gene alternatives, exosome-associated AAVs, that display improved infection rates and immune-tolerance, as well as Polyoma JC virus derived virus like particles (VLPs) enabling a

highly specific and targeted delivery of genetic cargos. (See section 3.1.1 for results on virus development.)

Second, we needed to establish protocols for transfecting larger volumes of cortical tissue in an as much as possible homogenous fashion. Since the brains and functional brain areas of rhesus monkeys are much larger than those of laboratory rodents, this is a specific challenge. Also, limited availability of genetic tools or published literature on optogenetic tools in NHPs required us to test the reagents and procedures before specific experimental application. As a first proof-of-concept for the usefulness of our injection protocols and the functionality of the viral constructs in NHP, we decided to apply the technique to an animal which was not used in a cognitive experiment, but was instead tested under anesthesia and investigated immunohistologically afterwards. The outcome of this proof-of-concept experiment with post-mortem histological analysis is detailed below (section 3.1.2) and provides evidence for a successful injection protocol, functionality of the viral constructs, and neuroanatomical detail needed for the interpretation of the physiological experiments.

Third, since a main idea of the project was to use light-induced neural (in-)activation to interfere with ongoing cognitive processes, we needed a way of applying laser stimulation to the cortical tissue in a safe, non-harming way, precisely timed and controlled in its light intensity. For this, we developed and integrated different laser stimulation protocols into our real-time experimental control software with which we conduct the cognitive experiments in awake NHPs. Since hardware and software are optimized for the specific research questions in each field of research, this integration in part had to be adapted separately to each experimental setting. As a result, we are now able to trigger a large variety of predefined sequences of laser pulses at millisecond precision and in sync with the animal's behavioral task, apply the laser beam to the neuropil via thin optical fibers at calibrated and selectable power of up to a few ten Milliwatt, sufficient for activation of the opsins in use, and choose between 3 different laser wavelength depending on the opsins in use. Optical fibers and microelectrodes are positioned stereotactically in the neuropil at micrometer precision with computer-controlled micromanipulators. This allows for configurations in which individual neurons are recorded in the awake animal conducting a behavioral task, while the laser illuminates the neuropil immediately surrounding the neurons recorded from (sections 3.1.3 & 3.1.4).

Fourth, we then transferred the methods to animals that were trained on specific cognitive tasks and from which we record with neurophysiological methods from individual neurons. In the first two use-cases, we apply light-driven inactivation of neural activity to interfere with long-range cortical communication underlying specific cognitive processes. As we assume that these processes depend on the transmission of neural signals (information) from frontal lobe to more posterior regions of the brain, like visual cortex or parietal association cortex, the idea in both projects is that we inactivate specifically these projections and study the impact of the disrupted signal flow on the neural dynamics in the receiving brain areas. Both projects are ongoing after the results from the first animal in each project provided extremely promising first results which now still need to be confirmed further before publication. The successful implementation of light-driven inactivation in NHPs conducting cognitive tasks will become evident from the neurophysiological results discussed in the context of two specific research questions below (sections 3.1.3 & 3.1.4).

Fifth, we also transferred the approach to an animal in which we tested larger-scale activation patterns across the whole brain when using broader light-driven activation of larger local volumes of brain tissue. For measuring large-scale brain activation, we established functional magnetic resonance imaging (fMRI) under light anesthesia/sedation. To effectively illuminate larger tissue volumes we established a technique for implanting an artificial dura (AD), which is made of biocompatible transparent silicone, and developed a novel skull-mountable chamber implant for attaching multiple optic fibers with defined geometric arrangement in an MR-compatible way. This allows us to investigate complex whole-brain responses to specific cortical network activation patterns in NHPs (sections 3.1.5).

2.2. Challenges

For in-house production of AAV viral constructs which satisfy all required specifications, the procedures had to be developed, implemented and optimized. This took significant time and effort. To avoid delays beyond the already long preparation phase of the test animals in the cognitive experiments with optogenetic manipulation, we used commercially available constructs in parallel.

2.3. Deviations from work plan

No major deviations from the work plan occurred, the projects have been implemented as planned.

2.4. Use of animals

All experimental procedures including animals have been approved by the responsible regional government office [Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES)] under permit numbers 33.92-42502-04-13/1100, 33.19-42502-04-18/2823, and 33.19-42502-04-14/1442, and comply with German Law and the European Directive 2010/63/EU regulating use of animals in research.

3. Results

3.1. Summary of research findings

3.1.1. Viral constructs

3.1.1.1. AAV engineering, production and quality assessment

Neuro-optogenetics, like all other techniques involving transgene expression, require vectors for the delivery of genetic information into cells and tissues of interest. We have selected the well-established Adeno-associated virus system (AAV) for the *in vivo* neuro-optogenetics in the NHP brain. Recombinant AAVs are a safe and relatively efficient tool to transduce cells *in vitro* and *in vivo*, they display no pathogenicity and low immunogenicity and a multitude of serotypes enables a selection of target tissues. We selected serotype 5 (rAAV5) for injections in NHPs and built a stock of alternative optogenetic tools including stimulatory and inhibitory opsins under control of mainly neuron-specific promoters and in combination with alternative fluorescence reporter genes. The list below displays ready-to-inject viruses that were provided by the UNC vector core. The names display: AAV-serotype/promoter/opsin gene (modification)/fluorescence reporter;

rAAV5/CaMKII-Jaws-KGC-GFP-ER2
rAAV5/CaMKII-hChR(H134R)-eYFP-WPRE
rAAV5/SSpEMBOL-CBA-GFP
rAAV5/CaMKIIa-eNpHR3.0-mCherry-WPRE
rAAV5/CaMKII-C1V1(E122T/E162T)-TS-mCherry
rAAV5/CaMKII-C1V1(E122T/E162T)-eYFP
rAAV5/CaMKII-hChR-ArchT3.0-eYFP
rAAV5/CaMKII-hChR(C128S/D156A)-eYFP

In parallel, we have set up a full production and quality assessment workflow to generate and test additional and purchased AAV vectors. As part of a PhD project (L Schiller) a quick cloning set-up was established to easily and rapidly exchange the functional elements of rAAVs, i.e. promoters, e.g. the constitutive CAG and Ef1a, or the neuronal CamKII or hSyn promoters, opsin genes, including hChR2, eNpHR, ArchT, eArch, Jaws or C1V1, multiple fluorescent reporters such as eGFP, eYFP, BFP, mCherry or tdTomato and regulatory elements of mRNAs like internal ribosomal entry sites (IRES) and the Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE). Combinations of these elements can quickly be generated in various AAV serotypes (rAAV1, rAAV1/2, rAAV2, rAAV5 and rAAV6) resulting in several hundred different rAAVs to choose from. Furthermore, new developments of more specific promoters, improved opsin genes etc. can easily be integrated into the existing system and allow speedy integration of technological advances.

To evaluate the functionality of self-made and purchased vectors we integrated analysis of rAAV by quantitative PCR (qPCR) to determine genomic titres and flow cytometry (FACS) to check functional titres on transduced cells from various origins, including human cell lines and primary neuron cultures from rodent CNS. Purity of viruses was confirmed by transmission electron microscopy (TEM) and biochemical methods, such as silver-stained SDS-PAGE and impurities were identified by mass spectrometry (MALDI-TOF). We identified ferritin complexes as minor impurity in our rAAV preparations. Prior to injection into the primate brain rAAVs were tested in vivo by injection into rodent CNS and if possible (depending on material availability) on organotypic cultures of brain slices from Rhesus macaques.

In addition to rAAVs, we explored further vector systems that enable accelerated in vitro testing, improved in vivo applicability or alternative injection routes per tissue targeting after systemic delivery (see below).

3.1.1.2. Alternative delivery vectors.

Viral vectors are the tools of choice for the delivery of transgenes but for a variety of reasons also non-viral vectors and delivery systems receive more attention. Lentiviruses (LV) are frequently used as an alternative to rAAVs and display long-lasting, stable transgene expression and capability to transduce non-dividing cells. We investigated the interplay of viral vectors with the cellular exosome machinery. Most human cell types and tissues secrete small, from intraluminal endosome-derived membranes (exosomes) with sizes of approximately 30-120 nm in diameter. We demonstrated that enhanced levels of the exosome-enriched tetraspanin CD9 led to increased levels of secreted exosomes in the respective vector producing cell lines and in turn to both a drastic increase of lentiviral infectivity and a boost of exosome associated AAVs (exo-AAV) production. The resulting viral vectors acted superior to standard vectors for cell and gene therapy in terms of transferring genetic material to many target cells.

3.1.1.3. Fourth generation lentiviral vectors

We drastically increased the expression level of CD9 in LV producer cell lines, determined the amounts of LV by genomic and physical titration as well as the transduction-efficiencies on various cell types by flow cytometry and microscopy using GFP as reporter gene. Overexpression of CD9 on LV producing cell lines did not increase the amounts of viral vectors, but the LV-CD9 showed a faster, higher expression of GFP on HEK293, HeLa, SH-SY5Y (human neuroblastoma derived), B- and T-lymphocytes. We demonstrated that enhanced CD9 enables lentiviral transduction in the absence of any pseudotyping viral glycoprotein or fusogenic molecule. The findings displayed an important role of CD9 for lentiviral vector and exosome biogenesis and pointed out a remarkable function of this tetraspanin in membrane fusion, viral infectivity, and exosome-mediated horizontal information transfer. In addition, these highly efficient LVs are subject of further developments on the course of a PhD project (R Rinaldi), which is addressing directed delivery in LV by including targeting molecules (TMs) in the viral envelope. This is achieved by integrating proteins or peptides with high affinity (e.g. minimalistic single-chain antibodies, scFv) to target-cell-exposed structures (e.g. surface receptors such as TrkB on neurons) by transmembrane anchors. In combination with fusogenic envelope-proteins, like CD9 or manipulated influenza hemagglutinin (HA) a highly cell specific vector can be achieved. As an additional benefit, TMs can be adopted for retargeting of exoAAVs or VLPs (below). The full study was published in *Molecular Therapy* (Böker et al. 2017).

3.1.1.4. Exosome associated AAV (exoAAV)

Exosome-associated adeno-associated viruses (exo-AAVs) are of particular interest for the past couple of years, because they introduced a new source of highly potent recombinant AAVs with improved features, including accelerated transduction rates and more efficient immune escape. However, key factors like the mode of action, efficiency of production, or engineering of exo-AAVs remain elusive to a large extent. AAV production in high CD9 cells, resulted in significantly higher numbers of AAVs that were associated to exosomes. A CD9-powered high-exosome environment was established during exo-AAV1 or exo-AAV2 production, and we could demonstrate that the yield of exo-AAVs dramatically increased when compared to standard exo-AAVs. Furthermore, we observed that exo-AAV-CD9_{GFP} was more efficient in transduction of cells in the same titer ranges as standard exo-AAVs. The results provide a technological approach for the generation of exo-AAVs with superior performance. To increase the target-specificity of exo-AAVs we will explore the integration of LV-approved TMs into the exosome-membranes. The study was published in *Molecular Therapy – Methods and Clinical Development* (Schiller et al. 2018).

3.1.1.5. Directed delivery in Polyoma JCV derived virus-like particles (VLPs)

A broader range of therapeutic cargos, including synthetic RNA or linear DNA cassettes can efficiently and cell-type specifically be delivered in engineered virus-like particles of the human polyoma JC virus (VLP). These non-viral carrier complexes have successfully been used *in vitro* and *in vivo*, e.g. for systemic RNAi against endogenous targets or reporter-gene delivery. Tissue-specific transfer of nucleic acids *in vivo* so far mostly relied on viral transfer systems. Due to the potential danger of recombination with cellular sequences, viral transfer systems bear a partly difficult to predict safety risk. Also, repeated *in vivo* administration of e.g.

adenoviruses and adeno-associated viruses is hampered because of their high immunogenicity in most individuals. In addition, due to the complex structure of the viruses it is only possible with considerable effort to deliver therapeutic nucleic acids to the target site at sufficient concentrations/levels and in suitable form. Another aspect is that viruses usually infect more than one type of cells. As an alternative, non-viral systems avoid most of these disadvantages but, in turn, and similar to retroviral systems, exhibit much lower transfer efficiencies and target-cell specificities. We have a long history in the research of polyoma JC virus (JCV) and the successful development of virus-like particles (VLPs). The need to provide clinically applicable effector molecules has been the motivation of the researchers to generate VLPs based on human JCV VP1 protein. JCV has been described as a suitable tool for transduction of genetic material in a variety of cell lines. Purified VP1 protein spontaneously forms stable homopentameric capsomers, which in turn assemble into VLPs. VLPs can be loaded in a single dissociation-reassociation cycle in which the cargo nucleic acids (here: siRNA, miRNA or linearized DNA) can be added in the dissociated stage. VLPs can then form around the nucleic acid cargo during slow dialysis versus physiological, non-reducing buffer conditions. As an example, using a siRNA to target the nuclear kappaB factor ligand RANKL in a rat osteoporosis model, significant silencing of RANKL with very low quantities of VLP could be achieved. Two studies on this gene therapy approach have been published in *Molecular Therapy – Nucleic Acids* (Hoffmann et al. 2016; 2018). Moreover, recovery of silencing by repeated VLP injections has been shown. In addition, the VLPs native tropism could be specifically altered by covalently linking a HER2/neu single chain antibody fragment onto the surface of the particles by different methods (see figure B). Thereby, transduction of HER2/neu positive breast- and colorectal-

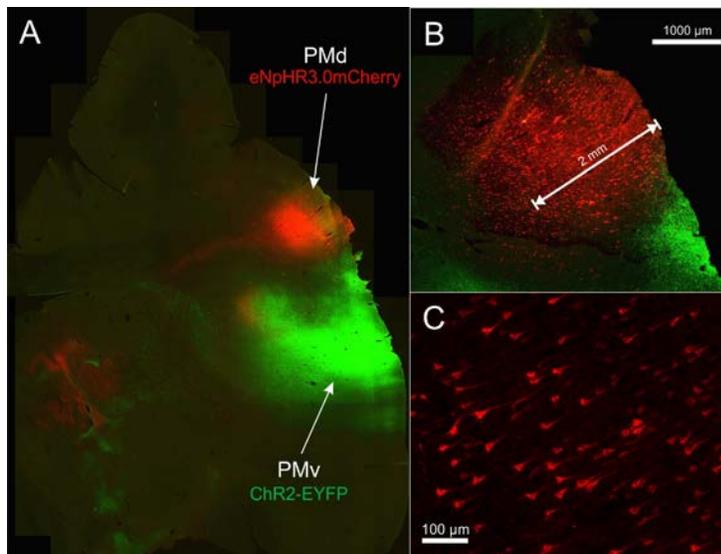


Figure 1 Opsin expression in the target areas. **A:** Coronal hemi-section from the frontal lobe at the level of dorsal (PMd) and ventral (PMv) premotor cortex. Colored in red are cells expressing eNpHR3.0-mCherry, in green are cells expressing ChR2-EYFP. Note axonal fibers leaving cortical area. **B:** eNpHR3.0-mCherry transduced cells in PMd indicating homogeneous expression throughout the depth of cortex. **C:** Higher magnification of transduced neurons with familiar pyramidal cell morphology.

cancer cells could be achieved and the natural tropism of the VLPs could be blocked.

3.1.2. Proof-of-concept and neuroanatomical considerations

Successful optogenetic experiments depend on a number of factors, including an appropriate viral vector (Lentivirus or AAV, serotype, etc.), genetic promoter and opsin used, but also the delivery method, which would result in a strong and specific expression of the opsin protein in a brain area and in the cells of interest. Optimal viral constructs and targeting strategies may vary widely between species, brain areas and specific experimental needs. As experiments in NHP are typically conducted with very low number of animals, and these are

usually behaviorally trained for periods of months or even years, we first evaluated the viability of chosen vectors prior to their use in experimental animals. The goal was to develop a protocol for effective larger-scale neuronal transduction. We focused our efforts/analysis on the frontal lobe areas PMd (dorsal premotor cortex; interest of the Gail lab), PMv (ventral premotor cortex; Scherberger), and FEF (frontal eye fields; Treue) and on the reciprocal connections to their parietal lobe targets. We chose the AAV2/5 vector, as it has been successfully used in primates before, and the CaMKII α promoter to limit opsin expression to excitatory pyramidal neurons, which are believed to provide long-range communication between cortical regions via their axonal projections. In our pilot study, we injected three different viral vectors (rAAV2/5-CaMKII α -hChR2(H134R)-eYFP, rAAV2/5-CaMKII α -eNpHR3.0-mCherry and in-house made rAAV2/5-CaMKII α -eNpHR3.0-eYFP) in on Rhesus monkey. Bolus injections of 1 μ l volume were spread over the 2mm depth of cortex at 1mm separation with multiple parallel injection tracks of approx. 2mm horizontal separation. The idea was to maximize the spatial spread of the virus without compromising tissue structure and effective opsin expression in the studied brain regions. Following 10 week incubation period, the animal underwent terminal experiment after which it was perfused and the brain was preserved for histological evaluation. We analyzed the injected areas, effective viral spread, expression in cell bodies, their axonal projections and the extent of retrograde transport.

First, we analyzed the injected areas for potential pathologies. Other than the minimal needle tract from the Hamilton injection syringe, no lesions or evidence of structural changes were observed as a result of our injection protocol. In the identified injection locations no apparent damage to the brain tissue was noticed (Figure 1A-B). The choice of virus and injection protocol yielded a high percentage of cortical pyramidal neurons expressing the opsin (Figure 1C).

We also quantified the effective viral spread. Our protocol led to a fairly uniform transduction of cortical neurons, throughout the depth of cortex. We estimated that the 1 μ l bolus volume of delivered virus resulted in an expression radius of about 1mm around each injection site (Figure 2).

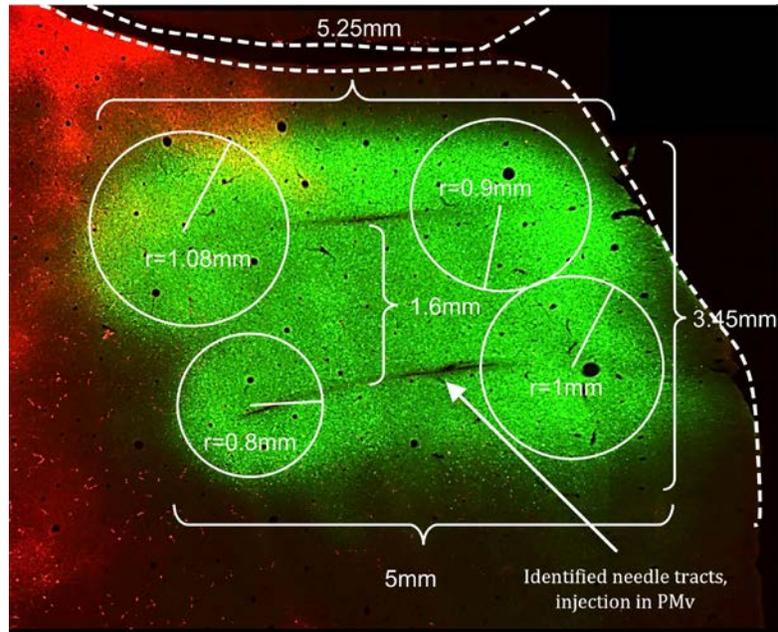


Figure 2 Assessment of the effective viral transduction. Identified injection location (see needle tracks) of the ChR2-EYFP virus in cortical area PMv. Basic size measurements indicate a spread with about 1mm radius for each bolus injection of 1 μ l volume. In green, cells and their fluorescent processes expressing ChR2-EYFP around the injection sites; in red, projection from the eNpHR3.0-mCherry transduced cells.

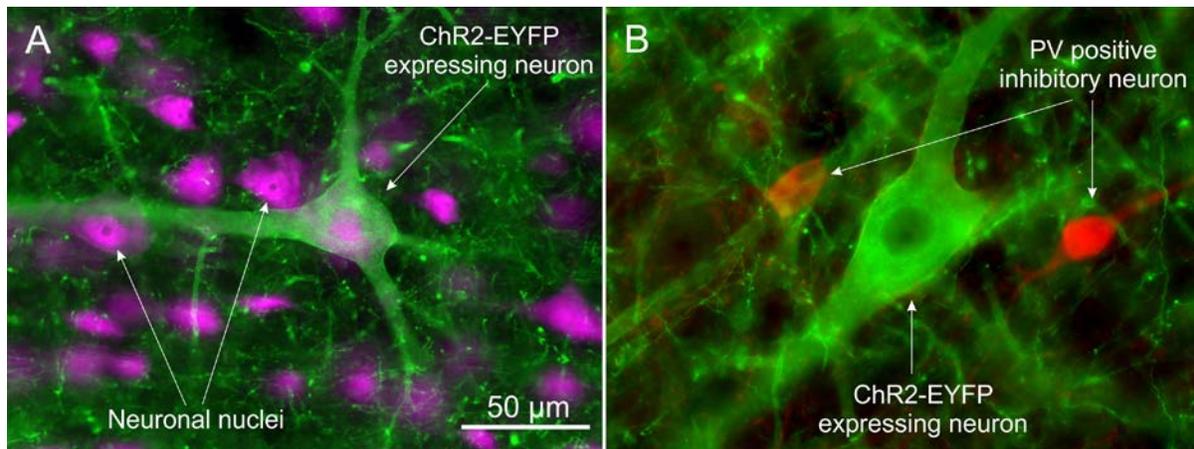


Figure 3 Phenotype of transduced cells. **A:** Expression of opsin proteins (ChR2-EYFP, green) was present only in neurons as evident by co-localization with a neuronal marker NeuN (magenta). **B:** The majority of transduced cells display pyramidal neurons morphology (ChR2-EYFP, green) and did not co-localize with Parvalbumin (PV, red) – a marker for subpopulation of GABAergic neurons. Note the highly efficient incorporation of ChR2-EYFP into cell membranes.

We also determined cell specificity of the expression and cellular distribution of the opsin protein on the cell membranes. Immunohistological staining with a neuronal marker (NeuN) revealed that the cells expressing the transgene were of neuronal phenotype (Figure 3A). The great majority of transduced cells display pyramidal neurons morphology (Figures 3B, 1C). Yet, Parvalbumin (PV), a marker for a subpopulation of inhibitory neurons, co-staining revealed that approximately 6% of mCherry positive cells in different areas (4100 cells counted) were also positive for PV (10150 cells counted).

Importantly, as one of the experimental objectives was the laser-mediated modulation of axonal processes at the target area (sections 3.1.3 and 3.1.4), we also examined whether opsins were efficiently transported along the axons of transduced cells. Axonal expression was easily visible, indicating strong transgene expression and efficient incorporation of the protein into the cell membrane (Figure 4). Moreover, we found axons positive for mCherry or EYFP in distant brain areas, specifically, long-range projections from area frontal area PMd and PMv to the parietal lobe, including axonal terminals in assumed target areas (Figure 4A,C). Occasional cell bodies of transfected neurons were also seen many millimeters away from the injection site (in parietal lobe), a fact that can only be attributed to the retrograde pickup of a virus vector (Figure 4C).

In summary, results of the test experiment showed that the chosen optogenetic tools and injection protocol can be used to efficiently target neurons of the fronto-parietal and fronto-visual (data not shown) cortical network. Laser stimulation experiments can be conducted both, in the injected areas as well as in distant areas that are targeted by axons of transduced cells.

3.1.3. Using optogenetics to elucidate the role of direct input to MT from the FEF for the attentional response modulation by spatial attention

Several studies indicate that the frontal eye fields (FEF) in the prefrontal cortex play a pivotal role in guiding attention and modulating neural activity in visual areas. Studies so far used methods (microstimulation and pharmacological intervention) that cannot disentangle the direct influence of FEF on attentional modulation in visual areas. The method of optogenetics provides

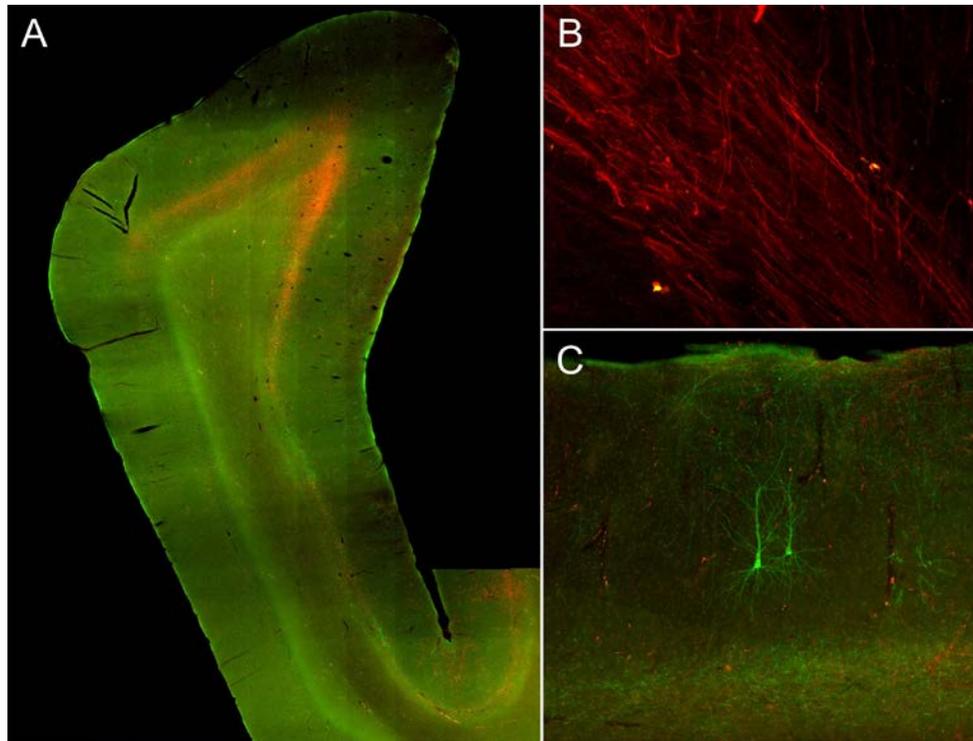


Figure 4 Axonal projections to posterior parietal cortex. **A:** An example illustrating eNpHR3.0-mCherry (red) and ChR2-EYFP (green) positive processes terminating in bottom cortical layers of the target areas in the parietal lobe (medial intraparietal area shown). **B:** Expression of eNpHR3.0-mCherry in axons originating in area PMd. **C:** Two retrogradely labeled pyramidal neurons in medial intraparietal cortex expressing ChR2-EYFP.

a new possibility to selectively manipulate neural projections between two areas. The aim of this project was to inhibit the direct anatomical projection from the FEF to visual area MT optogenetically and to evaluate the direct influence of the local FEF input on attentional modulation of MT neurons.

Therefore, we injected a viral vector (AAV5- α CaMKII-eNpHR3.0-mCherry) into the FEF of four rhesus macaques. The opsin of this viral vector is designed to be incorporated into the axons and presynaptic terminals of FEF projections. Two of the animals were used for histological confirmation that the inhibitory opsins are indeed expressed in FEF axons terminals in area MT (data not shown). The other two animals were trained in a spatial attention task (Figure 5). Here, two moving random dot patterns (RDPs) were presented at 2-10 degrees eccentricity relative to gaze direction. We manipulated the attentional focus of the animals by cueing the target

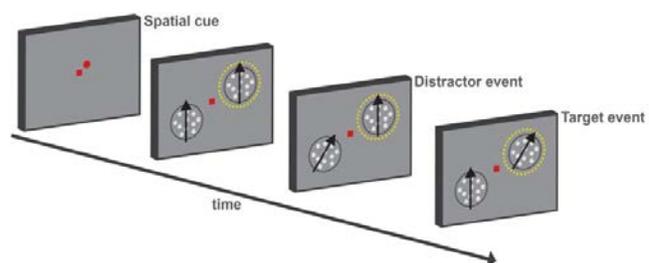


Figure 5 Behavioral task used in the study of the attentional network. Two attentional conditions share the same visual stimuli but differ by the attended location (inside or outside the receptive field of MT neurons) to investigate the effect of spatial attention on MT responses.

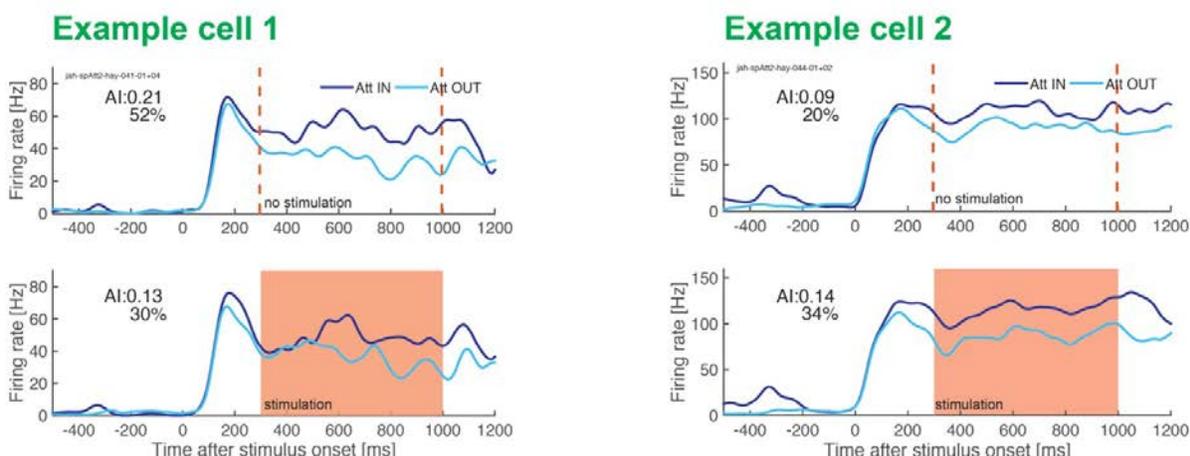


Figure 6 Time course of response to the presentation of a random-dot pattern (RDP) in the receptive field moving in a neurons preferred direction. All four panels plot the time course of response to the presentation of an RDP in the receptive field moving in the neuron's preferred direction. The dark blue traces represent trials where the stimulus in the receptive field was attended (the target), while the light blue traces represent trials where the stimulus inside the receptive field was unattended (the distractor). The two top panels show responses for trial without laser stimulation, while the two bottom panels show trials with laser stimulation (during the reddish segment of the time course).

stimulus at the beginning of each trial. Animals were rewarded for responding to a direction change in the target RDP while ignoring direction changes in the distractor RDP. Several months after virus injection, we recorded single neuron activity in area MT and laser-stimulated the vicinity of the recording electrode tip to inhibit FEF input during the sustained response of MT neurons. Trials with and without optical stimulation were randomly interleaved and not apparent to the animals by any obvious means.

The first example neuron (Figure 6, left) shows a decrease of the attentional modulation with laser stimulation of the surrounding tissue, i.e., a smaller difference between the two traces during the laser-stimulation (lower panel) compared to the difference without stimulation (upper panel) during the same time period. The second example neuron shows the opposite, i.e., the attentional modulation was increased by laser stimulation (Figure 6, right).

To quantify the effect of laser stimulation at the neural population level, we compared the attentional modulation index between trials with and without stimulation for all neurons recorded.

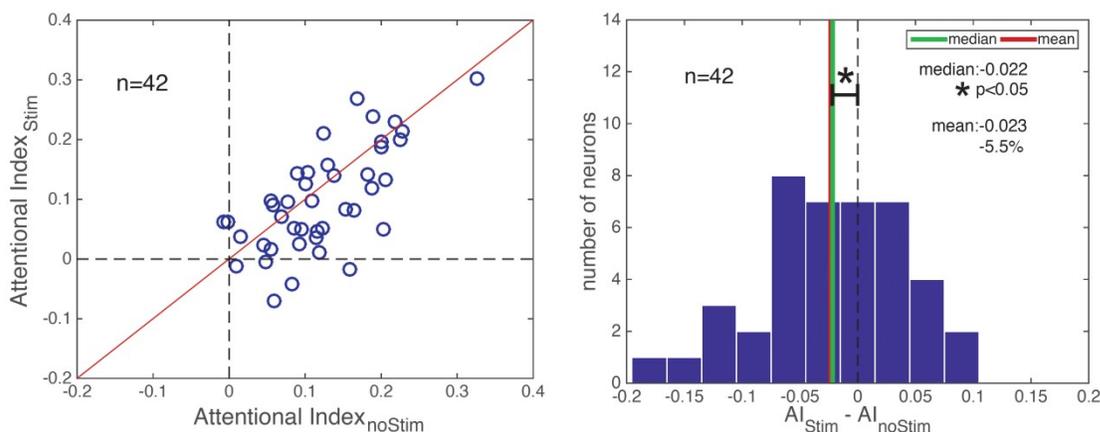


Figure 7 Effect of light-gated inactivation of FEF-to-MT projections on attentional modulation in MT.

The majority (62%) of the cells showed a decreased attentional modulation during stimulation (Figure 7, left). The mean attentional modulation across our population of cells was 29% without laser stimulation and dropped to 24% with laser stimulation. The distribution of differences of attentional modulations with and without stimulation is significantly shifted to negative values (Figure 7, right). It shows that the enhancement of firing rate caused by attention was reduced by 19% with laser stimulation. More specifically, firing rates were on average decreased when the RDP in the receptive field of the recorded neuron was the target, whereas firing rates were increased when the same stimulus was the distractor.

Our results reveal that the direct anatomical projection from the FEF to visual area MT plays a causal role in both, enhancing target responses and reducing distractor responses during a spatial attention task.

The FEF-MT project was started as a high-risk pilot project, as there had been no previous experience at the DPZ with applying optogenetic methods in NHPs. The optogenetic capacities needed for this were developed in the Neuro-Optogenetics project. The success shows not only that the viral vector we selected is suitable for inhibiting projections in macaque visual cortex, but also that such an optogenetic approach is very suitable as a complementary techniques to the neuropharmacological interventions used in traditional approaches. An open questions we plan to address in future is how this influence affects the direction tuning curves in MT, rather than just the preferred and non-preferred directions.

3.1.4. Modifying the frontoparietal reaching and decision making network with projection-specific optogenetic inactivation

Planning movements during rule-guided reaching involves areas in frontal and parietal cortex that are reciprocally connected. Simultaneous recording from the dorsal premotor area (PMd) in frontal cortex and the parietal reach region (PRR) in parietal cortex revealed coordinated activity between both areas. During rule-guided action selection, selectivity for motor

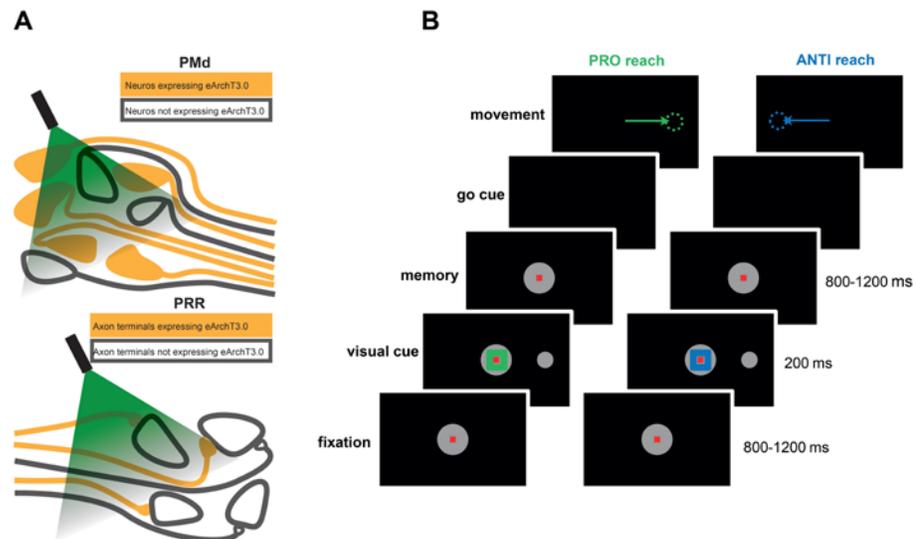


Figure 8 Experimental design for optogenetic manipulation of rule-guided sensorimotor transformations. **A:** Concept of optogenetic suppression of specific projections. In PMd (up), neurons expressing eArchT3.0 (hyperpolarizing opsins) are selectively inhibited by 532nm laser stimulation. In PRR (down), optical stimulation of eArchT3.0 expressed on axon terminals leads to the inactivation of presynaptic action potentials arriving from PMd. **B:** Memory-guided anti-reach task with two color-instructed spatial transformation rules. Laser stimulation was applied during the visual cue period.

goals occurred earlier in PMd than PRR when a visual target stimulus indicated an associated motor goal only indirectly. These findings support the idea that dynamic reorganization of network activity in PRR is contingent on projections from PMd.

To test for the putative causal dependency of PRR on PMd input neuronal inactivation of PMd activity is a viable strategy. But conventional methods (lesions, pharmacology) lack spatial and temporal specificity. In addition, these inactivation methods are not capable of targeting specific neural projections. Depolarizing/Hyperpolarizing opsins can be used to investigate the circuit-specific causality between brain areas at fine spatial and temporal scales, provided that the opsins are expressed on the axon terminals of long-range neural projections, following the same logic of section 3.1.3.

Here, we used optogenetic methods to phasically inhibit the presynaptic terminals of PMd projecting to PRR in the local vicinity of a recording microelectrode in PRR (Figure 8A). A viral vector with inhibitory opsin (rAAV5/CamKII α -eArchT3.0-eYFP) was injected into left PMd of a Rhesus macaque. After three months incubation, the recording electrodes combined with optical

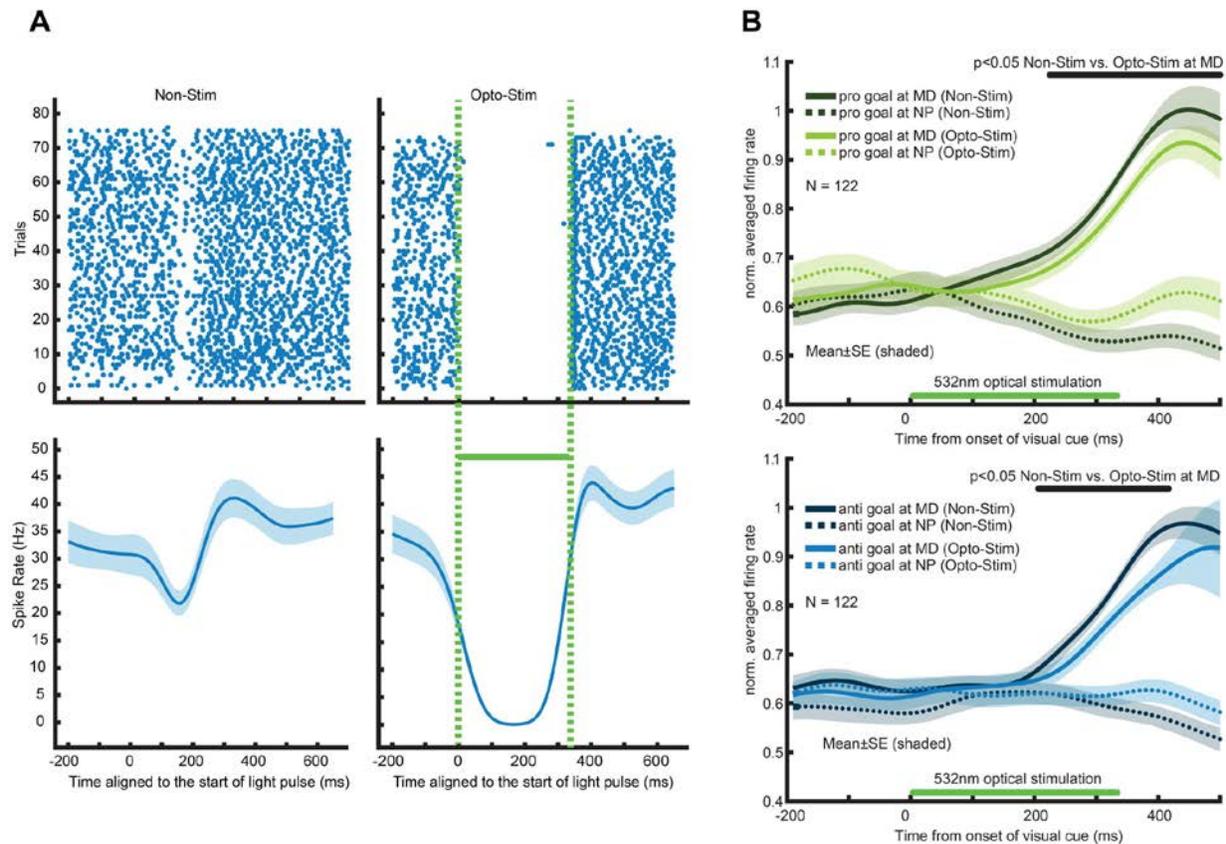


Figure 9 Effect of optogenetic manipulation on parietal motor goal encoding. **A:** Raster plots and peristimulus time histograms (PSTHs) of a PMd example neuron, aligned to the start of light pulses, demonstrating successful opsin expression and light-induced inactivation. **B:** Dynamics of motor-related tuning in PRR. PSTHs across neurons were computed as the average response in the direction of each neuron's maximal response (MD, solid) and in the opposite direction (NP, dotted). Mean \pm SE (shaded) of PRR normalized population activity in MD and NP were aligned to visual cue onset. Black bars indicate the significant ($p < 0.05$, bootstrap test) difference between trials without laser stimulation (non-stim) and trials with stimulation (opto-stim) in the direction of maximal response.

fiber were located in either PMd or PRR with inter-tip distances of 350-850 μ m. Single-neuron activities were recorded while the monkey conducted a pro/anti reach task on a haptic manipulandum (Figure 8B). The task required the monkey to map a spatial cue onto one of two reach goals, either at the location of the cue (pro) or opposite to it (anti), based on the instructed rule. The spatial transformation rule was instructed with a colored context cue. A continuous laser pulse (330ms duration, 532 nm, 12-16mW at the tip of optical fiber) was applied during display of the spatial and the context cue to the animal. Trials with and without optical stimulation were randomly interleaved.

In PMd, 78 of 116 neurons (67%) are modulated by optical stimulation, among which 63 neurons are completely silenced (Figure 9A), demonstrating effective opsin expression in PMd. Transient optical inhibition at the presynaptic terminals of PMd locally in PRR systematically changed neural activity of PRR, at the single-neuron and population levels. On average, motor goal representations in PRR are significantly attenuated by optogenetic inhibition, in both pro- and anti- reach conditions (Figure 9B). Our preliminary results reveal that the direct projections from PMd to PRR play a causal role in PRR neural responses to visual spatial input during motor goal formation and reach planning. This study shows that top-down signals from PMd influence motor goal encoding in the frontal–parietal circuit, the details of which mandate further analysis of the existing data and confirmation in a second animal.

3.1.5. Towards larger scale approaches of optogenetic manipulation

Optogenetics offers a possibility to noninvasively modulate an activity of defined neuronal populations, and we wanted to exploit this opportunity to better understand the connectivity, thus the function, of fronto-parietal cortical networks. However, the size of the primate brain, particularly in Rhesus monkeys, poses a major challenge as large volumes of the tissue need to be effectively transduced and subsequently illuminated with the laser light. Moreover, with classical electrophysiological approaches, one has to know what the target areas of a given

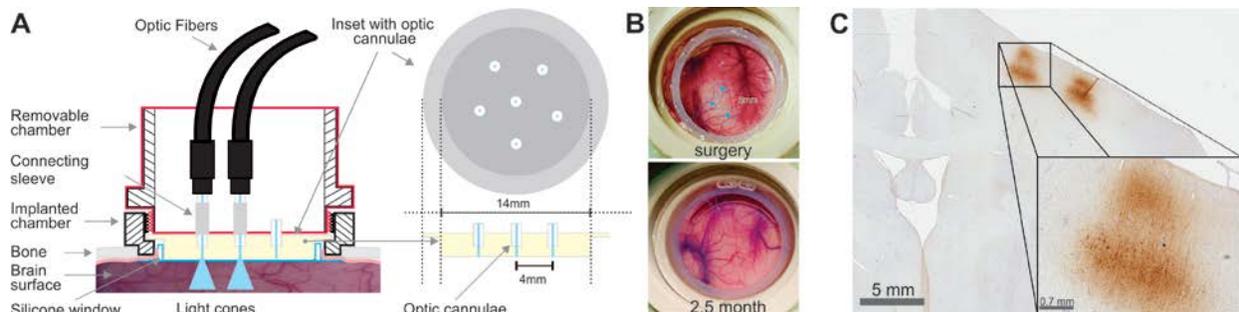


Figure 10 Towards large-scale optogenetic manipulation of cortical neural activity in rhesus monkey. **A:** Schematic of newly developed optogenetics chamber for simultaneous multi-site optical laser stimulation of cortical surface areas, like premotor cortex, via a detachable array of optic cannulae mounted into a chronic chamber implant. **B:** Chronically implanted silicone window below the cannulae array which serves as transparent artificial dura for transdural laser stimulation of the cortical surface layers over many months. Blood vessels serve as landmarks for reconstruction of intra-surgical injection sites (blue dots) of the viral vector (AAV-CaMKIIa-C1V1(E122T/E162T)-TS-mCherry; animal G). **C:** Immunohistological proof of successful transfection below the artificial dura after ending the opto-fMRI measurements.

population of neurons are, and accordingly record in these areas. Towards this goal, for example, we aimed for information about functional connectivity of PMd → MIP projections beyond the (still rather limited) knowledge about the anatomical connectivity. For this, we combined optogenetic excitatory stimulation with functional magnetic resonance imaging (opto-fMRI), first in a lightly anaesthetized/sedated rhesus monkey. To achieve this, we developed a novel system of a skull-mountable chamber implant for attaching multiple optic fibers with a defined geometric arrangement in an MR-compatible fashion (Figure 10A). Together with this, we established and implemented a technique for chronically implanting an artificial dura (AD) below the chamber implant. The AD is made of biocompatible, transparent silicone to allow direct access of light to the brain area of interest (Figure 10B). We injected an excitatory, red-shifted opsin C1V1 in discrete locations of PMd below the AD. Red-shifted light allows deeper penetration of the light into brain tissue with the same power due to lower light scattering, which is important since the optical fibers for laser stimulation are positioned above the AD outside the tissue. To test larger-scale activation patterns in response to light-driven PMd activation, we combined the laser stimulation with monitoring of the whole brain activity with functional magnetic resonance imaging (opto-fMRI). Preliminary results of this approach indicate that it is possible to map and study the functional connectivity of defined cortical areas (Figure 11). We observed remote activity patches which represent known/plausible targets of the PMd neural projections (e.g. FEF, PMv, M1, STS or MIP) and also point to other, not well studied, areas of the distributed network involved in the coordination of reaching.

3.2. Perspectives of application and potential follow-up projects

The Gruber lab will continue to utilize the developed gene delivery tools and focus on the applications of directed gene delivery tools. To date the first feasibility studies for systemic delivery of brain-targeting vectors are subject of a cooperation with an industrial partner (see section 4) and a grant addressing the development of transient, osteo-anabolic gene-therapy in

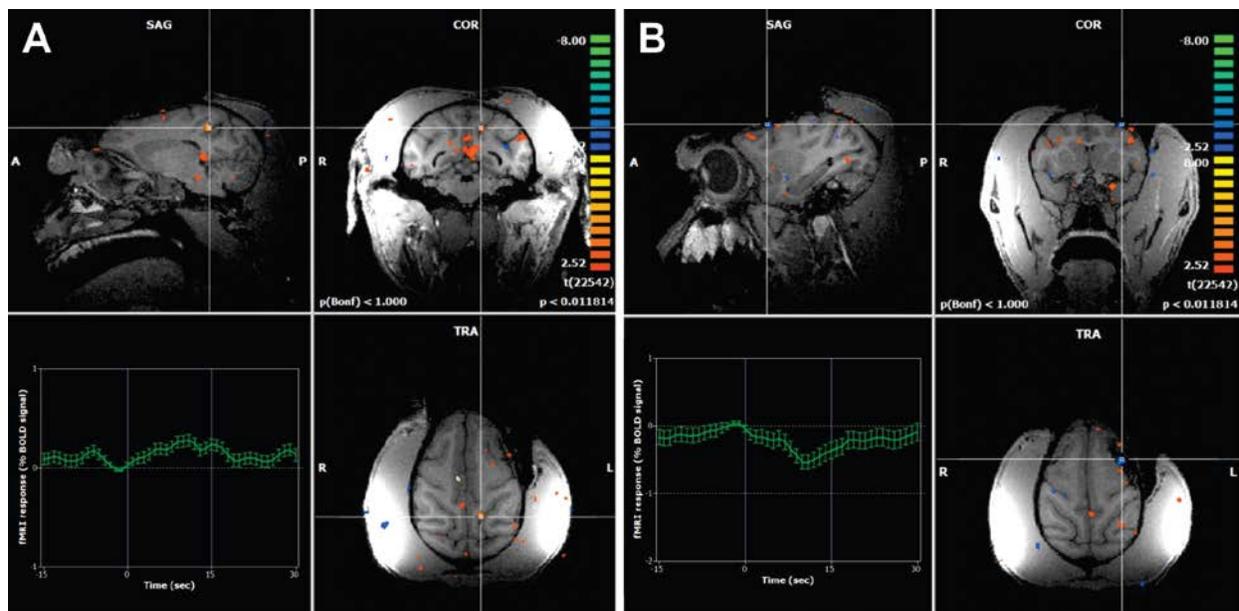


Figure 11 Optogenetic light-driven activation of area PMd (C1V1) combined with functional magnetic resonance imaging (opto-fMRI) in a sedated animal. The panels show examples of significantly up- (left) and down-regulated (right) regions, respectively.

collaboration with the University Medical Center Göttingen (UMG) has recently been awarded (Elsbeth Bonhoff Foundation grant No. 198).

For the Gail, Scherberger and Treue labs, the newly established optogenetic approach at the DPZ turned into an exciting and most useful tool, complementing correlative measures with a highly specific and precise inference measure. It will play a significant role in several future fundamental research projects on the role of cortical networks in cognitive processing. For example, each of the three labs included neuro-optogenetic approaches into their grant proposals submitted in 2018 in the context of the Sonderforschungsbereich SFB-889. This grant proposal was recently approved, allowing the three labs to follow-up on the projects initiated with the help of the Neuro-Optogenetics grant.

To give an impression about such follow-up projects, we highlight an example with a 3-year postdoctoral fellowship which was awarded by the Swiss National Science Foundation (grants P2FRP3_168460, P400PB_180818 / 1) to Dr. Anne-Dominique Gindrat (July 2017-June 2020). The project is conducted in the Scherberger lab, which has an ongoing interest in the fronto-parietal hand grasping network (ventral premotor area F5, primary motor cortex M1, anterior intraparietal area AIP). The project builds on the optogenetic approach developed in the Neuro-Optogenetic project to investigate this network in NHPs. The goal is to establish causal links between activity within the grasping network and specific hand grasping behaviors, and distinguishing the specific functional contributions of those areas in planning and executing grasping movements. The project will combine optogenetics, intracortical electrophysiological recordings with microelectrodes, and tracking of hand kinematics using a data glove in rhesus monkeys performing a visually-instructed delayed grasping task. The excitatory, red-shifted opsin C1V1 will be injected in area F5, and neuronal recordings will be conducted simultaneously with hand tracking while monkeys perform the grasping task with and without optogenetic stimulation. The effects of optogenetically activating area F5 will be examined with respect to the local F5 neuronal activity, the neuronal activity in remote, but directly connected areas M1 and AIP, and hand grasping performance. Pathway-specific optogenetic stimulation of the F5-to-M1 and F5-to-AIP projections will be applied. Test sessions with combined electrophysiological and hand kinematics recordings have been performed already in one behaving animal, while the second animal is still in training. We expect optogenetically-induced modulations of neuronal activity, and optogenetic stimulation in F5 may also subtly modify hand kinematics.

This follow up study, as an example, will very likely provide significant new insights on specific functional contributions of the hand grasping network in NHPs and their causal interconnections, which might ultimately contribute to the control of external devices, e.g., neuroprostheses.

4. Commercial applicability and transfer of knowledge

Related to the design of more specific gene-delivery systems targeting particular tissues, including, but not limited to the central nervous system, we continued the development of retargeting of Polyoma JC virus derived VLPs and related intact viruses through exterior attachment of targeting molecules. This was in part performed in an industrial collaboration with the Evotec AG and resulted in a joint patent application for a novel retargeting technology that

enables VLPs or intact viruses to reach cells and tissues with high specificity. This patent application was judged positively in all claims with respect to novelty, inventive steps and industrial applicability by the international search authority in August, 2018 (for details see https://worldwide.espacenet.com/publicationDetails/biblio?FT=D&date=20180809&DB=EPODOC&locale=en_EP&CC=WO&NR=2018141849A1&KC=A1&ND=6#).

Gruber J , Schneider S, Eckermann-Felkl E et al., 2018 (pending) Retargeting of Viruses or VLPs, PCT-Application PCT/EP2018/052523

5. Contributions of national and international collaboration partners

In the early phase of the project, we exchanged very useful practical information about viral application with Prof. Julio Martinez-Trujillo, Western University, Ontario, Canada, including a lab visit.

Continuously throughout the project, Prof. Tobias Moser, University Medical Center Göttingen, was involved in our discussions. He set up optogenetic stimulation in Marmoset monkeys in parallel, while being very experienced already with many years of optogenetic work in rodents. This collaboration also led to fruitful and practically relevant knowledge transfer.

Most intensively, our project benefits from a very fruitful collaboration with the laboratory of Prof. Jochen Staiger, also at the University Göttingen. Staiger, being an international renowned expert in neuroanatomy, provided access to his lab for our team to conduct important immunohistochemical staining and advanced microscopic imaging. Staiger will be co-authoring several of the planned manuscripts of this project. The collaboration with the Staiger lab was established in the context of this project but will serve as a very important partnership also for parallel projects. For example, the Gail, Treue, and Scherberger labs each collaborate with the Staiger lab in the context of the recently renewed DFG Sonderforschungsbereich SFB-889.

6. Student training and qualifying theses

Within the project, four PhD students (2 female, 2 male) were trained. Most of them finished their qualification within the runtime of this project. All students were/are enrolled in structured PhD programs at the Göttingen Campus, including thesis committees consisting of at least three faculty members, mandating regular meetings and reports, as well as a credit system for additional methods and skill training and career development measures.

PhD student Lara Schiller was focusing on AAV viruses and alternative vectors. Her thesis is entitled “Engineered delivery tools for gene therapy and optogenetics“ and was successfully finished early in 2018.

PhD student Rafael Rinaldi was responsible for improving target specificity of lentiviral vectors. The thesis entitled “Engineered genetic tools for directed gene regulation“ is scheduled for March, 2019.

PhD student Janina Hüer implemented the neurophysiological experiments of section 3.1.3. Her thesis “Top-down attention: neural pathways in the human and non-human primate examined by electrophysiology, optogenetics and psychophysics” was successfully defended in early in 2018.

PhD student Hao Guo focused on the development of multivariate neural state space approaches for quantifying the effects of optogenetic inactivation on neural dynamics in remote brain areas targeted by projections of the transfected neurons in rhesus monkeys (see 3.1.4). His thesis is scheduled for April, 2019.

7. Publications

The collaborative research project was started *de novo*, i.e., it was not building on an existing collaboration, and had to establish new techniques first. Preparation times for electrophysiological experiments in highly developed animals like NHPs, especially when requiring them to conduct complex cognitive tasks, take in the range of years. Hence, significant parts of the research that evolved in the three neurophysiology labs from this collaboration are not yet published as journal articles, but have been already successfully disseminated via conference contributions. Progress on viral vector development instead has been published in several articles.

7.1. Peer-reviewed journal articles (related to project)

Schiller LT, Lemus-Diaz N, Böker KO, Gruber J (2018) Enhancing exo-AAV production by CD9 over-expression. *Molecular Therapy – Methods and Clinical Development* 9:278-287. doi: 10.1016/j.omtm.2018.03.008

Hoffmann DB*, Gruber J*, Böker KO, Deppe D, Sehmisch S, Schilling AF, Lemus Diaz N, Komrakova M, Schneider S (2018) Effects of RANKL-Knockdown by Virus-like Particle mediated RNA-interference in a rat model of osteoporosis. *Molecular Therapy - Nucleic Acids* 12:443-452. doi: 10.1016/j.omtn.2018.06.001

Böker KO, Lemus-Diaz N, Rinaldi Ferreira R, Schiller LT, Schneider S, Gruber J (2017) The impact of the tetraspanin CD9 on exosome secretion and lentivirus infectivity. *Molecular Therapy* 26:634-647. doi: 10.1016/j.ymthe.2017.11.008

Hoffmann DB, Böker KO, Schneider S, Eckermann-Felkl E, Schuder A, Komrakova M, Sehmisch S, Gruber J (2016) In Vivo siRNA Delivery Using JC Virus-like Particles Decreases the Expression of RANKL in Rats. *Molecular Therapy – Nucleic Acids* 5: e298. doi: 10.1038/mtna.2016.15

7.2. Conference abstracts (related to project)

Hüer J, Saxena P, Fortuna MG, Guo H, Schiller LT, Gail A, Gruber A, Gruber-Dujardin E, Scherberger H, Staiger J, Treue S (2019) Pathway-specific optogenetic inhibition reveals that prefrontal area FEF has a direct influence on the attentional modulation of firing rates in visual area MT in the non-human primate, 13th Meeting of the German Neuroscience Society, Goettingen, 2019.

Fortuna M, Huer J, Guo H, Schiller LT, Treue S, Gruber J, Scherberger H, Staiger J, Gail A (2018) Optogenetic approaches to study frontoparietal networks in non-human primates. Society for Neuroscience Meeting. San Diego, USA

Hüer J, Fortuna MG, Guo H, Schiller LT, Gail A, Gruber J, Scherberger H, Staiger J, Treue S, (2018) Optogenetic inhibition reveals a causal role of the direct anatomical connection from the FEF to extrastriate visual area MT in mediating attentional modulation in non-human primates, Society for Neuroscience Annual Meeting, San Diego, USA

Gindrat ADG, Scherberger H, (2018) Causal investigation of the primate hand grasping network with pathway-specific neuro-optogenetics. Tübingen, Germany

Schneider S, Lemus-Diaz N, Schiller LT, Rinaldi FR, Boeker KO, Hoffmann DB, Gruber J (2018) Improving viral and non-viral vectors: adopting exosome biology for efficiency and hydrogels for controlled release. 26th Conference European Society for Gene and Cell Therapy, Lausanne, Switzerland. Published in Human Gene Therapy: doi.org/10.1089/hum.2018.29077.abstracts

Rinaldi FR, Lemus-Diaz N, Böker K, Schiller LT, Schneider S, Gruber J (2018) Improved viral vectors for transgene delivery. 11th FENS Forum of Neuroscience, Berlin, Germany.

Fortuna MG, Hüer J, Guo H, Schiller LT, Staiger J, Gruber J, Scherberger H, Treue S, Gail A (2017) Optogenetic tools to study frontoparietal networks in non-human primates – a histological analysis. 12th Meeting of the German Neuroscience Society. Göttingen, Germany

Hüer J, Fortuna MG, Guo H, Schiller LT, Gail A, Gruber J, Scherberger H, Staiger J, Treue S, (2017) Optogenetic inhibition of projections to area MT reveal a causal role of the FEF in the attentional modulation of visual cortex in non-human primates, Society for Neuroscience Annual Meeting, Washington, D.C., USA

Fortuna MG, Hüer J, Guo H, Schiller LT, Staiger J, Gruber J, Scherberger H, Treue S, Gail A (2017) Optogenetic tools to study frontoparietal networks in non-human primates – a histological analysis. Primate Neurobiology Meeting. Göttingen, Germany

Schneider S, Schiller LT, Lemus-Diaz N, Salguero GA, Gruber J (2017) Retargeted JCV derived virus-like particles as an efficient tool for directed delivery (P374). 25th Conference European Society for Gene and Cell Therapy, Berlin, Germany. Published in Human Gene Therapy: doi.org/10.1089/hum.2017.29055.abstracts

Gindrat ADG, Scherberger H, (2017) Probing the primate hand grasping network with neuro-optogenetics. Primate Neurobiology Meeting. Göttingen, Germany

8. Data management

We are committed to open, transparent and reproducible science, maintaining good scientific practice regarding acquisition, documentation, storage, archiving and publication of research data. Our data management conforms to the guidelines from the University of Göttingen and adheres to the FAIR principles (Findable, Accessible, Interoperable and Re-usable). We strive for maximal transparency and contribute to the Open Science movement.

Parts of the work on gene-transfer vectors appeared in open access journals, i.e. a study reporting improved methodologies for increased production rates of exosome-associated AAVs and two manuscripts on the *in vivo* utilization of virus-like particles in gene therapy settings (numbers 1, 3 and 4). The required biologics, i.e. plasmids encoding components of recombinant lentiviral vectors for exosome related viral vectors were deposited at the public service addgene (www.addgene.org) and are accessible free of charge (papers 1 and 2). The two studies on VLPs are closely related to patent applications and therefore the biologics are not public (number 3 and 4).

1. Enhancing exo-AAV production by CD9 over-expression. *Molecular Therapy – Methods and Clinical Development* 9:278-287. doi: 10.1016/j.omtm.2018.03.008
2. The impact of the tetraspanin CD9 on exosome secretion and lentivirus infectivity. *Molecular Therapy* 26:634-647. doi: 10.1016/j.ymthe.2017.11.008
3. Effects of RANKL-Knockdown by Virus-like Particle mediated RNA-interference in a rat model of osteoporosis. *Molecular Therapy - Nucleic Acids* 12:443-452. doi: 10.1016/j.omtn.2018.06.001
4. In Vivo siRNA Delivery Using JC Virus-like Particles Decreases the Expression of RANKL in Rats. *Molecular Therapy – Nucleic Acids* 5: e298. doi: 10.1038/mtna.2016.15

Further publications from the project will be made publicly available, either by publishing in Open Access journals or by releasing pre-print versions, unless considerations of intellectual property preclude this.

9. Press releases and media coverage

The development of the 4th generation lentiviruses from the Gruber lab was covered in a press release: “Gene taxi with turbo drive: Scientists at the German Primate Center improve DNA transfer in gene therapy” (<https://www.dpz.eu/en/home/single-view/news/gen-taxi-mit-turboantrieb-1.html>)