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A guide to *in vivo* optogenetic applications for cerebellar studies

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The mammalian cerebellum consists of a superficial cortex and centrally located output nuclei, which together with brainstem nuclei are organized in a modular fashion. Regardless of the function, these cerebellar modules consist of the same cell types and their connectivity has been unraveled to some detail using electrical stimulation experiments. To unravel the highest level of detail, cell-specific stimulation experiments are warranted, which cannot be accomplished using electrical stimulation. To reach this unprecedented level of specificity optogenetic applications are now being implemented in cerebellar studies. Due to the extensive knowledge about cell-specific markers in both the cerebellar cortex and the cerebellar nuclei, optogenetics can be applied cell-specifically. Ideally the anatomical and electrophysiological characteristics of the cerebellum can be utilized for designing future optogenetic studies. In this chapter we review the opportunities and pitfalls for optogenetic studies in the cerebellum. We provide insights into the technical issues at hand and which solutions are currently available.

3.1 Introduction: Shining light on cerebellar optogenetics

Since the beginning of electrical stimulation also the cerebellum has been probed for functional relevance. Early work from Morruzzi in the 1940-1950s indicated that motor responses and body posture could be precisely adapted by electrical stimulation of defined parts of the cerebellar surface [66]. It didn't take long before researchers started to evaluate the power of cerebellar stimulation when it comes to limiting brain perturbations, e.g. stopping epilepsy. Although the anti-epileptic effects of cerebellar stimulation were extensively probed in experimental animals and confirmed in initial clinical trials, subsequent double-blind studies revealed that the exact location of stimulation was affecting the efficacy (reviewed in[164]). The spatial precision of cerebellar stimulation has been of great importance for gaining insight in cerebellar functioning. Anatomical tracing studies in the late 1900s revealed that specific regions of the cerebellar cortex form functional modules together with downstream cerebellar nuclei and the inferior olive nuclei in the ventral brainstem [165, 166]. Especially in the cerebellar cortex this anatomical differentiation has been shown to be very precise and results in the need of neuromodulation techniques with high spatial resolution. Although novel electrode designs brought new options for neuromodulation on a micro-scale the recent development of optogenetics launched a new era of investigating brain functioning. Apart from the option of applying neurostimulation to a specific type of neuron rather than a volume of brain tissue, many light-sensitive ion channels have been described, which not only allow researchers to excite neurons, but also to inhibit their action potential firing (extensively reviewed in [167] and other literature). Alike for other brain areas also for cerebellar research optogenetics provide numerous opportunities. This chapter combines reports of optogenetic applications in the cerebellar field with a technical guideline for questioning cerebellar interactions with up- and downstream targets using optogenetics.

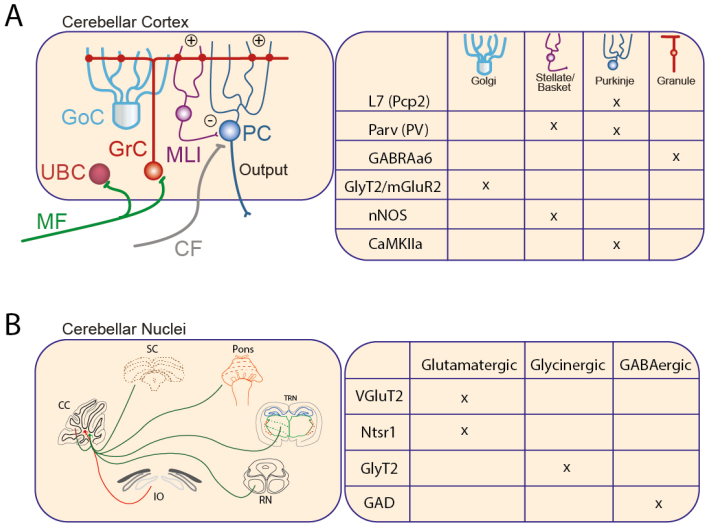
3.2 Optogenetic approaches in the Cerebellar Cortex

The foliated cerebellar cortex (CC) consists of 3 readily distinguishable layers of gray matter and a core of white matter, the latter of which is a combination of afferent mossy fibres and climbing fibres and efferent Purkinje cell axons. Adjacent to the white matter is the cytological diverse granular layer, which contains numerous granule cells, Golgi cells, Lugaro cells and unipolar brush cells. Distal to the granular layer a monolayer of Purkinje cells separates granule cells from the molecular layer, which contains molecular layer interneurons, i.e., stellate and basket cells. This outer layer also contains the dendritic trees of Purkinje cells and Golgi cells as well as the granule cell axons. In principle each of the cell-

types can be targeted using optogenetic tools (**Figure 1**), which would have been impossible using electrical stimulation techniques (juxta-cellular stimulation of single neurons is not discussed in this chapter, but could be used to increase action potential firing in a single neuron [168]). The increasing knowledge of anatomical connections of cortical neurons and their concurrent activity [169-171] enables one to control the activity in functionally distinctive modules or microzones using specific (sub-) populations of cerebellar neurons [154].

3.2.1 How to induce opsin expression

A common method to accomplish cell-specific expression of light-sensitive channels is the Cre-LoxP system, which is based upon the expression of Cre recombinase and its capacity to cause DNA synapsis and site-specific recombination of DNA strands at genetically engineered loxP sites [172].



	Golgi	Stellate/ Basket	Purkinje	Granule
L7 (Pcp2)			x	
Parv (PV)		x	x	
GABRAa6				x
GlyT2/mGluR2	x			
nNOS		x		
CaMKIIa			x	

	Glutamatergic	Glycinergic	GABAergic
VGluT2	x		
Ntsr1	x		
GlyT2		x	
GAD			x

Figure 1. Circuitry and cell-specific promotors of cerebellar neurons.

(A) (left) Schematic and simplified illustration of cerebellocortical circuitry. (right) Table with promotors identifying promotors for different cortical cells. (B) (left) Schematic and simplified illustration of cerebellar nuclei output and its downstream targets. (right) Table with promotors identifying promotors for different CN neurons. Apart from the listed promotors, several non-selective promotors like synapsin or CAG have been utilized in the cerebellum. See main text for references and examples (mGluR2 is specific for a subset of Golgi cells [261]. UBC = unipolar brush cell; GrC = granule cell; GoC = Golgi cell; MLI = molecular layer interneuron; PC = Purkinje cell; MF = mossy fiber; CF = climbing fiber; CC = cerebellar cortex; SC = superior colliculus; TRN = thalamic relay nuclei; RN = red nucleus; IO = inferior olive; L7-PCP2 = L7 Purkinje cell protein 2; Parv = parvalbumine; GABRA6 = alpha6-subunit of GABAA-receptor; GlyT2 = glycine transporter type 2; nNOS = neuronal nitric oxide synthase; and CaMKIIa = calcium-calmodulin-activated kinase type II alpha.

For optogenetics constructs the transgene can be preceded by a LoxP-flanked stop cassette and thus will only be expressed if the cell expresses Cre-recombinase. This commonly used strategy can be applied for instance, by cross-breeding a Cre-mouse line [173] (**Figure 1**) and any of the AI-mouse lines [174, 175]. Of these, the AI-27 or AI-32 mouse lines result in cell-specific expression of the channelrhodopsin 2-construct (ChR2), which encodes for light-sensitive ion-channels that mediate neuronal depolarization [167], combined with a red- or yellow-fluorescent protein construct, respectively. To drive ChR2-expression in Purkinje cells the most commonly used approach is to cross-breed with a transgenic mouse line in which the L7 (Purkinje cell protein 2) promotor for Purkinje-cell specific transgene expression [176], which is linked to Cre-recombinase [177] and thereby can be used for Purkinje cell-specific mutations (reviewed by [81]). For instance, L7Cre*Ai32 mutant mice show Purkinje cell specific ChR2-expression and can be identified by their YFP-positive identity (**Figure 2A**) [178]. Of note is that in several recent studies the specificity of the L7/Pcp2-Cre mouse lines has been questioned [123, 171] and warrants a detailed evaluation of the expression. To avoid the potential a-specific expression of Cre-lines, one option is to induce ChR2-transfection using *in utero* electroporation [179]. To direct the transfection specifically to cerebellar neurons, one option is to inject ChR2-encoding constructs into the 4th ventricle and electroporate the cerebellar progenitor cells in the rhombic lip or the subventricular zone near the 4th ventricle during embryonic days 10.5 and 12.5 [180] (**Figure 2B**). Novel electrode designs should warrant acceptable success ratios [181] and numerous constructs that can be used for excitatory or inhibitory optogenetics [175] should provide ample options for cerebellar optogenetics. One consideration is that the transfection rates of *in utero* electroporation typically fall short of those reached by cross-breeding transgenic mouse lines – a difference that can also be advantageous if mosaic but cell-specific expression is the goal. Another option is to transfect the cerebellum by cerebrospinal fluid injections, which results in mosaic expression throughout the whole CNS [182, 183].

To reach a more spatially restricted expression of opsins, often viral vectors are used. From the pre-optogenetics era of gene therapy it is known that various vectors can be used to transfect cerebellar cell types. Apart from Lenti-viral backbones (see for instance refs [184-186]), cerebellar tissue can be transfected using adeno-associated viral (AAV) vectors [187-189] (**Figure 2C**). As has been shown in other parts of the CNS [190], the various AAV-serotypes can lead to widely varying transfection efficiency rates and even serotype-specific tropism. Apart from the serotypes, also the promotors can be utilized to tune the transfection in cerebellar tissue. In case the location of transfection rather than the cell-type specificity is most important, human synapsin (hSyn) is commonly used and results

in neuron-specific staining. Another commonly used promotor is ubiquitous CMV and chicken-β-actin fused to CMV enhancer (CAG). When packaged in an AAV1-vector the CAG promotor showed an elevated specificity for Purkinje cells [187]. Another commonly used promotor is parvalbumine, which is endogenously expressed in Purkinje, stellate and basket cells [191-193]. Apart from these generally used promotors, cerebellar scientists have also been exploring the use of cell-specific markers. For cerebellar granule cells, the α6 subunit of the GABA-A receptors (GABRα6) is a selective marker [194].

Kim and colleagues produced an AAV1-GABRα6-GFP vector, which indeed resulted in selective expression of GFP molecules in granule cells [195]. In the same study, also an AAV1-vector was produced that packaged the αCaMKII-promotor, which is selective for Purkinje cells in the cerebellar cortex. Finally, the Augustine lab and collaborators generated a transgenic mouse in which ChR2 is expressed under a neuronal nitric oxide synthase promotor, which resulted in selective expression in molecular layer interneurons [193, 196, 197]. These results indicate that the viral constructs can be readily tuned to drive cell-specific transfection in the cerebellar cortex. Another approach to reach cell-specific expression is by injecting a transgenic mouse that expresses Cre-recombinase in a cell-specific manner with a viral vector that encodes the opsin-construct preceded by a floxed Stop codon (**Figure 2C**) (see for instance refs [111, 198]).

As has been pointed out previously [199], using the extensive knowledge of the anatomical connections within the cerebellum can help to study the functional connectivity of identified cell types. Great care needs to be taken that the volume, location and spread of the viral particles is restricted. Viral vectors can be injected using iontophoresis (see ref [200] for a functional protocol), although it is more common to use air-pressure. Pressure injections can be performed using injection systems that can be purchased from various vendors, or can easily be manufactured using an injection syringe, a piece of tubing and a glass injection pipette. Important for pressure injections is that the speed and volume are tightly controlled. Typically, the speed of injection is <50 nl/min and the volume for intracerebellar injections below 200 nl. Another measure taken to limit the spread of the viral vectors beyond the injected area is to leave the injection pipette in place for several minutes before retracting. To reach a stable opsin expression level, experiments are typically started after >3 weeks of incubation time, although the latency until stable levels have been reached could vary per serotype and promotor [190].

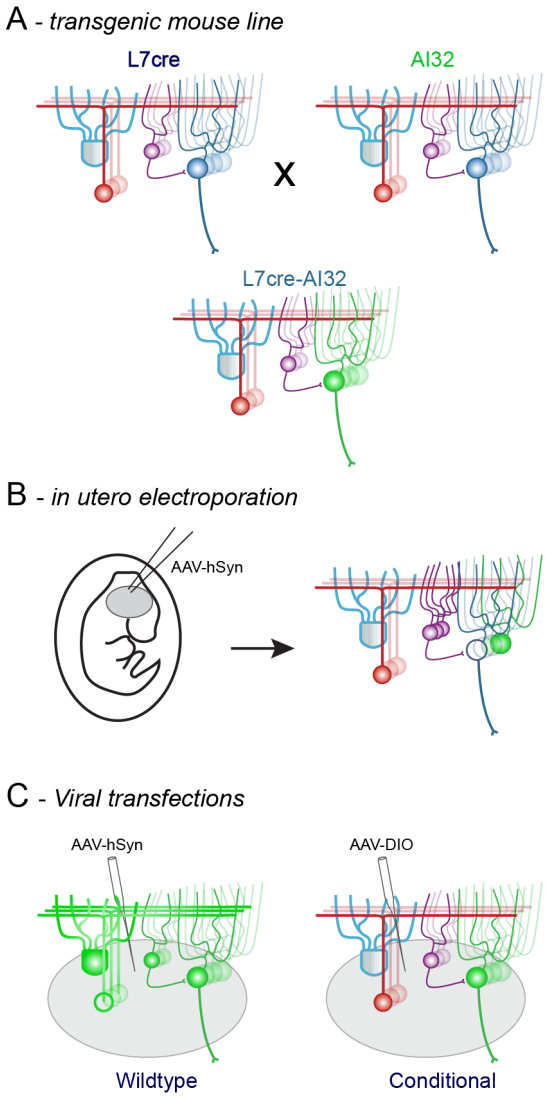


Figure 2. Transfection techniques for (non)specific expression.

(A) Cell-specific expression of ChR2 using a Purkinje cell-specific Cre-mouse line (L7cre) and a transgenic mouse in which the ChR2-construct and the fluorescent reporter are preceded by a floxed stop codon (AI32). Crossbreeding of these two lines results in Purkinje cell-specific expression of ChR2 throughout the whole cerebellum. (B) *In utero* electroporation using a-specific promotors like hSyn can still result in a cell-specific transfection due to the spatial and temporal specificity of the viral presence. (C) (left) Viral expression using a-specific promotors will result in opsin expressing in multiple cell types. (right) injecting a L7Cre mouse that expresses Cre-recombinase Purkinje cells only with a viral vector that encodes the opsin-construct preceded by a floxed Stop codon will result in Purkinje cell specific expression of ChR2, but only in the injected region.

3.2.2 Combining light and recording in the cerebellar cortex

Once the cerebellar tissue expresses opsins, the next step is to apply light to the brain. The intensity of light needed ranges between opsins, but for the commonly used variants of ChR2 1-5 mW/mm^2 is typically sufficient to evoke action potential firing [167, 201]. Although some experimental studies on ChR2-stimulation in the cerebellum aimed to record the spread of light in tissue and its effects on Purkinje cell action potential firing [115, 202] it is not clear whether the foliation and mixture of grey and white matter layers in the cerebellar cortex affect the spread of light. Although simplified calculation tools are readily available to the scientific community [203] these do not take into account that light attenuation coefficients [204] and other important characteristics vary widely in the brain [205]. Recently developed predictive models like the OptogenSIM platform by Liu et al [205] provide the scientific community with readily accessible tools for more realistic estimations of how far the light of particular wavelengths will travel. This could be particularly informative for experiments that report the physiological and behavioral responses following optical stimulation of the cerebellum with large (200-400 μm diameter) optic fibers (Figure 3A).

To limit the spread of light, small-diameter optic fibers (< 100 μm diameter) can be purchased from several vendors. Another option is to strip optic fibers and pull them to sharp-tip using heating and grinding (see for instance ref [193]). Once the diameter is sufficiently limited, they can be attached directly to recording electrodes to apply optical stimulation directly to the recorded area (Figure 3B). One important consideration for local optical stimulation is the fact that local optical stimulation causes stimulus artefacts evoked by photoelectrochemical effects that were first described by the French physicist Becquerel in 1839 and are considered as the main mechanism of stimulus artefact for conventional metal electrodes [206-208]. It is produced by photonic excitation of electrons at the electrode valence band that absorbs the photon energy; subsequently an electric potential is generated because these excited electrons leave their orbit [206, 208]. There are several ways to deal with this artefact. One of them is to place the recording electrode at a distance from the light source (Figure 3B) although this might not always be an option when recording and stimulating close to each other.

If possible another solution would be to switch to low energy photons (green light instead of blue) which in combination with decreasing light intensity to such levels that the amplitude of the artefact falls below the background noise might be a more reasonable solution. However opsins require a minimum light intensity to be activated so a more ideal solution would be an artefact free recording method. Several groups tried to eliminate

the photoelectric artefact by developing fully transparent electrodes with increasing manufacturing complexity [209-211]. The use of tin-doped indium-oxide and graphene as material does not seem favorable for (chronic) *in vivo* applications since indium tin oxide oxidizes over time and thereby alters the biocompatibility [212]. Still, a straightforward solution is to use the classic liquid filled glass pipettes and insert an optic fiber into the taper (Figure 3C,D). By placing a Pt or Ag/AgCl recording electrode at the back of the pipette, i.e. far from the light source, the charged ions move through the liquid instead of through the conduction band of the brain. This custom-made 'optrode' returns high quality single cell recordings virtually free of photoelectric artefacts. Note that this fiber-in-glass approach is not suited for chronic applications since the glass pipettes is very thin, fragile and are subject to increasing tip resistances over time that limit the recording quality.

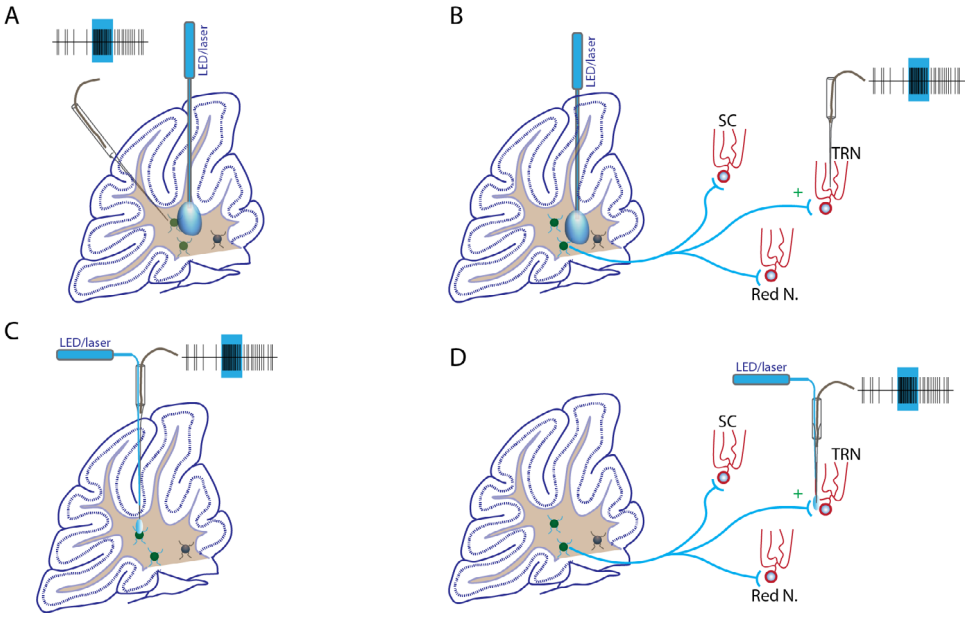


Figure 3. Stimulation and recording methods.

(A) Schematic for recording CN neurons (left top, schematic trace in which black vertical lines represent action potentials and the blue box the time of optical stimulation of ChR2) using a glass pipette (transparent tube including a wire) with a separate optic fiber (vertical blue probe) for light delivery. (B) Schematic for recording downstream targets with light delivery in CN. Note that this will also activate other pathways which might cause network effects. (C) Schematic for combined recording and light delivery through glass pipette. (D) Schematic for combined recording and light delivery at CN terminals in downstream nuclei to isolate the specific effect of cerebellar output on downstream targets without initiating network effect. SC = superior colliculus; TRN = thalamic relay nuclei; and Red N = red nucleus.

3.2.3 Optical stimulation – upper and lower limits

Ideally, optogenetics experiments are designed to encompass a recording electrode at the stimulation site. In this way the experimenters can tune their optical stimulation paradigm to the intended neuromodulation. Several landmark papers scrutinized the kinetics of opsin constructs and the neuronal responses [201, 213-218], which clearly indicate that optical stimulation cannot only be too low, but also too high. In the case of ChR2 over-stimulation would result in a decrease rather than in increase of action potential firing. Such an inhibitory effect is typically mediated by a 'depolarization block' caused by ChR2-mediated depolarization that inactivates sodium channels and subsequently inhibits action potential firing [219]. Obviously, such effects may lead to false assumptions, for instance, when correlating 'prolonged action potential generation' to a behavioral readout, while in fact the action potential firing was only initially elevated and then silenced. In a similar fashion the use of inhibitory opsins should be carefully considered. For instance, it was recently shown that when using chloride-conducting channelrhodopsins the expected inhibition of neurotransmitter release was effectively reversed [220]. To prevent previously mentioned over-activation artefacts, the maximum pulse length and the power should be critically considered. It is possible to prevent over-activation effects by implementing a duty cycle. This will limit the amount of light exposure but can also dramatically change the output compared to a continuous pulse of light. It is easy to recognize that a 200 ms long 50 Hz train of short (3 ms) light pulses (17 ms pause) and a 200 ms long continuous light pulse will have a completely different effect on the opsin-expressing neuron as well as on its downstream targets [219].

Sustained activation of any type of rhodopsin by light can also lead to heat induction. Heating depends on many properties such as power of light, duration and the wavelength, but also on (and not limited to) intrinsic brain properties, temperature loss (due to skull removal), blood flow etc. Commonly used light power densities for optical stimulation have been reported to marginally increase local temperatures near the stimulation site, leading to enhanced activity of neurons [221-223]. Recently it has been shown that prolonged light introduction of 10 mW can lead to temperature increases ranging between 1°C to 4°C across a large volume of tissue, which resulted in directly increased firing rates by up to 40% [224]. Another important issue is the diameter of optic fiber. It has been shown that the core peak temperature change for 62 μm fibers with the same light output (10 mW) is several °C higher compared to a 200 μm diameter fiber [224]. To control for the impact of such heating effects on neuronal firing and thereby on the study results, it is of upmost importance to include a set of control experiments in which the same optical stimulation is

applied in ChR2-negative animals. In case prolonged activation of the opsins is an absolute necessity a potential solution could be to use step-function opsins, which require only short light-pulse to activate long-lasting increases in action potential firing [167, 225].

3.2.4 Mediating Purkinje cell activity using opsins

It is of great importance to select the type of opsin and the optical stimulation paradigm that match the electrophysiological characteristics of the target cells. One of the main reasons for this is that opsins are membrane bound and thus the membrane surface area is linearly related to the number of light-sensitive ion channels that can be incorporated. For instance, granule cells, which are the smallest cerebellar neurons, can fit less opsin-channels than the larger Purkinje cells. The impact of activating light-sensitive ion channels is also related to the membrane properties – granule cells have a ~10-fold higher membrane resistance then Purkinje cells [125] and thus, the activation of a certain number of ion channels has a bigger impact on the membrane potential of granule cells than on Purkinje cells. Apart from the passive membrane properties it is likely that also the intracellular constellation has an impact on the neuronal response to opsin activation. For instance, Purkinje cells typically do not express voltage-gated sodium channels in their dendritic tree; activation of a high density of sodium-permeable ion-channels, i.e., ChR2-channels, in this membrane may induce pathophysiological processes. Indeed, in our hands the use of high density light stimulation on freshly prepared *in* vitro slices containing Purkinje cells expressing ChR2(H134R) can induce excitotoxicity (unpublished observation, F.E.H.).

Purkinje cells endogenously fire two types of action potentials (simple and complex spikes), which come about because of a delicately organized interaction between dendritic and somatic characteristics [226, 227], and thus the use of optogenetics directly in Purkinje cells to manipulate action potential firing is precarious. The available data on direct manipulation of Purkinje cell activity [45, 111, 171, 178, 185, 186, 197, 228], which has been achieved using a myriad of opsins, should be evaluated critically. First and foremost, Purkinje cells are characterized by an intrinsic pacemaking activity [229] that can easily be disrupted by, for instance, abnormal levels of excitatory inputs [230, 231], and result in abnormal firing patterns. Moreover, opsins' actions may depend on the viral vector used: Jackman and colleagues recently showed that when ChR2(H134R) is expressed by AAV1, 5, or 8 serotypes, repetitive stimulation resulted artificially in paired-pulse depression, whereas this was not the case when the same construct was encoded by AAV9 or by crossbreeding of mutant mouse lines [232]. Another important consideration for selecting suitable optogenetic constructs is the fluorescent tag that is typically coupled to the opsin.

Asrican and colleagues identified differences in light-evoked currents when tagging ChR2 with various fluorescent tags. Most notably, Purkinje cells that expressed ChR2-mCherry showed intracellular protein aggregates and the membrane potential did not respond to optical stimulation whereas Purkinje cells transfected with ChR2-YFP showed membrane-bound expression and responded with depolarizing currents upon optical stimulation [191] (but see refs [198, 220] for mCherry-containing constructs without aggregates). These findings underline the importance of selecting the optimal optogenetics construct and inclusion of controls for the efficacy of optical stimulation.

Most of the studies that allow critical evaluation of optogenetics concern activating opsins, i.e., ChR2 and correlates. Yet, a critical evaluation of the impact of inhibitory opsins, i.e. proton pumps [233], chloride channels [213, 234, 235] and light-gated chloride pumps [236, 237], is equally important. For the most widely used Halorhodopsin (eNpHR3.0) and Archaerhodopsin (eArch3.0) it was shown that short illumination (millisecond range) of axonal terminals attenuates synaptic transmission, albeit that eArch3.0 is a more potent inhibitor than eNphR3.0. Notably, such pulsed inhibition also resulted in potent rebound responses [220], which obviously is of importance for the interpretation of any readout. For longer inhibition (minute range) the use of eArch3.0 resulted in counterintuitively elevated presynaptic calcium concentrations and hence increased spontaneous neurotransmitter release [220]. For future investigations that require long-term inhibition of neuronal firing rates, other options include chemogenetic approaches that utilize G-protein-coupled receptors that can be selectively activated by designer drugs (e.g., excitatory hM3Dq [238] and inhibitory hM4Di [239]), photoswitchable tethered ligands linked to metabotropic glutamate receptors [240] and vertebrate rhodopsins [215, 241]. By applying the inhibitory compounds to specific cell types, like molecular layer interneurons [193, 196, 197] or to Purkinje cells directly [111, 228], the net effect on the cerebellar output can be tuned to the specific research questions.

3.3 Optogenetic manipulation of cerebellar nuclei activity

Apart from the vestibulo-cerebellar Purkinje cell projections to the vestibular nuclei, all Purkinje cell axons terminate in the cerebellar nuclei (CN). Most, if not all [242], CN neurons receive Purkinje cell input, which is GABA-mediated. The medial, interposed and lateral CN contain various types of neurons; up to 6 different types in the rodent lateral nucleus [243]. For several of these types of neurons, cell-specific markers have become available in the last years (see **Figure 1** for overview). Vesicular glutamate transporter type-II (vGluT2) is expressed in the CN solely by the glutamatergic projection neurons [244], of

which currently only a single type has been described despite the varying projection patterns (see for instance refs [88, 245]). Apart from using vGluT2-Cre transgenic mice, also the Ntsr1-Cre mouse line has been reported to selectively express in glutamatergic projection neurons of the CN [245]. The other cell types in the CN are inhibitory and release glycine, GABA, or both neurotransmitters from their terminals. By coupling Cre-recombinase to the glutamic acid decarboxylase (GAD), GABAergic neurons can be identified, just as glycinergic neurons can be tagged selectively by coupling Cre to glycine transporter type II (GlyT2) [198]. Ankri and colleagues recently dissected the various types of CN neurons that can be targeted using the GAD-Cre and GlyT2-Cre mice. They convincingly showed that GlyT2-Cre labelled a single type of neuron (based upon somatic diameter and dendritic morphology), which also expresses GAD, and that the GAD-Cre separately labels a smaller type of neuron that is GlyT2-negative and projects to the inferior olive [82, 198, 246, 247]. Using the GlyT2-cre mice it is in principle also possible to target the glycinergic projection neurons that selectively project to the brainstem's vestibular complex [248]. These cell-specific tactics provide researchers with a similar arsenal as previously mentioned for the cerebellar cortex. Importantly, also the same precautions for optical stimulation and recordings will have to be taken into account. Below we will review the currently available data important to design future optogenetic studies that can unravel the impact of afferents on CN neuronal activity and the impact of CN neurons on their downstream targets.

3.3.1 Manipulating afferents to Cerebellar Nuclei neurons

Neurons in the CN have been reported to receive a mixture of excitatory and inhibitory input from external inputs – for the current chapter we disregard the intra-nuclear axon collaterals and local interneurons (for review see ref [242]). The excitatory input may originate from the inferior olive and arrive as climbing fiber (CF) collaterals or from various reticular, pontine or spinal regions as mossy fiber (MF) collaterals [95]. It was concluded from electrical stimulation experiments performed *in vivo* that CF-inputs monosynaptically excited CN neurons [105, 108, 110, 249, 250]. Yet, a more recent study that used optogenetic approaches indicated that CF-evoked responses in CN could only be recorded in a few cells and if present, these responses were relatively weak [251]. A potential confounder for some of the electrical stimulation studies is that not only inferior olive neurons and their axons were stimulated, but also the neurons and axons passing nearby that may give rise to MF-evoked responses [105, 108, 251]. Electrical stimulation of isolated MF-afferents to CN neurons is mostly limited to *in vitro* studies in which the CN-surrounding white matter is stimulated and the excitatory responses are pharmacologically isolated by blocking

inhibitory currents (as reviewed by ref [252]). These data on putative MF-CN transmission reveal that the excitatory input is subject to synaptic plasticity and mediates also inhibitory transmission and cellular excitability levels. Applying selective optogenetic stimulation of MF-inputs will provide more insights in the differences between the MF- and CF-inputs to CN neurons.

For the other major input to CN neurons, the Purkinje cells, it was long thought that this GABA-mediated input had a purely inhibitory effect on CN action potential firing patterns. This thought was fueled by the extreme convergence of Purkinje cell axons onto single CN neurons, the perisomatic location of these axon terminals on CN neuronal membrane and the relatively slow time constants of the inhibitory postsynaptic potentials. However, the landmark paper by Person and Raman [83] revealed that synchronous Purkinje cell firing could elicit time-locked spiking in CN neurons, which can be used to design optogenetic paradigms for Purkinje cell stimulation. For instance, short pulses (1-2 ms pulse width) of 470 nm light on ChR2-expressing Purkinje cells could result in a short inhibition of CN spiking, followed by a well-timed action potential (see for instance refs [83, 105]). This will probably occur once a sufficient number Purkinje cells are synchronously entrained by the optical stimulation, i.e., when a sufficient level of Purkinje cell synchronicity is reached [83, 253]. By lengthening such potent optogenetic stimulation to tens or hundreds of milliseconds it may occur that CN neurons are depolarized to such levels that once the stimulation stops, the membrane potential shows a rebound depolarization (see also refs [105, 107, 108, 253, 254]). The accompanying increase in CN firing has indeed been shown in various optogenetic studies from several labs albeit ranging from a rather limited increases [115, 251] to more pronounced elevations that even accompanied behavioral responses [111, 193, 228]. These responses can be tuned quite precisely by varying the light intensity and/or location of optical Purkinje cell stimulation. It should be noted that the optical stimulation is merely enhancing the synchronicity of Purkinje cell firing for the duration of stimulation and that the local and downstream network will outlast the direct intervention.

3.3.2 Manipulating Cerebellar Nuclei neuron activity

The output neurons of the CN can be inhibitory or excitatory and thus the effect on downstream targets can increase or decrease local action potential firing. In general it is thought that the excitatory projection neurons connect to the di-, mes-, met- and myelencephalon and that the GABA-ergic and glycineric projection neurons project to the inferior olive and vestibular nuclei, respectively (**Figure 1B**). So far, a limited

number of optogenetic studies evaluated the impact of varying CN firing rates on these downstream targets. Nucleo-olivary neurons have been shown by electrical stimulation to provide a tonic and phasic inhibition to olivary neurons at the site of dendro-dendritic gap junctions [255]. When selectively activating these neurons using a GAD-Cre mouse and a double-floxed ChR2(H134R) viral construct, Lefler et al recently showed for the first time that this inhibitory input uncouples inferior olive neurons and thereby manipulates complex spike firing and Purkinje cell activity [247]. Moreover, it was recently shown for these nucleo-olivary neurons that optogenetic stimulation of Purkinje cells evoked post-synaptic responses that decayed 10-fold slower than in excitatory projection neurons [82]. The electrophysiological difference between these two types of neurons also extended into the sluggish action potential half width as well as a narrow dynamic range in action potential firing rates in nucleo-olivary neurons [82]. These apparent differences could be of importance for selecting optogenetic constructs to drive each of these neurons, or their afferents.

So far, the studies which have been applying optogenetics directly in CN to study the impact of glutamatergic neurons all injected a hSyn-promotor driven ChR2-vector into the cerebellar nuclei. In an effort to stop generalized spike-and-wave discharges in epileptic mouse models, Kros et al transfected interposed and lateral CN neurons, optically stimulated in these regions and recorded CN action potential firing patterns and the electrocorticogram [46]. Apart from the fact that the generalized episodes were reliably stopped, the responses recorded in the CN showed a mixture of increased and decreased firing during optical stimulation. This latter finding is most likely due to the fact that using the hSyn-promotor resulted in the transfection of inhibitory neurons, which synapse on the recorded cells. Also Chen et al transfected the lateral CN with the same a-specific promotor [256]. Although they did not extensively report on the CN activity during stimulation, the authors found that the electrophysiological responses in the striatum evoked by optical stimulation in the CN and those evoked by stimulation of the CN axon terminals in the centrolateral thalamic nucleus were in principle the same [256]. These results argue that although the transfection was most likely not specific for excitatory CN neurons the impact of optical stimulation is mediated by the excitatory input in the downstream target nucleus. Although the local stimulation of axon terminals may be an option for many studies, it could still be advantageous to study an isolated subgroup of CN terminals projecting to a particular downstream target. One way to do so is to use a retrogradely transported viral vector and inject it in the downstream nucleus targeted by the CN. Several vectors for such retrograde expression have been generated; currently it seems that AAV-retro provides the best options [257].

Using the cell-specific stimulation options, recently there have been several studies that revealed the functional importance for CN axonal connections back to the cerebellar cortex. These axonal tracts had been described in various anatomical papers, but due to the anatomical specifications it had not been possible to electrophysiologically isolate them sufficiently [258]. Using the a-specific hSyn promotor Gao et al transfected CN neurons with ChR2 and found that their fluorescent mossy fiber-like terminals evoked purely excitatory currents in granule and Golgi cells of the cerebellar cortex [259]. In another recent study, an inhibitory nucleo-cortical projection has been described from neurons that co-express GAD and GlyT2 and specifically target a subpopulation of cerebellar Golgi cells [198]. This latter study utilized GAD-Cre and GlyT2-Cre mice in combination with AAV-vectors encoding for floxed-ChR2 constructs, which warranted cell-specific manipulations of inhibitory neurons that are GABAergic and glycinergic (and excluded GABAergic nucleo-olivary and glutamatergic projection neurons) [198]. Such level of unprecedented anatomical, physiological and functional detail recently also lead to the characterization of Purkinje cell axon collaterals to granule cells and neighboring Purkinje cells in the adult brain [169, 171].

3.4 Final recommendations and conclusions

Utilizing optogenetics will have a positive impact on cerebellar studies. Novel research questions about the functional impact of particular connections can now be adequately addressed. The information content of the results from optogenetic studies will be unprecedented, but will require a multitude of controls and sensitive analyses to filter out artefacts and identify the true impact of optogenetic manipulations. For instance, to recognize subtle changes in action potential firing patterns evoked by the activation of a subset of presynaptic axon terminals, a sufficient number of stimulation repeats have to be recorded to allow for Monte Carlo bootstrapping and subsequently identification of significant changes in firing patterns. Another aspect of optogenetics in cerebellar network physiology that deserves attention is the fact that various recurrent loops exist that can affect the electrophysiological responses in addition to the direct effects of the optical stimulation. These loops come in various shapes and sizes that not only include cortical neurons (MF-granule cell-Golgi cell-granule cell; Purkinje cell-granule cell-Purkinje cell; etc), but also involve the CN neurons and inferior olive neurons (intra-CN, nucleocortical and nucleo-olivary) and thus can have a delayed effect on cerebellar activity patterns. Moreover, the glutamatergic CN output can also return to the MF or CF sources by thalamo-cortical networks and midbrain nuclei, or even via the sensory feedback triggered

by cerebellar-evoked motor responses. Although most of these potential feedback loops will be characterized by a fixed latency, it is very likely that the evoked network effects can last for extended periods and need to be considered when setting inter-stimulus intervals. Likewise, for most optogenetic constructs the inter-stimulus interval needs to be tailored to the desensitization and closed states [260]. Tuning these and other technical details will be decisive for the added value of optogenetics in cerebellar studies. Although the cerebellar field can ride on the constant wave of optogenetic innovations, this does not mean that optogenetic approaches can be bluntly copied from, for instance, the cerebral cortex field. By using the extensive anatomical knowledge of the cerebellar connectivity and cellular physiology the most effective and functionally relevant optogenetic approaches can be achieved.