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OPTOGENETIC CONTROL OF MOTOR COORDINATION BY $G_{i/o}$ PROTEIN-COUPLED VERTEBRATE RHODOPSIN IN CEREBELLAR PURKINJE CELLS.

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Running head: Expression of vertebrate rhodopsin in Purkinje cells

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G protein-coupled receptors (GPCRs) are involved in the modulation of complex neuronal networks in the brain. In order to investigate the impact of a cell-specific $G_{i/o}$ protein-mediated signalling pathway on brain function, we created a new optogenetic mouse model in which the $G_{i/o}$ protein-coupled receptor vertebrate rhodopsin (vRh) can be cell-specifically expressed with the aid of Cre recombinase. Here we use this mouse model to study the functional impact of Gi/o modulation in cerebellar Purkinje cells (PC). We show that *in vivo* light activation of vRh specifically expressed in PCs reduces simple spike firing that is comparable to 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 signalling in cerebellar PCs affects motor coordination and show a new promising approach for studying the physiological function of GPCR-mediated signalling in a cell-type specific manner.

The G protein-mediated signalling 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 signalling pathway is the primary role in which GPCRs 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 (GIRK) and voltage-gated Ca^{2+} channels via pertussis toxin-sensitive $G_{i/o}$ protein-mediated signaling (3). Therefore, vRh may allow for the precise spatio-temporal 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 are expressed throughout the brain and are 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 ($GABA_BR$) (6-8). $GABA_BR$ activation by application of the selective agonist baclofen, leads to a reduction in PC firing most likely due to membrane hyperpolarization induced by GIRK 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*, since $GABA_BRs$ and other Gi/o coupled receptors are expressed in various cell-types in the cerebellum and can only be activated by slowly diffusing drugs.

In order 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 cordination.

EXPERIMENTAL PROCEDURES

Generation and Screening of transgenic Mice- In order to generate a colony of *vRh-GFP*^{PC} transgenic mice, homozygous transgenic Purkinje cell specific CRE ($Tg^{Pcp2\text{-}cre}$) mice (13) were crossed with heterozygous vRh-GFP($Tg^{flvRh\text{-}GFP}$) 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. Following tissue dissolution, the mixture was heated to 99°C for 10 minutes and then cooled to room temperature. A PCR master mix contained either of the following oligos: vRh-GFP (5' CATGCTCACCACCGTCTGCT and 5' AAGATGGTGCGCTCCTGGAC) or Cre-Recombinase (5' TCTCACGTACTGACGGTGG and 5' ACCAGCTTGCATGATCTCC). The 50 ml final PCR reaction contained 1 ml gDNA, 1 ml of each primer, 1 ml dNTP mix (10 mM each of dATP, dTTP, dCTP, dGTP; New England Biolabs (NEB)), 5 ml 10X Thermopol II Reaction Buffer (NEB), 5 ml dimethyl sulfoxide, 0.5 ml Taq Polymerase (NEB) and 35.5 ml dH_20. PCR reactions were run on an Eppendorf thermocycler, using the following conditions: 92°C for 30 s, 60°C for 45 s and 72°C for 1 min run for 40 cycles or 95°C for 30 s, 55°C for 1 min and 72°C for 1 min 30 sec 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*^{PC} 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 anaesthetized with 0.2cc/g Avertin (tribromoethanol; Sigma) and transcardially perfused with 1X 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 minutes. Frozen, embedded brains (OCT, Tissue TEK) were cut into 25-30 micron section on a rotary microtome, mounted onto Superfrost/Plus Microscope Slides (Fisher), allowed to dry at room temperature for 1 hour and permeabilized with PBST (0.2% Triton X-100) for 15 minutes. Slices were incubated overnight with 1mg/ml X-gal staining solution (200 mM ferricyanate; Sigma, 200 mM ferrocyanate; Sigma, X-gal (40 mg/ml in DMSO); Sigma, 1 M $MgCl_2$; Sigma, 0.02% NP40; Sigma, and 1X PBS) at 37°C in a humid chamber.

Immunohistochemistry- Animals were deeply anaesthetized with 0.2cc/g Avertin (tribromoethanol; Sigma) and transcardially perfused with 1X 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 hour followed by a 30% sucrose solution for 24-48 hours. Frozen, embedded brains (OCT, Tissue TEK) were cut into 25-30 micron section on a rotary microtome, mounted onto Superfrost/Plus Microscope Slides (Fisher) and allowed to dry at room temperature for 1 hour. Sections were washed with 1X PBST for 15 minutes and blocked with 2% goat serum (1X PBST, 2 ml goat serum, Invitrogen) for 1 hour 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-$GABA_{B1}$ R, Novus Biologicals) were incubated on the sections overnight at 4°C, followed by three washes in 1X PBST for 15 minutes 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 hours at room temperature, followed by three rinses in 1X PBST for 15 minutes 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 hours. In order 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 minutes, 5% cresyl violet acetate for 3 minutes and 50% ethanol/acetic acid solution (approximately 4 drops) for 3 minutes with each step followed with a distilled water wash. Following the initial stain, slices were dehydrated by placing them into a 70% ethanol solution for 3 minutes, 96% ethanol for 3 minutes, two isopropanol washes for 3 minutes each. Two, 5 minute changes of xylene 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 an Zeiss Axiophot equipped with a CCD camera (SensiCam, PCO, Kelheim, Germany).

Stereotaxic Surgeries and Cannula Placement-Three to six-month old male *vRh-GFP^{PC}* and wild type littermates were the subjects of these experiments. All surgeries were performed under aseptic conditions. Rodents were anaesthetized using isoflurane for one hour 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's 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 regards to the apparatus). Fur from the top of 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 -5.88mm to -6.24mm 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, Rely-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 (3M) 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 regards to the *in vivo* electrophysiology, data were obtained by performing the above surgical procedure on three to six-month old *vRh-GFP^{PC}*, vRh-GFP($Tg^{flvRh\text{-}GFP}$) and C57/B6 mice. Surgeries were identical except that only one hole was drilled at Bregma points -5.88mm to -6.24mm. 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 mW, 473 nm, CrystaLaser, 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 second light pulse to the cerebellar region located directly under the cannula opening. Protocols for the *in vivo* recordings included a 26 second light pulse applied 10-20 seconds into each sweep (total sweep time approximately 1 minute).

Optrode Construction- A cleaved multimode glass optical fiber (50 mm core diameter, 0.37 numerical aperature, Thorlabs AFS50/125Y) was stripped of the outer polymer jacket and a glass coated tungsten microelectrode (Impedance 1-2.5MOhm) 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 anaesthetized 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 $CaCl_2$, 7 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 20 mM glucose bubbled with 95% O_2 and 5% CO_2 with a vibratome (VT1000S, Leica). Slices were kept for at least 1 hour at room temperature in a recording artificial cerebral spinal fluid composed of 124 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose bubbled with 95% O_2 and 5% CO_2. Slices were continuously perfused with an external solution containing 10 µM CNQX and 100 µM picrotoxin. Extracellular recordings from Purkinje cells were made at (temperature) with 10 mM baclofen being perfused during steady state firing. Patch pipettes (2-4 megaohms) 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 was further analyzed with Igor Pro 6.0 software (Wavemetrics).

In Vivo Recordings- Extracellular recordings were taken from vermal Purkinje cell layers of adult *vRh-GFP*^{PC} and wild type littermates that underwent stereotactic surgery. Recordings from actual Purkinje cells were confirmed by the presence of both complex and simple spikes. The custom optrode was lowered into the vermis and recordings were taken from cells ranging in depth from 1100 to 3200 mm below the surface. Activity was amplified and filtered (bandpass 0.5 to 9 kHz) with a multi-channel spike sorter (Plexon Inc., Austin, TX) and stored on a computer disk with a sampling rate of 32 kHz. During off-line analysis, simple spikes and complex spikes were discriminated using custom made software implemented in Matlab (MathWorks, Natick, MA). Single cell spike activity was used to calculate mean firing rates and inter spike intervals. The coefficient of variation (CV) of the simple spike interspike intervals was calculated to quantify the variability in spike activity.

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 + 50nA ejection pulses and retaining currents were -20nA. Baclofen or saline was applied for 26 seconds to the Purkinje cells that had both simple and complex spikes.

Lesion Studies- In order to confirm the position of the recording electrodes electrical microlesions were created at different sites following 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 minute via the recording electrode by using an A365 stimulus isolator (World Precision Instruments). Following lesions, mice immediately underwent a paraformaldehyde perfusion.

Behavioral Testing- Accelerating Rotarod- Mice were placed on a 3.0 cm x 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. While the mice were allowed to acclimate to the rotarod for 1 minute 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 second 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 time of fall were recorded. The runs were consecutively measured, three 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 baseline value of 5 seconds. The latency to fall and speed were recorded for each mouse. Data was averaged over 3 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, Fla). The instrument measures both fore- and hindlimb 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 approximately 2.5 cm/sec until release whereby the force gauge recorded the peak tension. Hindlimb grip strength was determined by similar means except that the hindlimbs were solely in contact with the pull bar. Measurements were averaged over 5 trials per mouse with and without light pulses and are recorded as the peak tension (g) and is 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 seconds. If the mouse was unable to descend completely and fell off the pole, a maximal default time of 120 sec was assigned to the animal. Experimental conditions included no light application and a 26 sec light pulse so that each animal was measured twice.

Balance Beam- In order 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 50 cm above the table surface. One end of the beam was mounted to a small, illuminated supportive area while the other end was fastened to an enclosed (20 cm^2) box. Mice underwent training on the beam for three 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 was averaged over 3 trials per mouse.

RESULTS

Creation of a new optogenetic mouse line vRh-GFP($Tg^{flvRh\text{-}GFP}$) for the controlled expression of vRh in a cell-type specific manner. To investigate the cell-type specific function of Gi/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 Gi/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 (Figure 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-cytomegalovirus enhancer. The vRh-GFP is only expressed when Lac-Z is excised by Cre recombinases, while LacZ is present throughout the central nervous system (CNS) when Cre is not expressed (Figure 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 (Figure 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-LacZ-vRh-GFP mice (Figure 1B) for the selective expression of vRh-GFP in cerebellar PCs (13). We call this mouse line *vRh-GFP*^{PC}. Immunohistochemical staining with GFP and calbindin antibodies verified that vRh expression was exclusive to PCs in mice that had undergone site-specific recombination (Figure 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$^{flvRh\text{-}GFP}$) mouse allows for the for cell-type selective, Cre recombinase mediated expression of vRh-GFP.

Gi/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 Gi/o pathway. To test our hypothesis, an optrode coupled to a laser delivered a 26 second 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 (Figure 2A). The recording paradigm consisted of an initial 10 sec recording of simple and complex spikes followed by a 26 sec light pulse and a post-light recording of 30 sec. *vRh-GFP*^{PC} mice exhibited an 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 sec light pulse was applied (Figure 2B and 2C). No change in the coefficient of variation (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*^{PC} before and after light, 0.57 ± 0.06 and 0.61 ± 0.07 (n=9), Figure 3D). Post-light recordings indicated that reduction of firing persisted for at least 30 sec after light was switched off (-28.4 ± 7.6% (n=9), Figure 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 Gi/o pathway within PC by an endogenously expressed Gi/o coupled GPCR such as $GABA_B$-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 $GABA_B$-R mediated Gi/o activation to vRh, we first compared the expression between vRh and $GABA_{B1}$-R in cerebellar PCs. Immunohistochemical staining of sagittal cerebellar sections revealed that $GABA_{B1}$-R expression is present in both granule and Purkinje cells and can be detected in cell bodies, dendrites and spines of PCs (Figure 3A) (12,15). Overlay studies revealed colocalization between GFP and $GABA_{B1}$-R expression in the soma and proximal dendrites in PCs (Figure 3A), suggesting the possibility that in these subcellular PC regions, Gi/o pathway activation by light could potentially activate $GABA_B$-R downstream targets but this idea needs to be further investigated.

In order to investigate how $GABA_BR$ activation influences the firing properties of PCs *in vivo,* we iontophoretically applied the $GABA_BR$ agonist baclofen in 3 month old mice. A 26s 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 (Figure 3B and 3C). No change in the CV was detected before and after application of baclofen or saline (Figure 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 sec after baclofen wash out (Figure 3B). In conclusion, $GABA_BR$ activation by baclofen in the PC layer of anaesthetized mice caused a reduction in the firing rate of PCs.

In order 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

CNQX 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) (Figure 3F and 3G). Again, no change in the CV was detected before and after baclofen application (Figure 3H; CV before and after baclofen application, 0.09 ± 0.014 and 0.09 ± 0.013 (n=5)). Thus, $GABA_B$-R activation by baclofen in PC *in vivo* induced a reduction in firing frequency as observed by light activation of vRh, suggesting that vRh and $GABA_B$-R activate a similar intracellular signalling pathway to modulate PC firing.

Photostimulation of vRh in Purkinje cells alters motor behavior. In order 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 (Figure 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*^{PC} adult mice after a 26s long light stimulus was applied to the vermis. Specifically, vRh positive mice either fell off the pole after light delivery (scored as 120 sec) or took at least twice as long to descend to the bottom of the pole (Figure 4B, wild type pre-pulse 17.51 ± 1.67 seconds; post-pulse 11.63 ± 0.87 seconds; *vRh-GFP*^{PC} pre-pulse 18.85 ± 2.87 seconds; post-pulse 102.1 ± 12.4 seconds; n= 10, ANOVA ***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*^{PC} mice stay on the accelerating rod for a shorter amount of time after light application in comparison to wild type mice (Figure 4C, wild type 93.25 ± 9.63 seconds versus *vRh-GFP*^{PC} mice 72.65 ± 13.7 seconds; n=10, ANOVA **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 seconds versus *vRh-GFP*^{PC} mice 101.61 ± 14.05 seconds n=10). Beam walk testing (Figure 4D) also revealed that the modulation on motor behavior was dependent on light (wild type pre-pulse 13.27 ± 2.14 seconds; post-pulse 10.72 ± 1.38 seconds; *vRh-GFP*^{PC} pre-pulse 7.43 ± 0.53 seconds; post-pulse 17.36 ± 4.37 seconds; 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 demonstrate that any 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 (Figure 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 n.s.). There were also no indications of changes in hind grip strength before and after light application (Figure 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 n.s.). Thus our results indicate that Gi/o-mediated modulation of PC firing is sufficient to alter motor coordination in behaving mice.

DISCUSSION

vRh-GFP(Tg$^{flvRh\text{-}GFP}$) mouse for the cell-type selective control of Gi/o signalling. The pursuit to gain a more thorough understanding of the physiological roles of cell-type specific GPCR signalling *in vivo* and *in vitro* has resulted in the development 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, which

are activated by inert chemical compounds (21,22). The second technique is a physical scheme that employs light-activated proteins to evoke intracellular signalling pathways, like PTX-sensitive $G_{i/o}$-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($Tg^{flvRh\text{-}GFP}$) mice were crossed with PCP2/L7-Cre recombinase ($Tg^{Pcp2\text{-}cre}$) mice for selective expression of vRh-GFP in PCs (13). The vRh expression was induced one week after birth following Cre-expression and was restricted to PCs of *vRh-GFP*^{PC} mice (Figure 1B). In order to visualize vRh-GFP after 1-3 months of age, an antibody against GFP had to be used, suggesting that the vRh-GPF 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 to the effects induced by application of $GABA_BR$ agonist, baclofen. As shown by the intense lacZ staining, especially within hippocampus and basal ganglia (Figure 1A), the vRh-GFP($Tg^{flvRh\text{-}GFP}$) mouse line is a promising tool that could be used in the investigation of $G_{i/o}$ signalling in other neuronal populations. According to our studies in PCs, vRh-GFP($Tg^{flvRh\text{-}GFP}$) 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. While 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 $G_{i/o}$ pathway has on motor control and the endogenous firing properties of PCs. Numerous examinations of the cerebellum and specifically the medial 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 Gi/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 seconds. 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 Gi/o signalling in vivo by light. While we have provided an effective means to modulate the activity of a single neuronal population and network, there are 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 noninvasive and light-dependent manner revealed that vRh promoted the modulation of GIRK and P/Q-type Ca^{2+}

channels via a functional coupling to the pertussis toxin-sensitive, Gi/o protein pathway (3). Because vRh couples to the G protein transducin, whereby the α subunit belongs to the G_i subfamily, these findings offer supporting evidence that mammalian rhodopsins are capable of coupling to other $G_{i/o}$ family members *in vitro*. In order to examine the possibility that vRh may also promote the precise spatio-temporal control of the $G_{i/o}$ 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 $G_{i/o}$ pathway in a membrane delimited way is the main inhibitory action of GPCRs on neuronal excitability (2). Many different transmitters, such as glutamate, acetylcholine (Ach), serotonin (5-HT) or GABA couple via specific GPCRs to the $G_{i/o}$ pathway, which are expressed throughout the brain. Among them, the $GABA_B$ receptor ($GABA_BR$) 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 $GABA_BRs$ 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 $G_{i/o}$ mediated signalling pathway in general. Up to this point, we have not detected the activation of other G protein pathways using vRh such as Gq or Gs in cellular or neuronal culture systems. Our attempt to control endogenous $G_{i/o}$-signalling by exogenously-expressed vRh still remains to be further developed. There are several matters to consider that may influence the feasibility of controlling Gi/o signalling and include the following: Firstly, $G_{i/o}$-coupled GPCRs have a variety of downstream signalling targets and have a binding preference to each of their respective targets. Secondly, more than one type of $G_{i/o}$-coupled GPCR is expressed in a single neuron and spreads in a specific distributing pattern. Lastly, some Gi/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 signalling domain of a specific GPCR and were able to control $5\text{-}HT_{1A}/G_{i/o}$ specific signalling 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 $GABA_BRs$ in dissociated PCs have been suggested to inhibit P/Q-type Ca^{2+} channels (31), 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 signalling 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 μm diameter) was coupled to a blue laser light (20 mW 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 optrode have been previously characterized in detail and it had been estimated that the fiber tip produces a total tissue volume experiencing $\geq$1 mW mm^{-2} light intensity to be ~0.5 mm^3 (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 Gi/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 Gi/o protein pathway in PCs has a significant functional influence on motor control and coordination.

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FOOTNOTES

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The abbreviations used are: 5-HT, 5-Hydroxytryptamin (Serotonin); ANOVA, analysis of variance; CV, coefficient of variation; DREADD, designer receptors exclusively activated by a designer drug; GFP, green fluorescent protein; GIRK, G protein inwardly rectifying potassium channel; GPCR, G protein coupled receptor; PC, Purkinje cell; PTX, pertussis toxin; S.E.M., standard error of the mean; TEA, triethanolamine; vRh, vertebrate rhodopsin.

FIGURE LEGENDS

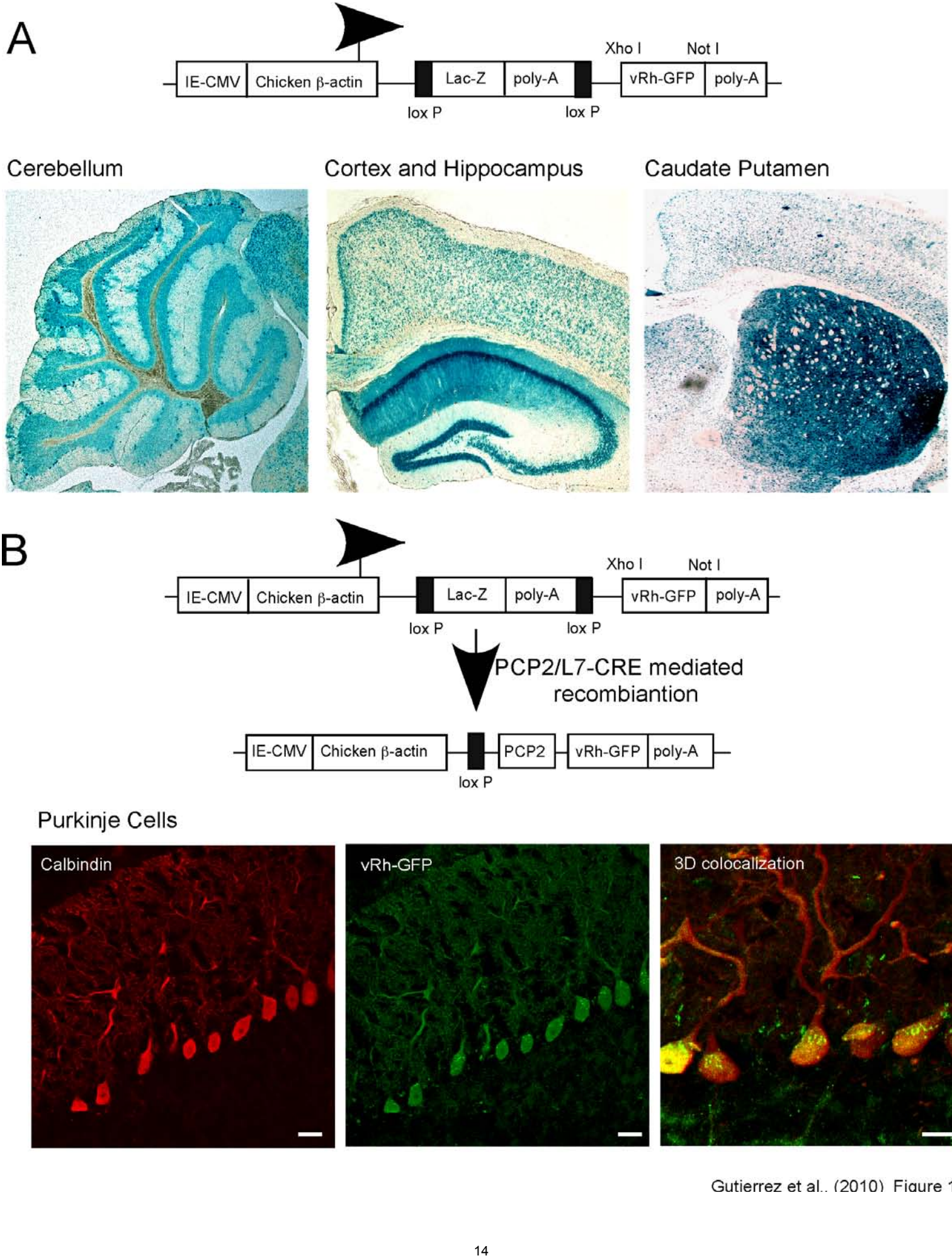
Fig. 1: Cell-type specific Cre recombinase-mediated expression of vertebrate rhodopsin in cerebellar Purkinje cells. (A) Schematic description of the construct used to create the transgenic animals expressing floxed vRh. vRh was cloned into the pCZW vector, which contains a CMV enhancer and β-actin promoter and a lacZ expression cassette, flanked by two loxP sequences. X-gal staining of sagittal brain slices from the vRh-GFP($Tg^{flvRh\text{-}GFP}$) mouse line shows β-galactosidase expression throughout the brain with robust expression localized in the cerebellum (left) and the hippocampus (middle) and caudate putamen (right). **(B)** Diagram revealing the results of Cre-mediated recombination events indicates an excision of the lacZ expression cassette and cell type specific expression of vRh-GFP driven in PCs. Cre recombinase-mediated induction of vRh-GFP expression in PCs was accomplished by crossing vRh-GFP($Tg^{flvRh\text{-}GFP}$) mice with Purkinje cell specific CRE ($Tg^{Pcp2\text{-}cre}$) mice. PC specific expression of vRh was verified by immunohistochemical staining for GFP (middle) and calbindin (a calcium binding protein associated with Purkinje cells, left). (Right) Three-dimensional reconstruction of confocal z-stack images revealing colocalization of vRh-GFP and calbindin in the soma and proximal dendrites of PCs. Scale bars (left and middle) 25 µm, (right) 10 µm.

Fig. 2: *In vivo* photostimulation of the cerebellar vermis of vRh-GFP^{PC} induces a reduction in the firing rate of Purkinje cells. (A) NISSL stain of sagittal cerebellum slices after electrolytic lesions indicate that the *in vivo* recordings and the application of baclofen (see Figure 3) were directed to the Purkinje cell layer. **(B)** PC firing rate recorded and calculated as percent change in firing before and after light application. The vRh-GFP^{PC} transgenic line demonstrated a significant reduction in firing during the light pulse that persisted after 30 second once light was switched off. **(C)** Representative firing rates (Hz) of individual PCs from control and vRh-GFP^{PC} mice with and without illumination reveal that only neurons from the vRh-GFP^{PC} line reveal decrease in the firing rate after light treatment. **(D)** Analysis of the coefficient of variation (CV) after light application indicates no significant difference between wild type littermates and vRh-GFP^{PC} mice. **(E)** Raw traces of control littermates and vRh-GFP^{PC} mice before and during the 473 nm light pulse reveals a reduction in the PC firing rate only in vRh-GFP positive transgenic mice. The presence of both simple and complex spikes as well as a comparative analysis of depth with a standard mouse brain atlas confirmed that recordings were taken from Purkinje cells. Statistical significance was evaluated with ANOVA. (** $P < 0.01$). Given values are mean ± S.E.M.

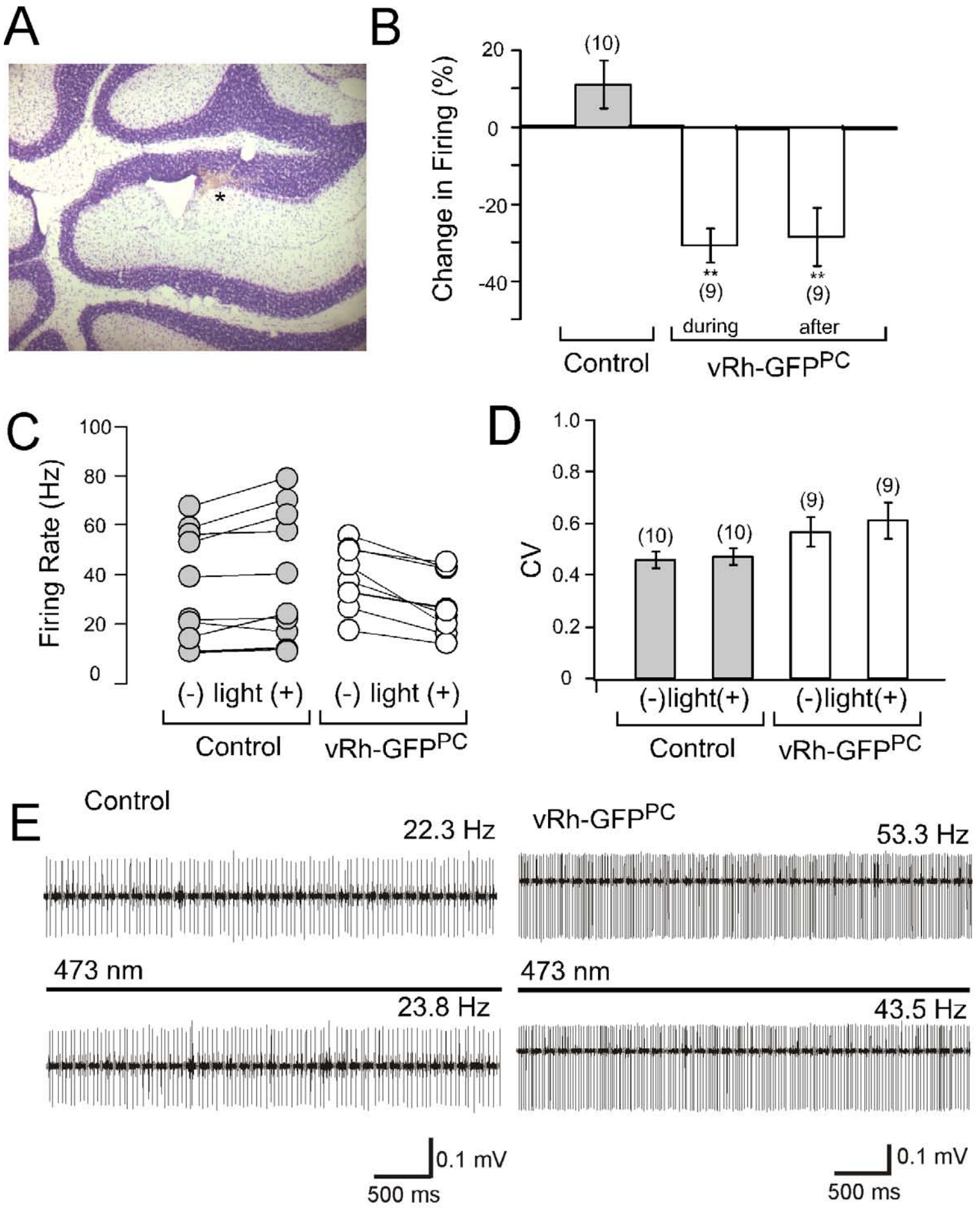
Fig. 3: *In vivo* and *in vitro* application of baclofen decreases the firing rate of cerebellar Purkinje cells. (A-E) *In vivo* recordings of cerebellar PCs before and after baclofen application. **(A)** Comparative immunohistochemical staining of $GABA_{B1}R$ (red) and vRh-GFP^{PC} (green) reveals the expression of $GABA_{B1}R$ in both PCs and cerebellar cortical neurons. vRh-GFP^{PC} expression is restricted to PCs and colocalized partly (yellow) with $GABA_{B1}Rs$. Scale bar 25 µm. **(B)** Percentage change in number of spikes for a 10 sec time interval during (t = 16-26 sec) and after (t = 20-30 sec) 1 mM baclofen application compared to control (saline application). Bar graphs indicate a significant decrease in the firing of Purkinje cells with baclofen application. **(C)** Spike frequency (Hz) before and after saline or baclofen application for each recorded PC demonstrates an overall reduction in firing that corresponds to baclofen administration. **(D)** Calculated values for the coefficient of variation (CV) between PCs treated with saline or baclofen reveals no significant difference in data dispersion between the two treatment groups. **(E)** Raw traces of PCs before and during either saline or baclofen treatment indicates reduced firing in the present of 1 mM baclofen. The presence of both simple and complex spikes as well as a comparative analysis of depth with a standard mouse brain atlas confirmed that recordings were taken from Purkinje cells. **(F-I)** Cerebellar slice recordings of PCs before and after baclofen application. **(F)** Percentage change in spike number during the 10 mM baclofen bath application displays reduced firing. **(G)** Modifications in the overall firing rate (Hz) for the recorded PCs during baclofen application. **(H)**

Calculated CV values for extracellular PC recordings indicate no significant difference before and during baclofen treatment. **(I)** Raw data traces demonstrating a decrease in PC firing with baclofen. Statistical significance was evaluated with ANOVA. (* $P < 0.05$). All values are mean ± S.E.M.

Fig. 4: Light activation of vertebrate rhodopsin expressed in Purkinje cells of the cerebellum induces changes in motor behavior. (A) Photograph demonstrating the permanent placement of the cannula light guide used for behavioral testing. **(B)** Pole test performance of control and vRh-GFP^{PC} mice (n=10), before and after a 26 sec light pulse. The light activation of vRh in vRh-GFP^{PC} mice results in either a fall (scored as 120 sec) 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^{PC} mice, before and after a 26 sec light pulse. Light activation of vRh in vRh-GFP^{PC} mice produces a significant decrease in rotarod performance when compared to wild type littermates. Performance between the two groups when no light pulse is applied reveals no significant difference. **(D)** Beam walk analysis demonstrates an increase in the time required to successfully cross the length of the beam after vRh activation in vRh-GFP^{PC} 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 sec. Additionally, a measurement of the number of paw slips reveals a significant increase after light application for the left side of the vRh-GFP^{PC} mice; whereas control littermates experienced no significant increase in slips post-light application. **(E)** Grip strength assessment of wild type and vRh-GFP^{PC} mice, before and after a 26 sec light illumination. No significant differences were observed for the grip strength of the front and hind paws between wild type littermates and vRh-GFP^{PC} 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 mean ± S.E.M.



Gutierrez et al., (2010) Figure 1



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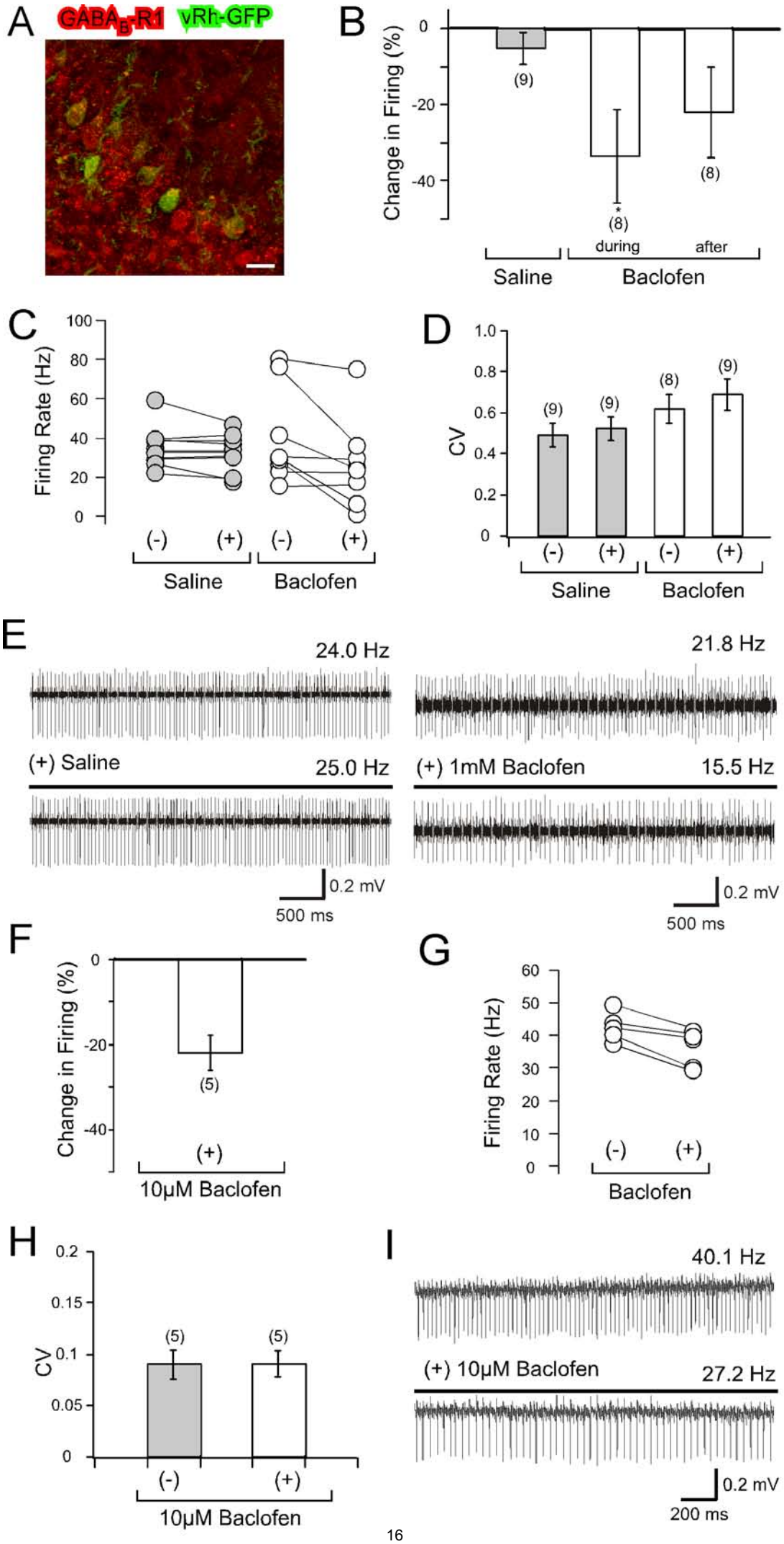

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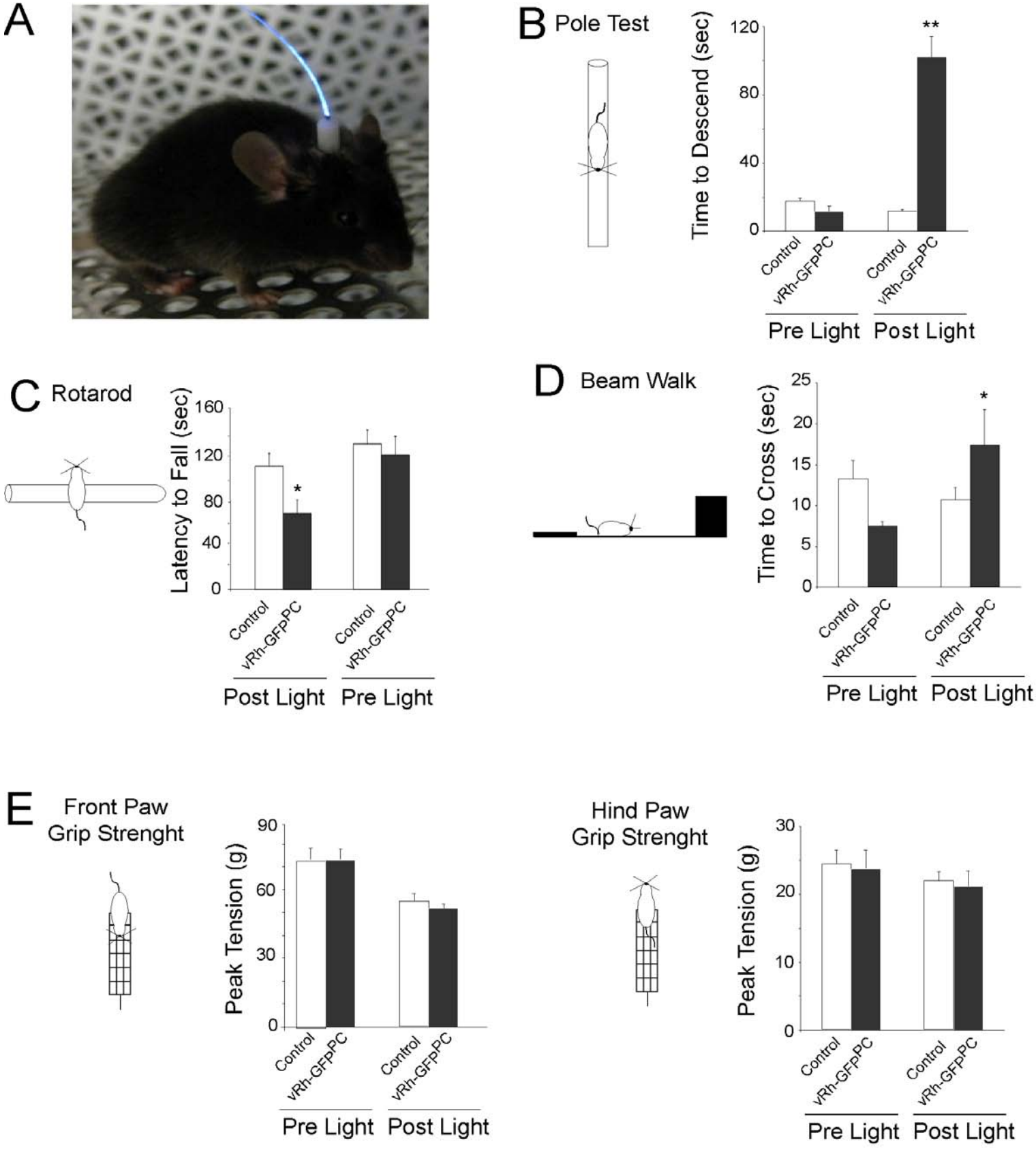

Gutierrez et al., (2010) Figure 4