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## **Optogenetics: a new method for the causal analysis of neuronal networks in vivo**

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### **Abstract**

The causal analysis of neuronal network function requires selective manipulations of genetically defined neuronal subpopulations in the intact living brain. Here, we highlight the method of optogenetics, which meets those needs. We cover methodological aspects, limitations, and practical applications in the field of neurosciences. The fundamentals of optogenetics are light-sensitive transmembrane channels and light-driven ion pumps, which can be genetically encoded, without requiring the application of exogenous cofactors. These opsins are expressed in neurons by means of viral gene transfer and cell-specific promoters. Light for stimulation can be non- or minimally invasively delivered by optical fibers. Illumination of opsins results in a depolarization or hyperpolarization of genetically modified neurons, depending on the type of optogenetic actuator. Strong expression levels and sufficient light densities provided, neuronal activity can be optically controlled in the intact network with millisecond precision. By applying fluorescent indicators of neuronal activity, an all-optical neurophysiological approach becomes reality.

## Introduction and scientific goal

The establishment of genetically encoded markers of various neuronal cell populations and the concomitant rapid development of novel imaging modalities in the last two decades revealed the unparalleled functional and morphological complexity of neuronal networks. This complexity arises from the heterogeneity of neuronal cell populations. In the last couple of years a considerable number of genetically and functionally distinguishable types of interneurons have been described. Up to now, no method to specifically stimulate those diverse network components had been available. Electrical stimulation of neurons leads to an unspecific excitation. In methods such as *uncaging* which combine pharmacological and optical strategies require the continuous application of substrates, rendering in vivo applications unfeasible. Already in 1979 Francis Crick called for novel methods to stimulate neurons, preferably light mediated manipulations of neuronal activity. Around this time the so called bacteriorhodopsins were described in prokaryotes. These bacteriorhodopsins are light sensitive proton pumps which convert light into chemical energy by creating a proton gradient. Over the years a wide range of light sensitive membrane proteins has been found. In 2003 the group of Ernst Bamberg characterised the light sensitive cation channel Channelrhodopsin-2 (ChR2) from the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2003). ChR2 comprises of an opsin with seven transmembrane helices forming a central pore and a covalently bound chromophore retinal. Upon absorption of a photon, the retinal undergoes a trans-cis-isomerisation to open the channel pore which allows cation influx according to the concentration gradient. This is in contrast to rhodopsin, the photoreceptor molecule in the vertebrate retina, a G-protein coupled receptor (GPCR). In case of GPCRs the conformational change activates a G-protein inducing a signal transduction cascade. Therefore channelrhodopsins are opsins of class 1 whereas GPCRs are class 2 opsins. In 2005 Karl Deisseroth's group used viral gene transfer to achieve ChR2 expression in mouse post mitotic neurons (Boyden et al., 2005). Critical for the success of this method in neuroscience was the fact that at least in the mammalian brain no exogenous application of Retinal is required as endogenous concentrations proved sufficient for the function of ChR2. This is an advantage over optochemical approaches reliant on exogenously added photosensitive ligands. In contrast, ChR2 is an entirely genetically encoded actuator, for the first time combining strategies of genetic expression with optical stimulation into a single-component-system, thereby justifying the term "optogenetics". Blue light illumination of ChR2-expressing neurons induces a cation (mainly Na<sup>+</sup>) influx which then causes a depolarisation of the neuronal cell membrane. If sufficiently depolarised the neuron may generate an optically induced action potential. Besides ChR2 many more depolarising cation channels with different spectra of excitation wavelengths and kinetics have been characterized over the last years. Light mediated inhibition of neuronal activity needs active ion transport carried out by ion pumps such as the light driven proton pump halorhodopsin from *Natronomonas pharaonis* (NpHR) (Gradinaru et al., 2010) and the chloride pump archaerhodopsin-3 (Arch, ArchT) from *Halobacterium sodomense* (Chow et al., 2010). Here we present the main working steps of an optogenetic experimental approach in the mammalian brain as well as some selected applications in rodents and primates. It is not our primary goal to present a complete overview of different expression strategies and optogenetic actuators. The main focus lies on a more comprehensive description of basic capabilities and limitations of this new method.

### **From correlative to causal analysis of neuronal network function**

Unlike the correlative descriptive analysis, a causal approach requires a direct and specific manipulation of neuronal network components, from single cell to the population level. Ideally the grade of specificity of manipulation tools should match the level of descriptive analysis. Differential gene expression is the key to morphological and functional diversity of neuronal networks. It represents the basis of descriptive analyses involving the use of fluorescent antibodies or transgenic strategies for differential, promoter-driven, expression of fluorescent proteins. Optogenetics provides specificity of manipulation by transferring the gene for the optogenetic actuator combined with a cell type specific promoter. It must be kept in mind, however, that a massive expression of exogenous membrane proteins may lead to a significant alteration of the structure and the dynamics of the neuronal cell membrane. Extensive control trials are therefore mandatory when choosing an optogenetic approach. Yet, optical stimulation is effective over relatively long distances. One can stimulate for example the pyramidal cells of the cortical layer V in mice by an optical fiber not penetrating the pia, over a distance of 600  $\mu\text{m}$ . Lensed optical fibers and holographic microscopic methods make it possible to define the stimulated region ranging from less than a micrometer to several millimetres. There are two main approaches to achieve opsin expression in neurons: viral gene transfer or transgenic methods. With viral gene transfer the spatial extent of the transduced area depends on the virus titer and the injected volume, whereas the transgenic method typically yields a brain-wide expression. This distribution significantly influences the specificity of the optogenetic stimulation. The most widely used transgenic mouse is the Thy1-ChR2 mouse line (line 18). In contrast to a local injection a whole network in these mice can be stimulated by single local stimulation.

Certainly, electrophysiological single cell recording remains so far unrivalled in terms of temporal precision and immediate manipulation of the membrane potential. Electrical stimulation entails however the crucial disadvantage of poor specificity. Although by accurately choosing the parameters the stimulation effects can be confined to selected neuronal elements. It has been shown that mostly axons are recruited during electrical stimulation with common parameters. Using optogenetics a considerably higher specificity of manipulation is possible, since the stimulation is limited strictly to expressing and illuminated neuronal elements (Fig.1). Another inevitable problem is the electrical artefact generated when using electrical stimulation. We can bypass this phenomenon by combining optogenetic stimulation with electrophysiological recording. Even so a photoelectrical artefact, the so called Becquerel effect, can occur. It is the consequence of photons hitting only a part of the recording metal of the electrodes. This creates a current between the non-uniform illuminated contacts leading to a voltage change. This problem can be avoided by fine-tuning the angle of light incidence relative to the electrode, or by using optical detection of neuronal activity (“all optical physiology”).

### **Optogenetic actuators**

All opsins have a common feature: they lack an endogenous fluorophore. Therefore, to determine the expression efficiency and the cellular localisation, opsins are fused with fluorophores such as GFP, YFP or mCherry, which are also genetically encoded. The activation threshold of ChR2 corresponds to a light density of  $1 \text{ mW}/\text{mm}^2$ . Normally this cannot be attained in a simple imaging experiment so that the illumination of the ChR2-YFP protein with 488 nm for the purpose of localisation is unlikely activating ChR2.

Genetically encoded optogenetic actuators can be subdivided into two classes, first those that are light sensitive depolarising ion channels, and secondly the light driven hyperpolarising ion pumps (table 1). Notably, the light driving the channels serves merely for the conformational change of the chromophore retinal. The opening kinetics are mainly determined by the protein structure of the channel. This should be considered when designing optogenetic stimulation parameters and choosing suitable actuators. For instance, stimulation pulses of 1-3 ms duration can already effectively activate the most widely used mutant ChR2(H134R). However this channel closes with an exponential time constant of 16 ms. Thus after this period still 37% of the channels are in the open confirmation confining the maximal effective stimulation frequency to 40 Hz in this mutant. In the author's view, it exhibits among all fast opsins the best compromise in terms of high expression level, low cytotoxicity and speed. Besides those fast channels, new variants have been generated by point mutation, resulting in time constants ranging from seconds to minutes: the so called step function opsins (SFO). These can for example be used to modulate the excitability of a neuronal network via subthreshold tonic depolarisation.

Hyperpolarising inhibitory ion pumps are able to transport ions against their concentration gradient during continuous absorption of light, requiring constant light illumination. Currently the chloride pump Halorhodopsin (eNpHR3.0) as well as the proton pump Archaeorhodopsin-3 (Arch, ArchT) are the most common hyperpolarising opsins. It is still a matter of debate, whether cellular physiology is disturbed more severely by a shift in the chloride equilibrium potential (NpHR) or by a change of local pH (Arch/ArchT).

### **Methods of cell specific expression of optogenetic actuators**

In the course of developing somatic gene therapy a multitude of viral vectors have been designed which are able to transfer exogenous DNA into postmitotic neurons.

The third generation adeno associated viruses (AAVs) have proven to be reliable and secure viral vector in rodents and primates. Most commonly serotype 2 is utilized. It can be pseudotyped with various other serotypes, changing the tropism of the virus. An example of nomenclature in case of AAV serotype 2, pseudotyped with serotype 5 is AAV2/5 or AAV5. In primates and rodents AAV2, AAV2/1 and AAV2/5 have been already tested. Yet, lentiviruses have been successfully used, too. However, on average they yield a lower expression and diffusion rate, probably due to the significantly larger diameter of the particle (100 nm instead of 20 nm in AAV) and their lower titers. Additionally, the lentiviruses exhibit a high immunogenic potential.

One of the most crucial prerequisites of a successful optogenetic experiment is a strong opsin expression level. Opsins need to be expressed in high numbers to achieve an effective *in vivo* stimulation/inhibition. On one hand, this depends on the tropism and the titre of the virus. On the other hand the strength of the promoter can be an important limitation. There is a great number of cell specific promoters, especially in case of interneurons (GAD67, 5HT3) which are convenient for descriptive analyses, applying fluorophore expression. However this is far from sufficient for a functionally effective optogenetic stimulation.

A limiting factor for the use of AAVs in optogenetics is the limited capacity of the vector with 4.7 kb at the maximum. Unfortunately this is not sufficient for many cell specific promoters. The following promoters, however, can be inserted upstream of the opsin gene yielding a specific and effective expression: CamKII (excitatory neurons), hSyn (all neurons), hThy-1 (human and murine version of the promoter, all neurons, lower expression than hSyn), Ef1a (all cells including microglia), CAG

(all cell including microglia, extremely high expression rate), SST (somatostatin-positive neurons), hypocretin (hypocretin-positive neurons).

The limitations imposed by the weakness of a promoter can be circumvented by using the Cre/loxP system (Abb. 2&3). Here, the fusion protein opsin/fluorophor and a strong ubiquitous promoter (EF1 $\alpha$ ) are inverted in a viral plasmid and the construct is double floxed with two loxP sequences. In a second viral plasmid the enzyme Cre recombinase is under the control of a cell specific promoter (e.g. GAD67, PV). Co-injecting both viruses results in a recombination in the target cell as even the limited strength of the cell specific promoter provides sufficient Cre-levels. After recombination, the opsin is expressed under the control of the strong ubiquitous promoter (EF1 $\alpha$ ) avoiding low expression by the cell specific promoter. Additionally to co-injecting two viral vectors, the floxed optogenetic vector can be introduced in one of the fast growing numbers of available transgenic Cre-mouse lines - an option which is widely used in the community.

### **Methods of optical Stimulation**

The next step in an optogenetic experimental approach is to choose a suitable light source. Depending on the opsin a local light density of 1-10 mW/mm<sup>2</sup> must be reached. Higher values lead to increased photon currents. However, all opsins within the illuminated area usually are already activated by 50 mW/mm<sup>2</sup>. Normally these high light densities can be obtained by coupling 50-100 mW lasers, either directly in an optic fiber or in a microscope system. When lasers are coupled in a glass fiber, the size of the illuminated area can be freely defined up to a diameter of 500  $\mu$ m. Lens based optic fibers allow illuminating an area of only a few micrometers. Optical fibers can be applied acutely or chronically (e.g. implanting a tip of an optic fiber, which can be attached to a long fiber with a connector). Whereas acutely inserted fibers also allow application of pharmacological manipulations at the same site, they break easily. By contrast, no pharmacological manipulations are possible with chronic implants (except for a cannula implanted directly next to the fiber). They are however far more robust and stay intact for weeks up to months. Laser diodes are a widely used light source and it is easy to couple them to optical fibers, yet up to now only laser diodes with wavelengths below 500 nm are commercially available. Light emitting diodes (LEDs) can be applied directly to the brain tissue, and many different wavelengths are available. It is important to note, however, that the produced heat must be dissipated to prevent tissue damage. LEDs can be coupled directly to a fiber with the disadvantage of significantly decreasing their light output.

For future applications it is worthwhile to employ small, completely implantable LEDs. Prototypes already exist but they have not been tested *in vivo* yet.

### **Applications in Neuroscience**

Taking advantage of the cell specificity of the optogenetic approach as described above, our understanding of the complexity of neuronal networks has considerably grown over the last years. This includes the causal analyses of circuit disorders like autism and schizophrenia (Yizhar et al., 2011), the demonstration of the mechanism of neurovascular coupling in fMRI (Lee et al., 2010), and the improved differentiation of stem cells into neurons (Stroh et al., 2011). Here we present three examples from this great variety of studies:

### **Combining optogenetic stimulation with optical methods for the detection of neuronal activity**

In addition to the optogenetic approach for functional manipulation, methods for optical detection of neuronal activity have been developed during the last years. For this purpose fluorescent indicators have been employed, changing fluorescence emission depending on the intracellular calcium levels. A neuronal action potential leads to a defined increase of the somatic intracellular calcium level, mainly due to the opening of voltage gated calcium channels in the neuronal cell membrane. Hence an increased emission of fluorescence by the calcium indicator can be regarded as the optical correlate of suprathreshold neuronal activity. In the presented study, ChR2 was expressed in the mouse visual cortex by means of viral gene transfer. Two weeks after virus injection the fluorescent calcium indicator Oregon-Green BAPTA1 (OGB1) was injected into the visual cortex. Via an optic fiber of 200  $\mu\text{m}$  diameter, blue light was applied to activate both the indicator and the opsin. As mentioned above, the activation of ChR2 requires a minimal effective light density of  $1\text{mW}/\text{mm}^2$ . To excite OGB1 a light density of  $0.01\text{ mW}/\text{mm}^2$  already suffices which is clearly below the ChR2 threshold. The intensity of the emitted light is detected by the optic fiber based detection setup, containing a photodiode allowing for a high temporal resolution in the kHz range. Thereby it is possible to measure the sum of neuronal activity of the neuronal population within the illuminated area (Grienberger et al., 2011). As a first step, spontaneous slow calcium waves have been detected in the visual cortex of anesthetized mice. This population activity is related to slow oscillations at a frequency of  $< 1\text{ Hz}$ , playing a mayor role in memory formation and control of fast oscillations. The origin of these oscillations had yet to be determined in a causal approach. Genetically defined pyramidal cells in the cortical layer 5 were activated by applying light pulses of high intensity, resulting in a global calcium wave. This provides causal evidence that a local cortical neuronal population is capable of inducing a global calcium wave, indistinguishable from spontaneous waves (Figure 4).

### **Optogenetics in freely moving animals, behavior**

Behavioural experiments combined with optogenetic manipulations offer key advantages concerning the temporal structure of an experiment. In contrast to the extended washout times of pharmacological reagents, the effects of light are immediately reversible which can reduce the duration of behavioural tests to only one day compared to the previously required several days. Testing the behaviour in an elevated plus maze combined with optogenetics allows for within-subject comparisons between conditions (light on/off) during a single experimental session (Tye et al., 2011).

Apart from these benefits of optogenetic stimulation there are several important aspects that need to be considered when applying this novel tool. An important limiting factor is the heat development due to the light exposure. High light intensities during constant or almost constant illumination can induce heat dependent alterations in the illuminated cells. The heat can alter the cell activity or lead to cell damage. Therefore every test group should include a number of control animals. Most suitable are YFP-controls, i.e. animals that are treated exactly like the test animals except for the injected virus which contains only the fluorophore gene and lacks the opsin gene. A further limiting factor in optogenetics is the potential toxicity of very high expression levels of the opsin and the fluorophore due to the use of strong promoters or very long expression times. If the expression exceeds certain levels, other important endogenous channels are at risk to be displaced from the cell membrane. In order to rule out this effect, intracellular recordings from brain slices performed under identical

conditions are recommended (i.e. same opsin, cell type, viral vector, amount of virus, virus titer, expression time, and light intensity).

### **Optogenetics in primates**

Non-human primates are the prime model of fine motor control and higher cognitive functions. It is therefore desirable to adapt the optogenetic techniques to this model. First attempts to establish them in rhesus monkeys have shown that the principles of optogenetic manipulation of neurons are also functional in primates and that the same vectors, opsins and promoters can be used (Diester et al., 2011).

Injecting AAVs into motor- and premotor cortex reliably results in a strong opsin expression in up to 80% of neurons. The hSyn promoter has proven to be particularly effective. Subsequent optical stimulation with blue light activates the ChR2 expressing neurons with millisecond precision. On the other hand, halorhodopsin expressing neurons can be completely turned off, i.e. spontaneous action potentials are suppressed. The step function opsin (SFO), a particularly light sensitive opsin with slow kinetics, can induce strong response frequencies. This increase in action potential generation can be immediately reversed by a yellow light pulse returning the opsin into its dark state.

Whereas the modulation of neuronal activity did not pose any problems, influencing behaviour in primates turned out to be very challenging. Directly adjacent to spots in motor cortex at which hand twitches could be evoked by electrical stimulation, optogenetic stimulation did not evoke movements. It remains to be determined whether this is only due to the bigger brain volume of primates as compared to rodents or whether more complex neuronal circuits and cognitive strategies might play a role. Nevertheless, by now a number of different studies have shown that optogenetic manipulations can influence the behaviour in non-human primates, too. For instance, the reaction times from an instructive cue to a reach are significantly increased by perturbing neural activity in premotor cortex. Furthermore stimulation of the frontal eye field (FEF) or of the primary visual cortex is able to influence the direction of eye movements. Common to all these successful applications is that they rather influence behaviour than causing it.

### **Preclinical applications**

We provide two examples of applications of optogenetic strategies which have a high likelihood of clinical implementation in the midterm. Probable targets include experimental therapies of retinitis pigmentosa and peripheral nervous system disfunctions.

Retinitis pigmentosa encompasses a diversity of hereditary diseases which lead to incurable blindness. Early degeneration of the highly sensitive rods is the common feature of all types of these degenerative diseases, whereas the more robust cones seem to persist longer. It has been demonstrated that expressing the opsin NpHR in the cones was able to restore the natural phototransduction cascade and therefore rescue to some extent the light sensitivity in the mouse model of retinitis pigmentosa. Moreover in *ex vivo* human retina the light sensitivity of photoreceptors was reactivated. A considerable number of patients may benefit from these therapeutic options (Busskamp et al., 2010).

Another possible clinical application is based on the stimulation of the peripheral nervous system. Electrical stimulation has been used for this purpose so far. However, it recruits particularly the large, fast fatiguing motor units if a weak electrical stimulation is applied. This reverse order of natural recruitment acts as a limiting factor for therapeutical application. In contrast, optogenetic

stimulation of peripheral nerve fibers has been shown to avoid this problem increasing the effectiveness of this stimulation.

It is still far from clear if optogenetic manipulations will find their way into clinics. Advancing strategies for safe somatic gene transfer is in our view mandatory for this step.

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**Conflict of interest.** On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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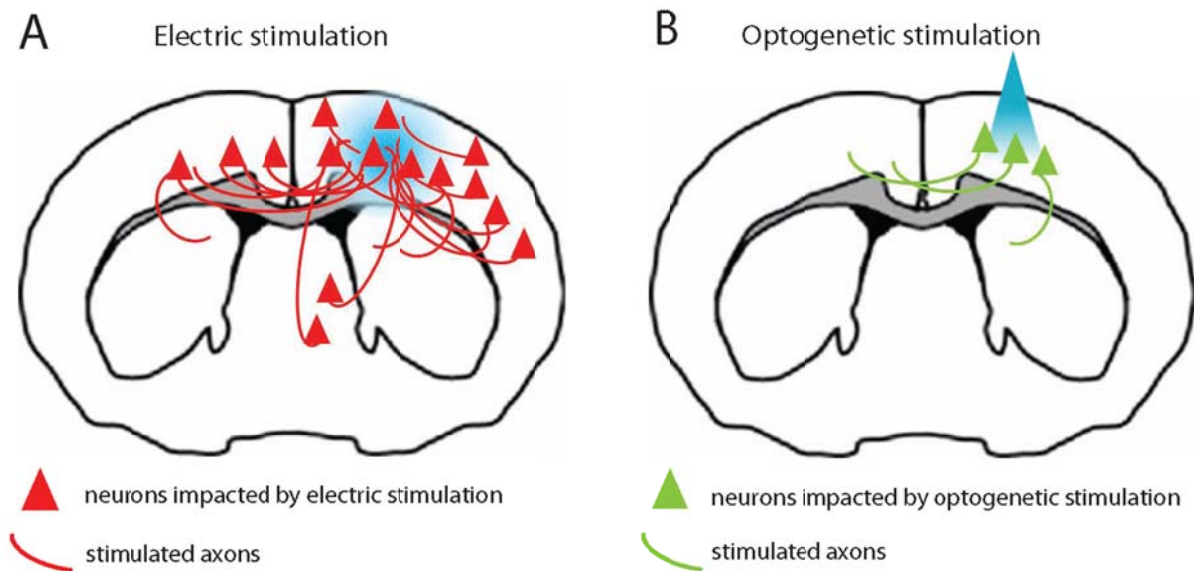


## Table and Figures

Opsin	Activation ( $\lambda$ )	Kinetics ( $\tau$ )	References
<b>Depolarizing, fast closing channels</b>			
ChR2(H134R)	470 nm	18 ms	Nagel et al. ( <i>PNAS</i> , 2003)
ChIEF	450 nm	10 ms	Lin et al. ( <i>Biophysical Journal</i> , 2009)
ChETA	470 nm	4 ms	Gunaydin et al. ( <i>Nat. Neurosci.</i> , 2010)
VChR1	545 nm	133 ms	Zhang et al. ( <i>Nat. Neurosci.</i> , 2008)
C1V1	540 nm	34 - 58 ms	Yizhar et al. ( <i>Nature</i> , 2011)
<b>Depolarizing, step function opsins (SFO)</b>			
ChR2-SFO	470 nm Akt. 590 nm Deakt.	2 s - 29 min	Berndt, et al. ( <i>Nat. Neurosci.</i> 2009)
<b>Hyperpolarizing, ion pumps</b>			
Arch/ArchT (Chlorid)	566 nm	9 ms	Chow et al ( <i>Nature</i> , 2010)
eNpHR3.0 (Protonen)	590 nm	4 ms	Gradinaru et al. ( <i>Cell</i> , 2010)

**Table 1 Characteristics of selected excitatory and inhibitory opsins. For details please see (Mattis et al., 2012).**

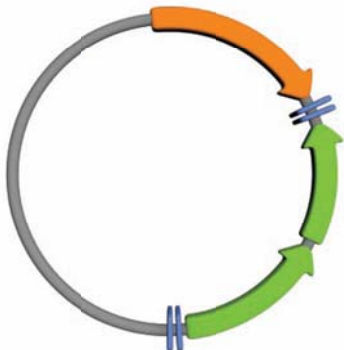
Neuroforum Stroh/Diester Optogenetik Abbildung 1



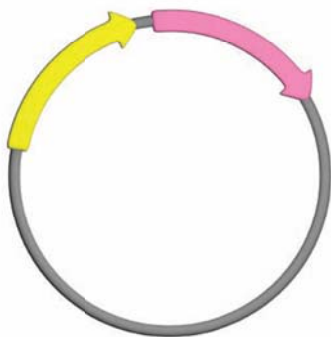
**Figure 1 Electrical and optogenetic activation patterns of neuronal networks.** (A) Electrical stimulation in cortex with conventional parameters (e.g. 100 $\mu$ A, 300 Hz, biphasic 150 $\mu$ s long pulses) activates a wide spread network, which is mainly based on the stimulation of afferent or passing by axons. (B) Optogenetic stimulation of a local opsin-expressing area leads to a locally limited activation of neurons, due to the limited spread of light. While axons usually can be stimulated optogenetically, non-expressing axons are not activated, because they originate from a non injected area. *Diester*

Neuroforum Stroh/Diester Optogenetik Abbildung 2

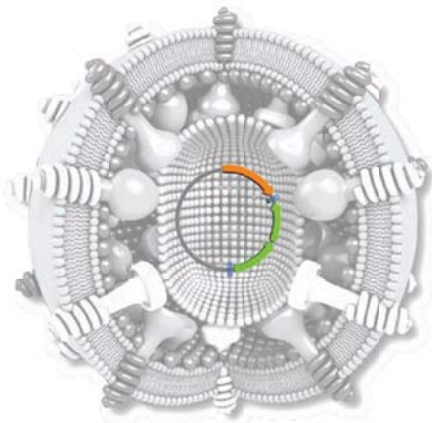
**A** EF1 $\alpha$ -floxed-ChR2-YFP Plasmid



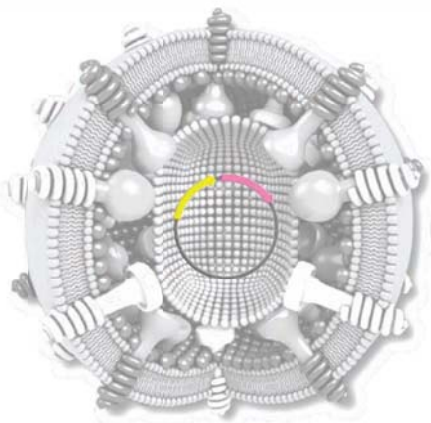
**B** PV-Cre Plasmid



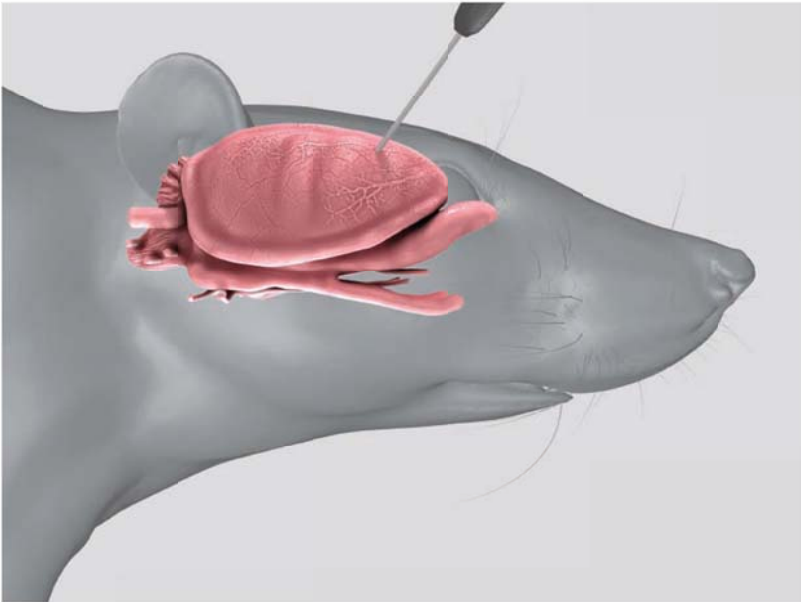
**C** EF1 $\alpha$ -floxed-ChR2-YFP Virus



**D** PV-Cre Virus



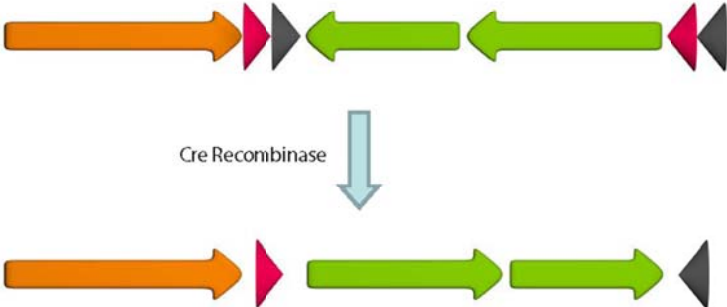
**E** Stereotactic injection of both AAVs in rodent brain



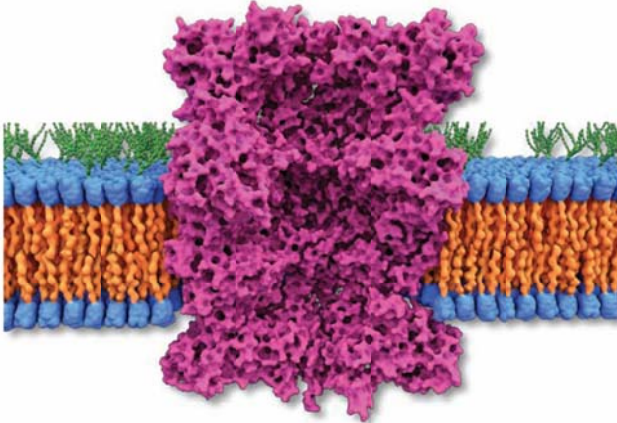
**Figure 2 Basic principles of an optogenetic approach (1).** (A) Cloning of a viral plasmid with sequences of a double-floxed inverted opsin and of the fluorophore (ChR2-YFP) (green), as well as the ubiquitous promoter (EF1 $\alpha$ ) (orange). (B) Sequence of the Cre recombinase (pink) and the cell type specific promoter Parvalbumin (PV) (yellow) in a second viral plasmid. (C, D) In helper cells and via additional viral plasmids two viruses are produced *in vitro*. (E) Stereotactic injection of both viruses (typically in a 1 (Cre): 4 (ChR2-YFP) ratio) into the rodent brain, with a volume of 0,1 – 0.5  $\mu$ l. Tobias Jung, Jena University.

Neuroforum Stroh/Diester Optogenetik Abbildung 3

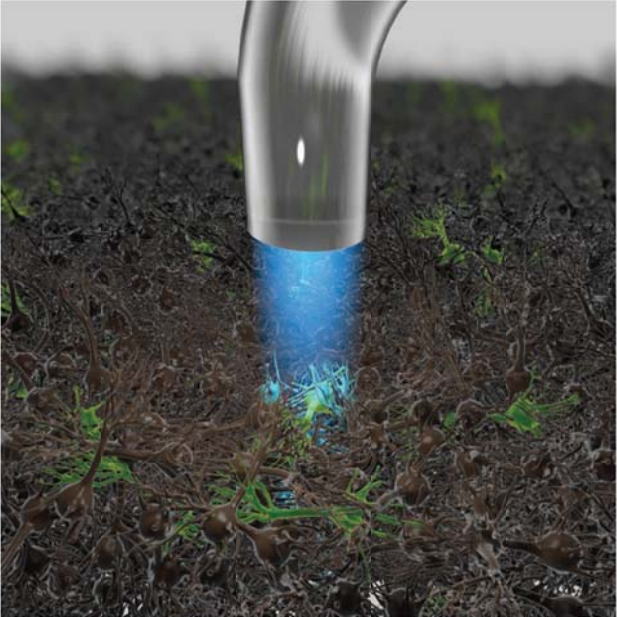
A Specific recombination in genetically defined cells



B Expression of ChR2-YFP, Integration into neuronal membrane

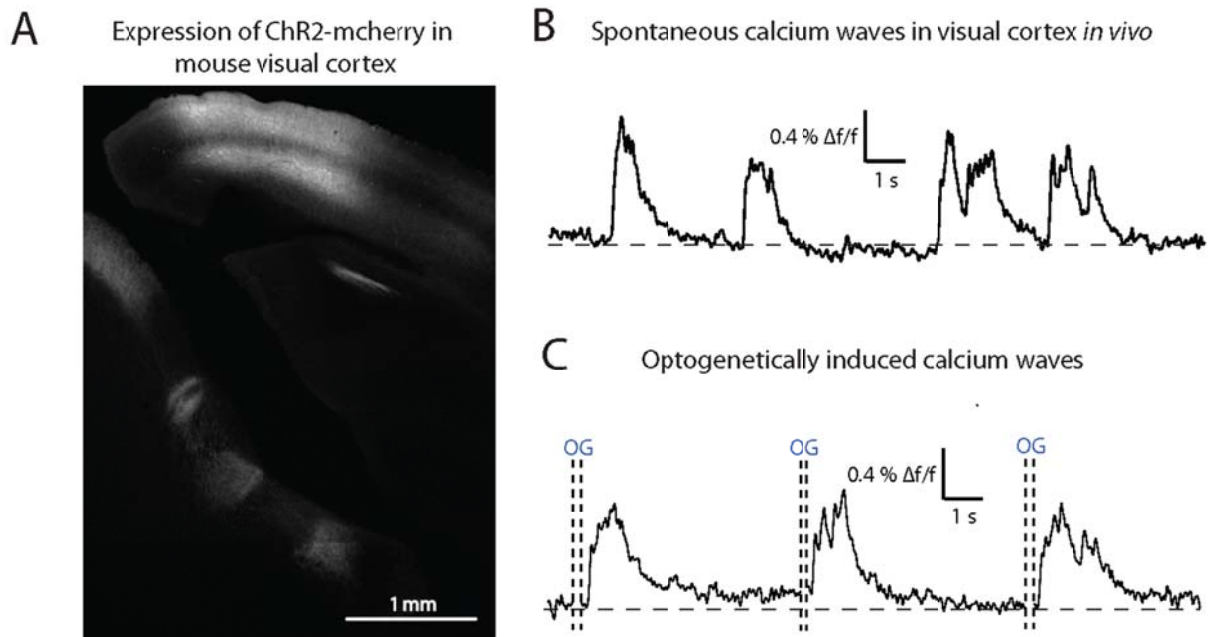


C Selective stimulation of PV interneurons by an optic fiber *in vivo*



**Figure 3 Basic principles of an optogenetic approach (2).** (A) Over the course of 5-10 days post injection only PV expressing interneurons infected with both viruses express Cre-recombinase, resulting in a recombination of the ChR2-YFP sequence. (B) Controlled by the strong EF1 $\alpha$  promoter the ChR2-YFP sequence is transcribed and translated. The ChR2-YFP fusion protein is integrated into the neuronal cell membrane. (C) After implantation of an optical fibre and with illumination of blue light only PV interneurons are selectively stimulated *in vivo*. Tobias Jung, Jena University Jena

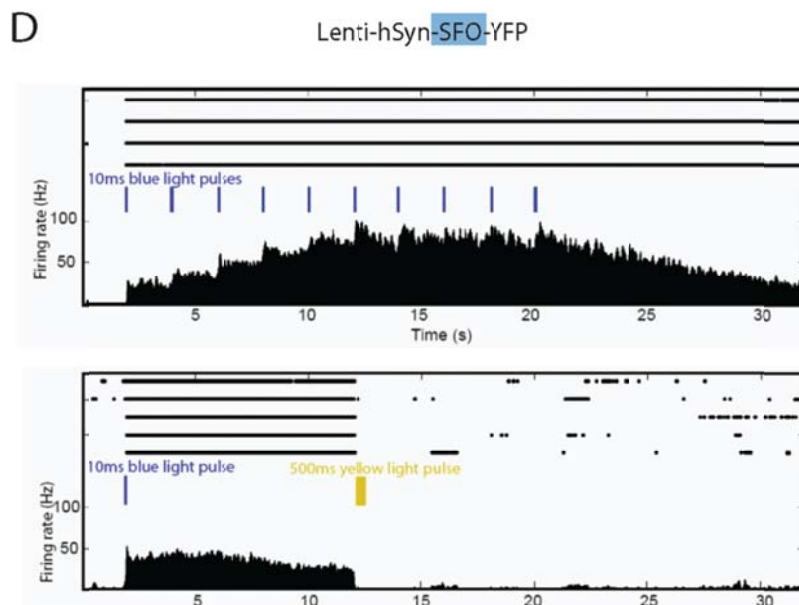
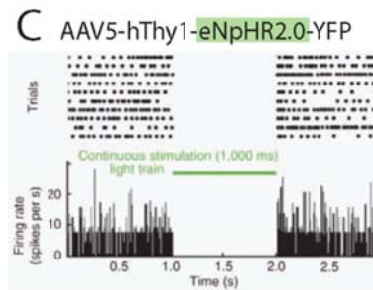
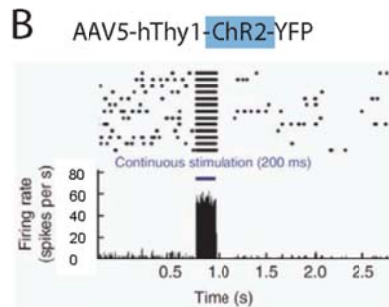
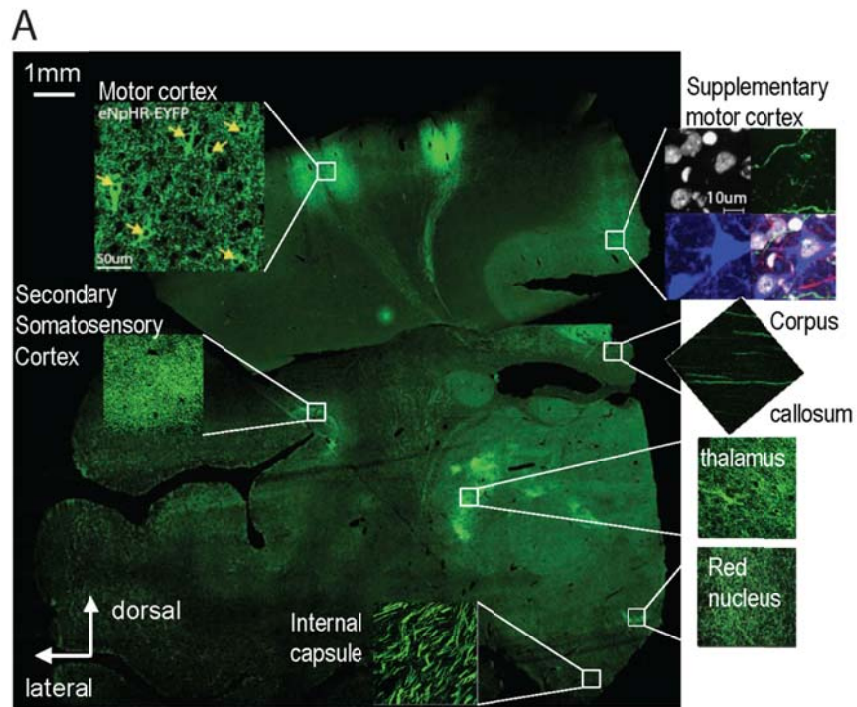
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**Figure 4 *In vivo* induction of calcium waves by optogenetic stimulation of cortical layer V in mice.** (A) Sagittal section visualized with a confocal fluorescence microscope. Stereotactic injection of ChR2-mCherry AAVs into the visual cortex of a mouse leads to a strong expression in layer V. Besides the somatic cell membranes, anterograde axonal projections into the visual thalamic nuclei, as well as apical dendrites are also visualized. (B) After local staining of the visual cortex *in vivo* with the calcium indicator Oregon Green an optic fiber is implanted. During illumination of the indicator with low intensity light and detection of the fluorescence emission via the optical fiber, spontaneous slow calcium waves can be observed in the anaesthetized mouse. (C) Stimulation of ChR2 expressing pyramidal cells of layer V with short high intensity light pulses induces slow calcium waves. The optogenetically induced waves cannot be distinguished from spontaneously occurring waves. This demonstrates the ability of defined local cortical cell populations to generate global calcium waves, in a causal approach. Stroh, Adelsberger, Ruehlmann, Konnerth.



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**Figure 5 Histological and electrophysiological results in optogenetically modified rhesus monkeys. (A)** Histological study. The motor cortex of a rhesus monkey was injected twice, leading to two local expression sites. Single opsin-expressing neurons are distinguishable in magnified images. Outgoing axons are also stained by the fluorescence protein EYFP. Besides the efferent axons arching down directly from motor cortex, other labelled axons end in the supplementary motor areal (SMA), the somatosensory cortex (S2), the red nucleus, the thalamus, the corpus callosum, containing axons connecting the hemispheres and the internal capsule, containing axons leading to the spinal cord. **(B-D)** Electrophysiological documentation of light evoked and light inhibited action potentials. **(B)** A blue light pulse generates action potentials in a ChR2 expressing area. Millisecond precise control is possible; a 200 ms light pulse is depicted. Typically shorter pulses of 1-2 ms are used. **(C)** Green light suppresses action potentials in a NpHR2.0 expressing cell. **(D)** The so called Step Function Opsin (SFO) is stimulated by a single short pulse of blue light (10ms). Repeated pulsing leads to an accumulation of the effect until a plateau of neuronal activity is reached. The effect of a single light pulse can last several seconds, in newer versions of the opsin up to 30 min. This effect can be stopped by a yellow light pulse. *Diester et al.*