Optogenetic Control of Motor Coordination by $G_{i/o}$ Protein-coupled Vertebrate Rhodopsin in Cerebellar Purkinje Cells*

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G protein-coupled receptors are involved in the modulation of complex neuronal networks in the brain. To investigate the impact of a cell-specific $G_{i/o}$ protein-mediated signaling pathway on brain function, we created a new optogenetic mouse model in which the $G_{i/o}$ protein-coupled receptor vertebrate rhodopsin can be cell-specifically expressed with the aid of Cre recombinase. Here we use this mouse model to study the functional impact of $G_{i/o}$ modulation in cerebellar Purkinje cells (PCs). We show that in vivo light activation of vertebrate rhodopsin specifically expressed in PCs reduces simple spike firing that is comparable with the reduction in firing observed for the activation of cerebellar $G_{i/o}$-coupled GABA$_B$ receptors. Notably, the light exposure of the cerebellar vermis in freely moving mice changes the motor behavior. Thus, our studies directly demonstrate that spike modulation via $G_{i/o}$-mediated signaling in cerebellar PCs affects motor coordination and show a new promising approach for studying the physiological function of G protein-coupled receptor-mediated signaling in a cell type-specific manner.

The G protein-mediated signaling pathway provides a pivotal module for the adjustment of neuronal networks against physiological or behavioral tasks on a second to minute time scale (1). Among G proteins, the $G_{i/o}$-mediated signaling pathway is the primary role in which GPCRs² mediate their inhibitory action on neuronal excitability (2). The processes and importance of such modulation in cellular and network functions has mainly been investigated with the application of drugs, activating or inhibiting more or less specifically a certain GPCR pathway. Recently, we demonstrated that light-activated vertebrate rhodopsin (vRh) is a suitable alternative to control ion conductances such as G protein-coupled inward rectifying K⁺ channel and voltage-gated Ca$^{2+}$ channels via pertussis toxin- sensitive $G_{i/o}$ protein-mediated signaling (3). Therefore, vRh may allow for the precise spatiotemporal control of $G_{i/o}$-mediated pathway in vivo, leading to an investigation that focuses on the function of this pathway in animal behavior or brain functions such as motor coordination.

The cerebellum plays a central role in overall motor coordination and motor learning. An extensive array of GPCRs is expressed throughout the brain and is believed to be involved in the modulation of network activity and synaptic plasticity. It has been recognized that the code for motor coordination and balance lies within the firing cadence and output pattern of cerebellar PCs, which are the sole-output neurons from the cerebellar cortex (4, 5). PCs integrate a range of cortical, vestibular, and sensory information via excitatory synaptic input from parallel and climbing fiber pathways and inhibitory synaptic input originating from neighboring interneurons. The PC firing pattern is determined by several factors that include the interplay between excitatory and inhibitory synaptic inputs, several ion channel conductances that support intrinsic firing properties, and modulation by postsynaptic GPCRs like the GABA$_B$ receptor (GABA$_B$R) (6–8). GABA$_B$R activation by application of the selective agonist baclofen leads to a reduction in PC firing most likely due to membrane hyperpolarization induced by G protein-coupled inward rectifying K⁺ channel activation (9–12). The exact mechanism in which $G_{i/o}$-mediated GPCR modulation may occur within PCs and how such modulation may influence the single spike pattern and motor coordination has been difficult to address in vivo, as GABA$_B$Rs and other $G_{i/o}$-coupled receptors are expressed in various cell types in the cerebellum and can only be activated by slowly diffusing drugs.

To overcome the kinetic and spatial issues that the pharmacological approach presents and to investigate the functional impact of $G_{i/o}$ protein-mediated modulation on cerebellar function via spike modulation in cerebellar PCs, we created an optogenetic mouse model for the cell type-specific expression of vRh and demonstrated that spike modulation of PCs affects motor coordination.

**EXPERIMENTAL PROCEDURES**

*Generation and Screening of Transgenic Mice—To generate a colony of vRh-GFP<sub>PC</sub> transgenic mice, homozygous transgenic Purkinje cell specific CRE (Tg<sub>Pcp2-cre</sub>) mice (13) were crossed...
with heterozygous vRh-GFP(Tg<sup>ΔvRh-GFP</sup>) mice. Routine screening of all transgenic mice was accomplished by adding either tail or toe tissue to 0.3 ml of lysis buffer containing 100 mM Tris, pH 8.5, 5 mM EDTA (disodium salt), 0.2% SDS, and 200 mM NaCl. Twenty microliters of proteinase K (20 mg/ml, Roche Diagnostics) was added to the lysis buffer, and the mixture was shaken overnight at 55 °C. After tissue dissolution, the mixture was heated to 99 °C for 10 min and then cooled to room temperature. A PCR master mix contained either of the following oligos: vRh-GFP (5′-CATGCTCACCCACCGTCGCT and 5′-AACAGTTGAGCTCCTGGAAC or Cre-Recombinase (5′-TCTCA-CGTTACTGACGGTGG and 5′-ACCCAGTTGCAGTATGCTC). The 50-ml final PCR reaction contained 1 ml of gDNA, 1 ml of each primer, 1 ml of dNTP mix (10 mM each of dATP, dTTP, dCTP, dGTP; New England Biolabs (NEB)), 5 ml of 10× Thermopol II reaction buffer (NEB), 5 ml of dimethyl sulfoxide, 0.5 ml of Taq Polymerase (NEB), and 35.5 ml of distilled H<sub>2</sub>O. PCR reactions were run on an Eppendorf thermocycler using the following conditions: 92 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min run for 40 cycles or 95 °C for 1 min run for 40 cycles or 95 °C for 1 min and 72 °C for 1 min 30 s for 40 cycles to detect vRh-GFP or Cre-Recombinase, respectively. PCR products were analyzed on a 1% agarose gel utilizing standard electrophoresis conditions. Positively identified vRh-GFP<sub>pc</sub> mice expressed both the vRh and Cre recombinase genes. Wild type littermates were distinguished as being negative for either vRh or Cre recombinase or both.

**β-Galactosidase Staining**—Animals were deeply anesthetized with 0.2 cc/g Avertin (tribromoethanol; Sigma) and transcardially perfused with 1× PBS followed by a neutral-buffered formalin solution (4% paraformaldehyde). Upon complete perfusion, brains were isolated and post-fixed in the same paraformaldehyde solution for 15 min. Frozen, embedded brains (OCT, Tissue Tek) were cut into 25–30-μm sections on a rotary microtome, mounted onto Superfrost/Plus Microscope Slides (Fisher), allowed to dry at room temperature for 1 h, and permeabilized with PBST (0.2% Triton X-100) for 15 min. Slices were incubated overnight with 1 mg/ml X-gal staining solution (200 mM ferricyanide, Sigma; 200 mM ferrocyanate, Sigma; X-gal (40 mg/ml in DMSO), Sigma; 1 mM MgCl<sub>2</sub>, Sigma; 0.02% Nonidet P-40, Sigma; 1× PBS) at 37 °C in a humid chamber.

**Immunohistochemistry**—Animals were deeply anesthetized with 0.2 cc/g Avertin (tribromoethanol; Sigma) and transcardially perfused with 1× PBS followed by a neutral-buffered formalin solution (4% paraformaldehyde). Upon complete perfusion, brains were isolated and post-fixed in the same paraformaldehyde solution for 1 h followed by a 30% sucrose solution for 24–48 h. Frozen, embedded brains (OCT, Tissue TEK) were cut into 25–30-μm sections on a rotary microtome, mounted onto Superfrost/Plus Microscope Slides (Fisher), and allowed to dry at room temperature for 1 h. Sections were washed with 1× PBST for 15 min and blocked with 2% goat serum (1× PBST, 2 ml goat serum, Invitrogen) for 1 h at room temperature. Primary antibodies (1:200 anti-GFP (Synaptic Systems) and 1:200 anti-calbindin (Swant) or 1:200 anti-GFP (Millipore) and 1:200 anti-GABA<sub>B1</sub>R (Novus Biologicals) were incubated on the sections overnight at 4 °C followed by 3 washes in 1× PBST for 15 min per wash. Anti-species-specific secondary antibodies (anti-mouse Alexa 546 and anti-rabbit Alexa 488 or anti-rabbit Alexa 546 and anti-mouse Alexa 488, Invitrogen) were incubated on the sections for 2 h at room temperature followed by 3 rinses in 1× PBST for 15 min per wash. Images were taken utilizing standard epifluorescence microscopy and processed with Volocity software.

**Nissl Staining**—Sagital sections (30 μm) of transcardially perfused brains were mounted onto Superfrost/Plus Microsoft slides and allowed to air dry for 24 h. To stain and remove the lipids and residual fixation solutions from the tissue, slices were placed into a 1:1 chloroform/ethanol solution for 45 min, 5% cresyl violet acetate for 3 min, and 50% ethanol/acetic acid solution (~4 drops) for 3 min with each step followed with a distilled water wash. After the initial stain, slices were dehydrated by placing them into a 70% ethanol solution for 3 min, 96% ethanol for 3 min, 2 isopropyl alcohol washes for 3 min each. Two 5-min changes of xylened made any unstained parts of the tissue transparent. Finally, coverslips were mounted onto the slides with DePeX mounting medium and allowed to dry overnight. Images were taken on a Zeiss Axioptoph microscope equipped with a CCD camera (SensiCam, PCO, Kelheim, Germany).

**Stereotaxic Surgeries and Cannula Placement**—Three-to-six-month-old male vRh-GFP<sup>pc</sup> and wild type littermates were the subjects of these experiments. All surgeries were performed under aseptic conditions. Rodents were anesthetized using isoflurane for 1 h or less. Sedation was verified by using the gentle toe pinch withdraw reflex. A lubricating ophthalmic ointment was applied to prevent corneal drying during surgery. Mice were mounted into the stereotactic frame (Narishige Group, Model SR-6M) by placing non-rupture ear bars into the ear canals and gently tightened into place. Confirmation of correct ear bar placement was dependent upon complete lateral immobilization of the head. The rodent mouth was secured by using the incisor adapter on the anterior mount of the apparatus. The nose was placed into the nose clamp, and the head was checked for a level position (in regard to the apparatus). Fur from the top of the head was removed and cleaned with 70% ethanol and 10% povidone-iodine. A midline incision was made, and all soft tissue from the skull surface was removed. One 1-mm-wide hole was drilled through the skull with a battery-operated drill at Bregma points −5.88 to −6.24 mm, and two additional 1.5 mm, anterior-lateral holes were drilled for mounting screws. The dura was manually removed. The modified, flanged cannula guide (Plastics One) and skull screws (Plastics One) were cleaned in ethanol and saline and vertically lowered into their correct coordinates. The flanged cannula guide was kept in place by two 2.4-mm long, 1.57-mm wide mounting screws. A cap of dental cement (3M, Rel-X Luting) was applied on top of the head and surrounded the cannula guide. The wound was permanently closed by applying a thin layer of Vetbond tissue adhesive (3 M) to each side of the scalp. Animals were subcutaneously administered 1 ml of sterile saline and recovered on a heating pad in individual cages. In regard to the <i>in vivo</i> electrophysiology, data were obtained by performing the above surgical procedure on three-to-six-month old vRh-GFP<sup>pc</sup>, vRh-GFP(Tg<sup>ΔvRh-GFP</sup>), and C57/B6 mice. Surgeries were identical except that only one hole was
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drilled at Bregma points −5.88 to −6.24 mm. Upon completion of the recording session, mice were euthanized by cervical displacement.

Fiber Optics and Photostimulation—For blue-light photostimulation through the modified cannula guide, a diode-pumped crystal laser (20 milliwatts, 473 nm, CrystalLaser, Reno, NV, BCL-473-020) was coupled into a multimode hard polymer-clad fiber (200-μm core diameter, 0.37 numerical aperture, Thorlabs BFL37–200). The animal behavior photostimulation protocol involved applying a 26-s light pulse to the cerebellar region located directly under the cannula opening. Protocols for the in vivo recordings included a 26-s light pulse applied 10–20 s into each sweep (total sweep time ~1 min).

Optrode Construction—A cleaved multimode glass optical fiber (50-mm core diameter, 0.37 numerical aperture, Thorlabs AFF50/125Y) was stripped of the outer polymer jacket, and a glass-coated tungsten microelectrode (Impedance 1–2.5 megaohm) was attached to the stripped end of the optical fiber with epoxy. The optrode was coupled to a blue laser (Crystal Laser BCL-473–020). Triggering of the laser was controlled by a custom-made Matlab program and a corresponding D/A card.

Electrophysiological Analysis; Brain Slice Recordings—Sagittal sections (250 μm thick) were cut from the cerebellum of P21, C57/B6 mice. Mice were anesthetized with isoflurane and decapitated. The cerebellum was dissected out, cooled, and sliced in an ice-cold solution containing 87 mM NaCl, 75 mM sucrose, 2.5 mM KCl, 0.5 mM CaCl2, 7 mM MgCl2, 1.25 mM NaH3PO4, 25 mM NaHCO3, and 20 mM glucose bubbled with 95% O2 and 5% CO2 with a vibratome (VT1000S, Leica). Slices were kept for at least 1 h at room temperature in a recording artificial cerebral spinal fluid composed of 124 mM NaCl, 3 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.23 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose bubbled with 95% O2 and 5% CO2. Slices were continuously perfused with an external solution containing 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione and 100 μM picrotoxin. Extracellular recordings from Purkinje cells were made at 35 °C with 10 mM baclofen perfused during steady state firing. Patch pipettes (2–4 megohms) were filled with an internal solution comprised of 140 mM potassium methyl sulfate, 4 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM Tris-phosphocreatine, pH 7.3 (KOH). Membrane voltages were recorded with an EPC10/2 amplifier (HEKA). PatchMaster software (HEKA) was utilized to control voltage and data acquisition. Data were further analyzed with Igor Pro 6.0 software (Wavemetrics).

In Vivo Recordings—Extracellular recordings were taken from vermal Purkinje cell layers of adult vRh-GFPPC transgenic mice with an internal solution comprised of 140 mM potassium chloride, 95% O2 and 5% CO2 with a vibratome (VT1000S, Leica). The animal behavior photostimulation protocol involved applying a 26-s light pulse to the cerebellar region located directly under the cannula opening. Protocols for the in vivo recordings included a 26-s light pulse applied 10–20 s into each sweep (total sweep time ~1 min).

Baclofen Application—A Union-40 iontophoresis pump (Kation Scientific) was used for the extracellular delivery of 1 mM baclofen (dissolved in 150 mM NaCl, pH 3.5) or saline through a Carbostar-3 (Kation scientific) carbon electrode, which includes 2 barrels for microiontophoresis. Baclofen was delivered by +50-nA ejection pulses, and retaining currents were ~20 nA. Baclofen or saline was applied for 26 s to the Purkinje cells that had both simple and complex spikes.

Lesion Studies—To confirm the position of the recording electrodes, electrical microlesions were created at different sites after the completion of the in vivo recordings. Lesion areas were at least 700 μm apart. The designated regions received a 10-μA anodic current for 1 min via the recording electrode by using an A365 stimulus isolator (World Precision Instruments). After lesions, mice immediately underwent a paraformaldehyde perfusion.

Behavioral Testing-Accelerating Rotarod—Mice were placed on a 3.0 × 9.5-cm rotating drum of an accelerating rotarod (Columbus Instruments, Rotamex-5 Rotarod). The rod was elevated 44.5 cm above the floor of the apparatus. Although the mice were allowed to acclimate to the rotarod for 1 min before beginning the experimental protocol, no formal prior training was introduced into the testing paradigm. Testing conditions included application of either no light or a 26-s light pulse. Upon receiving photostimulation, mice were promptly placed onto the rotarod where the duration and speed of the run were recorded. Constant acceleration of 40 rotations/min was applied until the mouse fell from the rod and activated the infrared beam. The running duration and rotarod speed at the time of fall were recorded. The runs were consecutively measured 3 times with a 5-min rest period between each run. In the case of no light pulse, animals were allowed to rest for 1 min in between each run. If mice were unable to stay on the rotarod, they were assigned a base-line value of 5 s. The latency to fall and speed were recorded for each mouse. Data were averaged over three trials per mouse.

Grip Strength Test—The muscle strength of wild type and transgenic mice was assessed utilizing the Chatillon DFE Series Digital Force Gauge (AMETEK TCI Division-Chatillon Force Measurement Systems, Largo, FL). The instrument measures both forelimb and hind-limb grip strength in laboratory rodents by employing an electronic digital force gauge that directly calculates the animal’s peak force value exerted upon a pull bar. To measure forelimb grip strength, animals were held by the tail base and lowered at an angle onto the flat wire mesh of the pull bar so that the forelimbs would be exclusively examined. The mouse was slowly pulled away from the bar at ~2.5 cm/s until release, whereby the force gauge recorded the peak tension. Hind-limb grip strength was determined by similar means except that the hind limbs were solely in contact with the pull bar. Measurements were averaged over 5 trials per mouse with and without light pulses and recorded as the peak tension.
(g) and calculated from the force applied to the bar when grasp is released.

**Pole Test**—Balance and motor coordination were examined by calculating the capacity of the mice to navigate an angled pole. Mice were held by the tail and lowered, head-upward onto the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm). The time required for descent to the base of the apparatus was recorded with a maximal duration of 120 s. If the mouse was unable to descend completely and fell off the pole, a maximal default time of 120 s was assigned to the animal. Experimental conditions included no light application and a 26-s light pulse so that each animal was measured twice.

**Balance Beam**—To assess fine motor coordination and balance abilities, the capability to cross a narrow beam onto an enclosed platform was analyzed for each mouse. The horizontally placed, 70-cm long beam was 7 mm in diameter and situated 55 cm above the table surface. One end of the beam was mounted to a small, illuminated supportive area, whereas the other end was fastened to an enclosed (20 cm²) box. Mice underwent training on the beam for 3 days (3 trials a day) before data collection. Briefly, the mouse was placed at an illuminated end of the beam, and the time required to traverse the beam to the safety platform was recorded. In addition to recording the latency, hind-feet slips were also noted. Measurements were taken both with and without light pulses. Data were averaged over three trials per mouse.

**RESULTS**

**Creation of a New Optogenetic Mouse Line vRh-GFP(Tg^vRh-GFP^) for the Controlled Expression of vRh in a Cell Type-specific Manner**—To investigate the cell type-specific function of G_{i/o} pathway activation within neuronal networks *in vivo* and to analyze the functional impact of pathway activation on mouse behavior, we created transgenic mice to specifically activate the G_{i/o}, coupled, light activated GPCR vRh by Cre recombinases. We first identified positive pCZW-fl-Lac-Z-vRh-GFP transgenic founders by genotypic analysis and examination of β-galactosidase expression (Fig. 1A). In this construct, Lac-Z is flanked by loxP sites and followed by the vRh-GFP. The expression of Lac-Z and vRh-GFP is under the control of the ubiquitous chicken β-actin promoter-cytomegalo virus enhancer. The vRh-GFP is only expressed when Lac-Z is excised by Cre recombinases, whereas LacZ is present throughout the central nervous system (CNS) when Cre is not expressed (Fig. 1A) (14). By performing β-galactosidase staining of both coronal and sagittal sections, we were able to visualize abundant LacZ expression throughout the CNS. Staining was especially robust in the cerebellum, hippocampus, and caudate putamen (Fig. 1A) and was also detected in other tissues outside the CNS such as gut, pancreas, and stomach. To demonstrate that vRh-GFP expression can be induced cell type-specifically, we crossed mice that expressed Cre recombinase under the PCP2/L7 promoter with pCZW-fl-Lac-Z-vRh-GFP mice (Fig. 1B) for the selective expression of vRh-GFP in cerebellar PCs (13). We call this mouse line vRh-GFP<sub>PC</sub>. Immunohistochemical staining with GFP and calbindin antibodies verified that vRh expression was exclusive to PCs in mice that had undergone site-specific recombination (Fig. 1B). Upon closer examination, we detected vRh in the PC soma in a punctate pattern and in the proximal dendrites. Thus, the vRh-GFP(Tg<sup>vRh-GFP</sup>) mouse allows for cell type-selective, Cre recombinase-mediated expression of vRh-GFP.

**G_{i/o} Pathway Activation by vRh in Vivo Reduces the Frequency of PC Firing**—We first examined if activation of vRh by light would modulate PC firing as would be expected from GPCRs coupling to the G_{i/o} pathway. To test our hypothesis, an optrode coupled to a laser delivered a 26-s 473-nm light pulse to vermal PCs *in vivo*. Throughout the experiments PCs were selected by their characteristic regular spiking pattern and by the occurrence of complex and simple spikes. Additionally, we confirmed the location of the *in vivo* recording site by an electrolytic lesion at the end of the experiments (Fig. 2A). The recording paradigm consisted of an initial 10-s recording of simple and complex spikes followed by a 26-s light pulse and a post-light recording of 30 s. vRh-GFP<sup>PC</sup> mice exhibited a 30.8 ± 4.5% (n = 9) reduction in the spontaneous firing rate in comparison to a 10.9 ± 6.3% (n = 10) increase in firing in control mice when the 26-s light pulse was applied (Fig. 2, B and C). No change in the CV was observed before and after light stimulation (control before and after light, 0.46 ± 0.03 and 0.47 ± 0.03 (n = 10); vRh-GFP<sup>PC</sup> before and after light, 0.57 ± 0.06 and 0.61 ± 0.07 (n = 9); Fig. 3D). Post-light recordings indicated that reduction of firing persisted for at least 30 s after the light was switched off (−28.4 ± 7.6% (n = 9); Fig. 2D). Thus, our data show that light activation of vRh, selectively expressed in PC, reduce the firing frequency of PCs *in vivo*.

**In Vitro and In Vivo Application of Baclofen Reduces the Spontaneous Firing Rate of Purkinje Neurons**—We next investigated if the activation of the G_{i/o} pathway within PC by an endogenously expressed G_{i/o}-coupled GPCR such as GABA<sub>B</sub>R would induce comparable modulation of PC firing as observed for the light activation of vRh. Because we are interested in comparing the effects of GABA<sub>B</sub>R-mediated G_{i/o} activation to vRh, we first compared the expression between vRh and GABA<sub>B</sub>R in cerebellar PCs. Immunohistochemical staining of sagittal cerebellar sections revealed that GABA<sub>B</sub>R expression is present in both granule and Purkinje cells and can be detected in cell bodies, dendrites, and spines of PCs (Fig. 3A) (12, 15). Overlay studies revealed colocalization between GFP and GABA<sub>B</sub>R expression in the soma and proximal dendrites in PCs (Fig. 3A), suggesting the possibility that in these subcellular regions, G_{i/o} pathway activation by light could potentially activate GABA<sub>B</sub>R downstream targets, but this idea needs to be further investigated.

To investigate how GABA<sub>B</sub>R activation influences the firing properties of PCs *in vivo*, we iontophoretically applied the GABA<sub>B</sub>R agonist baclofen in 3-month-old mice. A 26-s lasting iontophoretic application of 1 mM baclofen led to a reduction in the firing frequency by 33.6 ± 12.3% (n = 8), which was significantly different from the 5.3 ± 4.1% (n = 10) reduction in firing frequency when saline was applied (Fig. 3, B and C). No change in the CV was detected before and after application of baclofen or saline (Fig. 3D; CV before and after saline application, 0.49 ± 0.06 and 0.52 ± 0.06 (n = 10); CV before and after 1 mM baclofen application, 0.62 ± 0.07 and 0.69 ± 0.08 (n = 8)). A 22.0 ± 11.9% (n = 8) reduction in firing frequency was still
observed 30 s after baclofen wash out (Fig. 3B). In conclusion, GABABR activation by baclofen in the PC layer of anesthetized mice caused a reduction in the firing rate of PCs.

To investigate if the reduction in firing frequency is caused by intrinsic or extrinsic PC modulation, we performed extracellular recordings of PC firing in cerebellar slices from 4-week-old mice and blocked the inhibitory as well as excitatory inputs into PCs with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione and 100 μM picrotoxin. We concentrated on tonically firing PCs and excluded PCs demonstrating a trimodal spiking activity. Application of 10 μM baclofen reduced the AP firing by 21.9 ± 4.1% (n = 5) (Fig. 3, F and G). Again, no change in the CV was detected before and after baclofen application (Fig. 3H; CV before and after baclofen application, 0.09 ± 0.014 and 0.09 ± 0.013 (n = 5)). Thus, GABABR activation by baclofen in PCs in vivo induced a reduction in firing frequency as observed by light activation of vRh, suggesting that vRh and GABABR activate a similar intracellular signaling pathway to modulate PC firing.

Photostimulation of vRh in Purkinje Cells Alters Motor Behavior—To investigate the functional consequence of Gi/o-mediated modulation of PC firing, we implanted a laser guide positioned on top the cerebellum to illuminate the cerebellar cortex (Fig. 4A). We chose the anterior vermis as the specific illumination area because it is known to be involved in balance, equilibrium, and motor execution (16–19). In all motor tests administered, a significant difference was detectable between wild type and transgenic vRh-GFP(Tg<sup>pcP2-cre<sup>) adult mice after a 26-s long light stimulus was applied to the vermis. Specifically, vRh-pos-
itive mice either fell off the pole after light delivery (scored as 120 s) or took at least twice as long to descend to the bottom of the pole (Fig. 4B, wild type pre-pulse 17.51 ± 1.67 s; post-pulse 11.63 ± 0.87 s; vRh-GFP<sup>PC</sup> pre-pulse 18.85 ± 2.87 s; post-pulse 102.1 ± 12.4 s; n = 10, ANOVA; ***<sup>,</sup> p < 0.0001). The accelerating rotarod test was administered by delivering a pulse of light at the beginning of the experiment followed by a performance evaluation without light application. This behavioral paradigm was designed this way to control for the possibility that the duration of time spent on the rotarod would increase because of the acquisition of motor skill learning regardless of transgenic expression and could potentially mask any effects that light activation of vRh may have on firing and behavioral output (20). Accelerating rotarod testing revealed that the vRh-GFP<sup>PC</sup> mice stay on the accelerating rod for a shorter amount of time after light application in comparison to wild type mice (Fig. 4C, wild type 93.25 ± 9.63 s versus vRh-GFP<sup>PC</sup> mice 72.65 ± 13.7 s; n = 10, ANOVA, **<sup>,</sup> p < 0.001). There was no significant difference in the time spent on the rotarod without any light application between the two groups of mice (wild type 109.99 ± 10.57 s versus vRh-GFP<sup>PC</sup> mice 101.61 ± 14.05 s n = 10). Beam walk testing (Fig. 4D) also revealed that the modulation on motor behavior was dependent on light (wild type pre-pulse 13.27 ± 2.14 s; post-pulse 10.72 ± 1.38 s; vRh-GFP<sup>PC</sup> pre-pulse 7.43 ± 0.53 s; post-pulse 17.36 ± 4.37 s; n = 10, ANOVA; *, p < 0.05).

As an additional control for each behavioral test, grip strength for both hind and front paws was analyzed before and after light treatment. These tests were performed to demon-
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A. GABA<sub>δ</sub>-R1 and VRh-GFP

B. Change in Firing (%)

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C. Firing Rate (Hz)

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D. CV

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E. 24.0 Hz

(+ Saline

25.0 Hz

(+ 1 mM Baclofen

15.5 Hz

0.2 mV

500 ms

F. Change in Firing (%)

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G. Firing Rate (Hz)

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H. CV

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I. 40.1 Hz

(+ 10 μM Baclofen

27.2 Hz

0.2 mV

200 ms
GABAB1R in both PCs and cerebellar cortical neurons. vRh-GFP(TgflvRh-GFP) mice were crossed with PCP2/L7-Cre to guarantee precise temporal control, which cannot be achieved (3, 23, 24). The advantage of using light-activated proteins is the concept of two new approaches that circumvent the use of traditional receptor-specific agonists and antagonists. The first consists of a chemical approach that utilizes engineered GPCRs such as DREADDs (designer receptors exclusively activated by a designer drug) that are activated by inert chemical compounds (21, 22). The second technique is a physical scheme that employs light-activated proteins to evoke intracellular signaling pathways, like PTX-sensitive G1/o-coupled vRh in neurons (3, 23, 24). The advantage of using light-activated proteins is the guaranteed precise temporal control, which cannot be achieved with application of chemical compounds. To further develop and utilize this tool for cell type-specific applications, we created mice whose expression of vRh-GFP was dependent upon the use of cell type-specific expression of Cre recombinase. The vRh-GFP(TgflvRh-GFP) mice were crossed with PCP2/L7-Cre recombinase (Tg^Pcp2-cre) mice for selective expression of vRh-GFP in PCs (13). The vRh expression was induced 1 week after birth when Cre expression was found to be restricted to PCs of vRh-GFP^PC mice (Fig. 1B). To visualize vRh-GFP after 1–3 months of age, an antibody against GFP had to be used, suggesting that the vRh-GFP concentration within PCs is low. Despite the potential lower expression levels, light stimulation of vRh in vivo led to a significant reduction of AP firing in PCs that was comparable with the effects induced by application of GABA_β_2R agonist, baclofen. As shown by the intense lacZ staining, especially within hippocampus and basal ganglia (Fig. 1A), the vRh-GFP(Tg^flvRh-GFP) mouse line is a promising tool that could be used in the investigation of G1/o signaling in other neuronal populations. According to our studies in PCs, vRh-GFP(Tg^flvRh-GFP) mice provide a new optogenetic tool for the analysis of in vivo function of GPCRs.

**Modulation of Simple Spikes in the Medial Cerebellar Regions Leads to Changes in Motor Behavior**—One of the surprising findings of our study was that a ~20–30% reduction in vermal PC firing was sufficient to cause motor deficits in freely behaving mice. This finding was especially remarkable because the expression level of vRh appeared to be relatively low and limited throughout cerebellar PCs. Although no quantitative measurement was taken of expression levels, vRh was only visible with antibody application. The seemingly restricted vRh concentration in vermal PCs not only exhibits the necessity to create an alternative and optimized method for in vivo expression but also highlights the magnitude of influence that the G1/o pathway has on motor control and the endogenous firing properties of PCs. Numerous examinations of the cerebellum and specifically the vermal cerebellar region have indicated that this area plays a pivotal role in regulating extensor tone, sustaining upright stance, and dynamic balance control (18, 19, 25). It is thought that the cerebellum employs anticipatory and feedback mechanisms to maintain balance during locomotion and that failure in these systems induce an ataxic-like phenotype (19, 26, 27). Behavioral testing revealed that the photostimulation of positive vermal PCs in vRh-GFP^PC mice induced changes in motor output. Specifically, an overall lack of balance, coordination, and performance was quite apparent with positive transgenic mice that significantly differed from control littermates. These results are consistent with prior studies that have examined the correlation between vermal lesions and gait ataxia, postural defects, and motor coordination difficulties and highlight the importance of G1/o modulation of PC firing for motor control. As a side note, an early examination of light delivery to positively expressing vRh PCs indicated that the optimal length of activation was around 20 s. Similar behavioral responses could be elicited with longer light pulses but was ultimately found to be unnecessary. Furthermore, brief pulses of light were unable to reliably evoke changes in the intrinsic firing properties of vermal PCs.

**Considerations for Controlling G1/o Signaling in Vivo by Light**—Although we have provided an effective means to modulate the activity of a single neuronal population and network, there are significant differences between wild type and positive transgenic mice detected throughout the behavioral tests were attributable to the photoactivation of vRh and are not a result of insufficient strength or muscle ability. Measurements of front grip strength revealed no significant difference between the two groups both before and after light application (Fig. 4E; wild type pre-pulse 73.0 ± 4.99 g; post-pulse 54.9 ± 4.55 g; vRh-GFP^PC pre-pulse 73.0 ± 3.14 g; post-pulse 51.9 ± 1.76 g; n = 10, ANOVA; not significant). There were also no indications of changes in hind grip strength before and after light application (Fig. 4F; wild type pre-pulse 24.5 ± 1.9 g; post-pulse 22.0 ± 1.26 g; vRh-GFP^PC pre-pulse 23.67 ± 2.82 g; post-pulse 21.1 ± 2.31 g; n = 10 ANOVA; not significant). Thus, our results indicate that G1/o-mediated modulation of PC firing is sufficient to alter motor coordination in behaving mice.

### DISCUSSION

vRh-GFP(Tg^flvRh-GFP) Mouse for the Cell Type-selective Control of G1/o Signaling—The pursuit to gain a more thorough understanding of the physiological roles of cell type-specific GPCR signaling in vivo and in vitro has resulted in the development of new tools that circumvent the use of traditional receptor-specific agonists and antagonists. The first consists of a chemical approach that utilizes engineered GPCRs such as DREADDs (designer receptors exclusively activated by a designer drug) that are activated by inert chemical compounds (21, 22). The second technique is a physical scheme that employs light-activated proteins to evoke intracellular signaling pathways, like PTX-sensitive G1/o-coupled vRh in neurons (3, 23, 24). The advantage of using light-activated proteins is the guaranteed precise temporal control, which cannot be achieved with application of chemical compounds. To further develop and utilize this tool for cell type-specific applications, we created mice whose expression of vRh-GFP was dependent upon the use of cell type-specific expression of Cre recombinase. The vRh-GFP(Tg^flvRh-GFP) mice were crossed with PCP2/L7-Cre recombinase (Tg^Pcp2-cre) mice for selective expression of vRh-GFP in PCs (13). The vRh expression was induced 1 week after birth when Cre expression was restricted to PCs of vRh-GFP^PC mice (Fig. 1B). To visualize vRh-GFP after 1–3 months of age, an antibody against GFP had to be used, suggesting that the vRh-GFP concentration within PCs is low. Despite the potential lower expression levels, light stimulation of vRh in vivo led to a significant reduction of AP firing in PCs that was comparable with the effects induced by application of GABA_β_2R agonist, baclofen. As shown by the intense lacZ staining, especially within hippocampus and basal ganglia (Fig. 1A), the vRh-GFP(Tg^flvRh-GFP) mouse line is a promising tool that could be used in the investigation of G1/o signaling in other neuronal populations. According to our studies in PCs, vRh-GFP(Tg^flvRh-GFP) mice provide a new optogenetic tool for the analysis of in vivo function of GPCRs.
FIGURE 4. Light activation of vertebrate rhodopsin expressed in Purkinje cells of the cerebellum induces changes in motor behavior. A, shown is a photograph demonstrating the permanent placement of the cannula light guide used for behavioral testing. B, shown is pole test performance of control and vRh-GFP<sup>PC</sup> mice (n = 10) before and after a 26-s light pulse. The light activation of vRh in vRh-GFP<sup>PC</sup> mice results in either a fall (scored as 120 s) or an increase in the time required to descend from the pole. Light application to control littermates did not initiate any significant difference in the time required to descend the pole. C, rotarod performance of wild type littermates (n = 10) and vRh-GFP<sup>PC</sup> mice before and after a 26-s light pulse is shown. Light activation of vRh in vRh-GFP<sup>PC</sup> mice produces a significant decrease in rotarod performance when compared with wild type littermates. Performance between the two groups when no light pulse was applied reveals no significant difference. D, beam walk analysis demonstrates an increase in the time required to successfully cross the beam after vRh activation in vRh-GFP<sup>PC</sup> mice. Conversely, the time needed to cross the beam decreases in control littermates regardless of the light pulse. Falls were assigned a value of 120 s. Additionally, a measurement of the number of paw slips reveals a significant increase after light application for the left side of the vRh-GFP<sup>PC</sup> mice, whereas control littermates experienced no significant increase in slips post-light application. E, grip strength assessment of wild type and vRh-GFP<sup>PC</sup> mice before and after a 26-s light illumination is shown. No significant differences were observed for the grip strength of the front and hind paws between wild type littermates and vRh-GFP<sup>PC</sup> mice before and after light application. Statistical significance in all behavior experiments was evaluated with ANOVA (*, p < 0.05; **, p < 0.01). Shown values are the mean ± S.E.
several concerns associated with this study that may be influenced by the overall methods utilized. These potential issues include the extent and range of light penetration within the cerebellum and the presence of any plausible variables related to light delivery that may influence the in vivo behavioral and/or electrophysiological testing. Previous studies investigating the feasibility of controlling neuronal excitability in a non-invasive and light-dependent manner revealed that vRh promoted the modulation of G protein-coupled inward rectifying K\(^+\) and P/Q-type Ca\(^{2+}\) channels via a functional coupling to the pertussis toxin-sensitive, G\(\alpha_{i/o}\) protein pathway (3). Because vRh couples to the G protein transducin, whereby the \(\alpha\) subunit belongs to the G\(\alpha_i/o\) subfamily, these findings offer supporting evidence that mammalian rhodopsins are capable of coupling to other G\(\alpha_i/o\) family members in vitro. To examine the possibility that vRh may also promote the precise spatiotemporal control of the G\(\alpha_i/o\) pathway in vivo, we established an investigation that focused on the function of this pathway in animal behavior and system coordination such as motor control. Activation of the G\(\alpha_i/o\) pathway in a membrane delimited way is the main inhibitory action of GPCRs on neuronal excitability (2). Many different transmitters, such as glutamate, acetylcholine, serotonin (5-HT), or GABA couple via specific GPCRs to the G\(\alpha_i/o\) pathway, which are expressed throughout the brain. Among them, the GABA\(\beta\)R is widely distributed throughout the brain including the cerebellum (28) and is located in the granule cell, PC, and molecular layer. Within the molecular layer, GABA\(\beta\)Rs are found at the presynaptic terminals of parallel fibers and at the PC dendrites and spines (15, 29, 30). Taking all of this into consideration, our in vivo data seem to suggest that the photoactivation of vRh in vermal PCs acts via the G\(\alpha_i/o\)-mediated signaling pathway in general. Up to this point, we have not detected the activation of other G protein pathways using vRh such as G\(\alpha_i\) or G\(\alpha_o\) in cellular or neuronal culture systems. Our attempt to control endogenous G\(\alpha_i/o\) signaling by exogenously expressed vRh still remains to be further developed. There are several matters to consider that may influence the feasibility of controlling G\(\alpha_i/o\) signaling and include the following. First, G\(\alpha_i/o\)-coupled GPCRs have a variety of downstream signaling targets and have a binding preference to each of their respective targets. Second, more than one type of G\(\alpha_i/o\)-coupled GPCR is expressed in a single neuron and spreads in a specific distribution pattern. Last, some G\(\alpha_i/o\)-coupled receptors can form heterodimers with other types of GPCRs. Although we recently demonstrated the ability to target and modify GPCRs by tagging vRh with the C-terminal signaling domain of a specific GPCR and were able to control 5-HT\(_{1A}/G\alpha_i/o\)-specific signaling properties of neurons (24), further ingenuity is required to overcome the intrinsic issues presented thus far. An additional point of contention surrounds the idea that GABA\(\beta\)Rs in dissociated PCs have been suggested to inhibit P/Q-type Ca\(^{2+}\) channels (31) and establish a heterodimeric functional coupling with mGluR1 at postsynaptic sites of the PF-PC synapse (11, 32) and are thought to be involved in synaptic plasticity (33, 34). Therefore, future studies should be focused on investigating which downstream signaling pathway is activated and whether discrete motor learning tasks can be modulated by the photoactivation of vRh. An additional concern focuses on the delivery of a maximal but specific and controlled amount of light to the brain tissue. To achieve this goal, a stripped, multimode optical fiber (200 \(\mu\)m diameter) was coupled to a blue laser light (20 milliwatts of power at 473 nm) and affixed above the cerebellar vermis. It is understood that the light-scattering properties within the brain are influenced by species and age, incident wavelength, and physiological characteristics of the tissue (35–38). Specifically, the blue laser light utilized (473 nm) for this study has been described as having a high propensity for scattering within the brain and is also weakly absorbed (36–38). The specifics of this optode have been previously characterized in detail, and it had been estimated that the fiber tip produces a total tissue volume experiencing \(\approx 1\) milliwatt mm\(^{-2}\) light intensity to be \(\sim 0.5\) mm\(^2\) (36). These fiber optic specifics correlate with our data in that the most significant decrease in the firing rate of vermal PCs was elicited in neurons located at more superficial tissue depths, thereby supporting the notion that increase tissue depth corresponds to a lower level of light intensity.

In summary, we generated a new mouse line that allows for the cell type-specific activation and modulation of the G\(\alpha_i/o\) pathway through vRh and demonstrated the feasibility of modifying the firing properties of a single neuronal population through the utilization of light. Thus, for the first time our experimental results revealed that the in vivo modulation of the G\(\alpha_i/o\) protein pathway in PCs has a significant functional influence on motor control and coordination.

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REFERENCES


Expression of Vertebrate Rhodopsin in Purkinje Cells

# In vivo delivery

- In vivo delivery method
- Fiber optic specifics
- Power usage
- Light intensity

# In vivo behavior

- Motor learning tasks
- Light delivery
- Feasibility of controlling neuronal excitability

# References

Expression of Vertebrate Rhodopsin in Purkinje Cells
