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Article

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Posted Date: February 17th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1344254/v1>

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Optogenetic control of mGluR1 signaling modulates synaptic plasticity and cerebellum driven learning

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Summary

Neuronal plasticity underlying cerebellar learning behavior is strongly associated with type 1 metabotropic glutamate receptor (mGluR1) signaling. This receptor is located at perisynaptic sites at cerebellar Purkinje cells (PCs) and detects glutamate spill over. Activation of mGluR1 leads to activation of the $G_{q/11}$ pathway, inducing synaptic plasticity at the parallel fiber-Purkinje cell synapse (PF-PC) in form of long-term depression (LTD). To optogenetically modulate mGluR1 signaling we fused mouse melanopsin (OPN4) that activates the $G_{q/11}$ pathway to the C-termini of mGluR1 splice variants (OPN4-mGluR1a and OPN4-mGluR1b). We provide the prove-of-concept approach to modulate synaptic plasticity via optogenetic activation of OPN4-mGluR1a inducing LTD at the PF-PC synapse *in vitro*. Moreover, we demonstrate that light activation of mGluR1a signaling pathway by OPN4-mGluR1a in PCs leads to an increase in intrinsic activity of PCs *in vivo* and improved cerebellum driven learning behavior.

Keywords: optogenetics, synaptic plasticity, cerebellum, mGluR1, motor learning, LTD

Introduction

Glutamate is the main excitatory neurotransmitter in the CNS and activates ionotropic and metabotropic glutamate receptors (mGluRs). While ionotropic glutamate receptors encode the transmission rate of a signal, mGluRs dynamically regulate transmission strength between synapses. As such they are expressed throughout the CNS. Their absence or malfunction can lead to the development of disorders such as schizophrenia, Parkinson's disease, anxiety disorders and dyskinesias – making them prime candidates for therapeutic drug development¹. mGluRs belong to the superfamily of G protein coupled receptors (GPCRs), which activate different G protein pathways on a timescale of milliseconds to seconds² and are categorized into three different groups. These groups mainly couple to the $G_{q/11}$ pathway (group 1 with mGluR1,5) and to the $G_{i/o}$ pathway (group 2 with mGluR2,3 and group 3 with mGluR4, 6-8).

Among the mGluR family members, mGluR1 is extensively expressed in PCs, located at the perisynaptic sites of PC dendritic spines in the cerebellar cortex. Roughly 100,000 parallel fibers (PFs) and one climbing fiber (CF) converge on the dendritic branches of every single PC³. The simultaneous arrival of PF and CF input is detected by mGluR1 as glutamate spill over and decoded by the PC as mismatch signals or errors⁴. mGluR1 initiates long-term depression (LTD) by coupling to the $G_{q/11}$ pathway, activating phospholipase C_β (PLC_β), which hydrolyses phosphoinositides into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). This leads to mobilization of intracellular calcium as well as protein kinase C (PKC_α) activation resulting in phosphorylation and endocytosis of AMPA receptor GluR2 subunits⁵. Various mGluR1 splice variants exist, which differ in their length of the C-termini. The C-termini of mGluR1 contain binding sites for a variety of intracellular scaffolding and signaling proteins; e.g., the long C-terminus of mGluR1a but not of mGluR1b contains consensus sequences for the interaction with Homer, PDZ domain binding proteins, and calmodulin⁶. Only mGluR1a, but not mGluR1b, induces LTD⁷.

Cerebellar disorders resulting from disruption of mGluR1 signaling have been described in a heterogeneous group of autoimmune, neoplastic, and genetic human disorders such as paraneoplastic degeneration (PCD) and spinocerebellar ataxia (SCA)⁸. The involvement of mGluR1 in these human diseases has been studied in mouse models. Deletion of mGluR1 in mice causes ataxic gait and motor discoordination as well as impairment of cerebellar driven motor learning including motor impairments on the rotarod and learning deficits in the Morris watermaze^{9,10}. Notably, introduction of the splice variant mGluR1a but not mGluR1b was sufficient to rescue the phenotype⁷.

Although compelling evidence shows that mGluR1 is indispensable for normal cerebellar function, current techniques lack spatiotemporal or activity-dependent precision to resolve modulatory qualities of mGluR1 signaling. To understand neuronal network modulation by GPCRs, various optogenetic and pharmacogenetic approaches have been developed. For optogenetic modulations light-activated GPCRs such as vertebrate rod and cone opsins, parapinopsin, melanopsin and jellyfish opsin have been established to control the $G_{i/o}$, $G_{q/11}$ and G_s pathways, respectively^{11, 12, 13, 14, 15, 16}. To achieve highly customized GPCR specific targeting and downstream signaling, chimeras of suitable opsins and GPCR domains of choice have been

engineered by exchanging the intracellular domains between the light-activated and ligand-gated GPCRs^{17, 18, 19, 20, 21}. Here, we developed an optogenetic chimera by fusing the C-termini of the two predominantly expressed mGluR1 isoforms of PCs, the mGluR1a and mGluR1b, to vertebrate mouse melanopsin carrying mCherry in the third intracellular loop for receptor localization²². Using these chimeric light-activated GPCRs we demonstrate mGluR1a isoform specific induction of LTD at the PF-PC synapse *in vitro*, increase in PC spiking activity *in vivo*, and involvement of mGluR1 signaling in cerebellum-driven learning behavior.

Results

Design and Characterization of OPN4-mGluR1 Variants

We created two optogenetic tools to control the function of $G_{q/11}$ pathway activation in splice variant specific mGluR1 domains. We used vertebrate mouse melanopsin (OPN4) as a light-activated GPCR for controlling the $G_{q/11}$ pathway¹⁶. For monitoring the localization of OPN4, mCherry was inserted into the third intracellular loop of OPN4²². Onto the C-terminus of OPN4 the mGluR1 C-termini of mGluR1a (long, 355aa) or mGluR1b (short, 67aa) were introduced as neuronal trafficking signals (Figure 1a,b). To test $G_{q/11}$ dependent increase in intracellular Ca^{2+} concentration, we expressed these constructs together with GCaMP6m in HEK293 cells and recorded Ca^{2+} activity over 90 s (Figure 1c). As indicated in Figure 1d, light-mediated activation of OPN4-mGluR1a and OPN4-mGluR1b induced intracellular Ca^{2+} responses with comparable kinetics (Figure 1f-h). OPN4-mGluR1a Ca^{2+} responses are similar to pharmacological activation of mGluR1a (Figure 1e, pink) and can be blocked by YM-254890, a $G_{q/11}$ -protein inhibitor (Figure 1e, green). Taken together, OPN4-mGluR1 variants were both functional and induced robust calcium signals in HEK293 cells mediated by $G_{q/11}$ protein activation.

Modulation of Synaptic Plasticity by OPN4-mGluR1 Variants *in vitro*

mGluR1 activation induces cerebellar LTD (Figure 2a). Interestingly, in mGluR1 (-/-) mice only mGluR1a but not mGluR1b rescues synaptic LTD at the PF-PC synapse⁷. To explore effects of OPN4-mGluR1 on cerebellar plasticity, both variants - OPN4-mGluR1a and OPN4-mGluR1b (Figure 2b, c) - were virally expressed in PCs. OPN4-mGluR1a showed high overlap with endogenous mGluR1a expression patterns (Figure 2c top). In contrast, OPN4-mGluR1b was only sparsely expressed in PC dendrites (Figure 2c bottom). For LTD induction, we combined light pulses (470nm, 400ms) with electrical PF-stimulation (75x, 0.5 Hz). As indicated in Figure 2d, light stimulation in the presence of OPN4-mGluR1a, but not OPN4-mGluR1b (Figure 2e) or the control mCherry (Figure 2f) induced robust LTD. Short-term plasticity indicated by the paired-pulse ratio (PPR) was not altered across all conditions (Figure 2d-f IV). These data reveal that light-dependent activation of OPN4-mGluR1a but not OPN4-mGluR1b is sufficient to induce long-term plasticity in form of LTD at the PF-PC synapse.

Modulation of Purkinje Cell Simple Spikes by OPN4-mGluR1a *in vivo*

mGluR1 also modulates the intrinsic simple spike firing frequency of cerebellar PCs. We next investigated whether OPN4-mGluR1a modulates the intrinsic firing activity of PCs *in vivo* (Figure 3a). Application of mGluR1 agonist (DHPG) as well as light-pulse stimulation increased simple spike firing frequency (Figure 3b-d). Light-induced simple spike firing frequency increased with longer light-pulse duration (Figure 3e), indicating possibilities to induce graded signaling

Revealing PC Activity Using Task-related Labeling

mGluR1a and cerebellar LTD are important for motor learning³ as well as procedural memory in spatial learning. Global loss of mGluR1 leads to motor impairment, which can be rescued by selective expression of mGluR1a/b in cerebellar PCs⁷. We screened for regions of the cerebellum that are active during motor learning on the accelerated rotarod or spatial learning in the Morris watermaze using the Fos-TRAP mouseline²³ crossed with LSL-tdTomato mice. In these mice, 4-hydroxy-tamoxifen (4-OHT)-dependent Cre recombinase is expressed under control of the c-Fos promotor, allowing cre-mediated recombination and identification of active tdTomato positive neurons following 4-OHT administration (Figure 4a). For motor learning, rotarod experiments were performed, followed by 4-OHT injection 30 min after the last trial (Figure 4b,c). Mice successfully learned to balance on the accelerating rotarod (Figure 4 e). Comparison of active regions between rotarod and home-cage littermates revealed high activity in various cerebellar lobules, especially posterior vermis (Figure 4d,f). For spatial learning, mice had to find a submerged platform in the Morris watermaze during five consecutive days (D1-D5). Mice were separated in two groups, one group received 4-OHT injection 30 min after the last trial of D1, whereas the other received 4-OHT injection on D5, respectively (Figure 4g). All mice showed shorter swimming distances over the training period (Figure 4 i). Comparison of active regions between mice labeled on D1 and D5 revealed high activity in posterior vermis (in particular lobe VIII), and the hemispheres (in particular Curs I) (Figure 4h,j). Posterior lobes VIII and IX were involved in both paradigms.

OPN4-mGluR1a Activation in Purkinje cells *in vivo* Promotes Procedural Learning

To modulate mGluR1 signaling in identified cerebellar regions, we expressed OPN4-mGluR1a or mCherry (as control) in pcp2-cre mice (Figure 5a). To investigate motor learning, mice underwent training sessions for two consecutive days on the accelerated rotarod. After the first trial without stimulation, mice received continuous photo-stimulation during the time on the rotarod for all consecutive trials (Figure 5b). Motor performance and learning rate of OPN4-mGluR1a mice in comparison to mCherry controls did not reveal differences at D1 of acquisition, but the learning rate increased for OPN4-mGluR1a compared to mCherry expressing mice at D2 (Figure 5c, left and middle). Performance in starting trials (without photo-stimulation) did not differ between groups (Figure 5c, right). In the retention test session without photo-stimulation on D8, groups did not differ in performance and learning rate (Figure

5d). No differences were observed between groups regarding the general performance improvement (Figure 5e) and motor skill retention (Figure 5f).

To investigate spatial learning, mice were trained for five consecutive days (D1-D5, 4 trials per day) and received photo-stimulation before training (Figure 6a). Both groups learned the task, indicated by moved distance over time (Figure 6b left), swim patterns (Figure 6c) and relative time spent in the target quadrant (Figure 6d). OPN4-mGluR1a mice swam significantly shorter distances compared to mCherry controls. Additionally, enhanced spatial learning of OPN4-mGluR1a mice is accompanied by a rapid increase in spatial strategies (Figure 6e, red) and simultaneous decrease in repetitive looping strategies (grey) starting at D3. Swimming speed and thigmotaxis (indicated by time spent in periphery) did not differ between groups (Figure 6b middle, right). Taken together, electrophysiological results and behavioral experiments suggest that light-activation of the $G_{q/11}$ pathway in mGluR1a receptor domains by OPN4-mGluR1a modulates long-term synaptic plasticity in cerebellar PCs and can enhance cerebellum-driven motor and spatial learning.

Discussion

We designed an optogenetic GPCR to control mGluR1a signaling domains via OPN4-mGluR1a. Our results show that optogenetic stimulation of OPN4-mGluR1 activates $G_{q/11}$ protein signaling cascades. OPN4-mGluR1a, but not OPN4-mGluR1b induced LTD of PFs evoked EPSCs in cerebellar PCs *in vitro*. *In vivo* single cell recordings verify, that light activation of OPN4-mGluR1a and pharmacological activation of mGluR1 signaling by the mGluR1 agonist DHPG increased spontaneous neuronal activity. Finally, we could confirm that activation of OPN4-mGluR1a in cerebellar regions involved in rotarod and watermaze performance improves cerebellar learning in both tasks without changing the basic behavior performance.

It has been proposed that LTD induced by coincident parallel and climbing fiber activation is a critical component of cerebellar learning mechanism. More recent studies promoted the critical role of mGluR1 in LTD induction in the cerebellum and hippocampus^{24,25,26,27}. These *in vitro* studies show that the mGluR1 agonists, DHPG, induced LTD-like persistent reduction of hippocampal and of cerebellar PF-PC synaptic transmission. We now established a method to control the mGluR1-induced LTD optogenetically. We show that light-dependent activation of OPN4-mGluR1a, but not OPN4-mGluR1b induces LTD at the PF-PC synapse. This result suggests that splice variant dependent-targeting of OPN4 is important for anchoring the construct at synaptic sites, at which LTD occurs. In addition, we discovered *in vivo* that OPN4-mGluR1a increase spontaneous simple spike firing comparable to native mGluR1 activation by DHPG. These findings are in line with published *in vitro* and *in vivo* studies showing that application of mGluR1 agonists such as DHPG enhances the simple spike rate of PCs in current clamp and extracellular recordings^{28,29,30}.

We demonstrated that light-dependent activation of OPN4-mGluR1a expressed in PCs induces an increase in spontaneous firing and synaptic depression at the PF-PC synapse. If and how these two mGluR1a mediated effects on PCs are causally linked is unclear. One hypothesis is

that intracellular Ca^{2+} concentration is responsible for the increased intrinsic firing of PCs. It has been shown, that PF stimulation induces an mGluR1 mediated slow EPSP, which is dependent on a transient rise in intracellular Ca^{2+} ³¹. Moreover, mGluR1 contribute to the basal activity of PCs, where mGluR1 activation induces an inward cation current and an increase in spiking of PCs *in vitro* and *in vivo*³². The activation of mGluR1 by light stimulation of OPN4-mGluR1a similarly increases the intracellular calcium concentration resulting in increased intrinsic firing of the PCs. Thus, OPN4-mGluR1 allows optogenetic controlled activation of $\text{G}_{q/11}$ protein induced increase in neuronal activity, and precisely regulation of synaptic plasticity in a subcellular localization-dependent manner.

Theoretical work by Marr and Albus hypothesizes that PF-PC LTD in the cerebellum implements motor learning^{33,34}. This hypothesis has been supported by the experimental data of Ito and colleagues forming the basic concept of cerebellar learning. Cerebellar learning requires cerebellar plasticity and involves various synapses within the cerebellar neuronal network. Cerebellar plasticity depends for example on the composition and spatial distribution of different channels and receptors, local concentrations of calcium and nitric oxide and the timing of the incoming inputs³⁵. However, there is a long-standing debate whether an increase or decrease in synaptic strength at the PF-PC synapse underlies motor learning. For example, it has been shown that mice deficient in cerebellar LTD have strong negative effects in Morris watermaze performance but no effects in the starmaze or rotarod task^{36,37}. Other studies using mutant mouse lines with deficits in LTD and LTP inductions argue that LTP rather than LTD is the key mechanism for cerebellar motor learning^{38,39}. Recent studies using optogenetic control of synaptic AMPA receptor endocytosis demonstrate that LTD in the cerebellum indeed is responsible for motor learning underlying adaptation of the optokinetic response and vestibulo-ocular reflex⁴⁰. Another strong correlation between cerebellar LTD induction, motor coordination and spatial learning has been found in mGluR1-deficient mice. These mice show impairments in LTD in the cerebellum and deficits in several behavior motor tasks including the rotarod and Morris watermaze performances. In a subsequent study Ichise and colleagues showed that while mGluR1 deficient mice failed on the rotarod, mGluR1 rescue mice showed normal LTD and normal motor behavior⁴¹. The role of mGluR1 signaling for motor behavior could further be shown in $\text{G}_{q/11}$ protein deficient mice, that had no cerebellar LTD and were significantly impaired on motor behavior including the rotating rod⁴². In our study we show that light-dependent activation protocols of $\text{G}_{q/11}$ signals in mGluR1 domains in cerebellar PCs, which induce LTD *in vitro*, improves learning performance in the Morris watermaze and on the rotarod. Thus, our data support the correlation between LTD induction at the PF-PC synapse and enhanced motor learning.

Several human disorders such as paraneoplastic degeneration (PCD) and spinocerebellar ataxia (SCA) have been associated with pathological changes of mGluR1 signaling induced by autoimmune, neoplastic and genetic dysregulations^{3,8,43}. Pharmacological treatment that enhances mGluR1 signaling using positive allosteric modulators resulted in a significant long-lasting improvement of motor performance in the SCA mouse model^{44,44}. On the other hand, mGluR1-negative allosteric modulator rescued moderate ataxia in an early SCA1 mice model⁴⁵.

The underlying temporal and spatial mechanism are still under debate. However, the evidence that hypofunction and hyperfunction of mGluR1 signaling underlies certain types of human SCAs⁸ suggests modulation of mGluR1 signaling, as a promising therapeutic target for cerebellar ataxic diseases. Thus, our new optogenetic tool OPN4-mGluR1a, opens new possibilities to study/improve different types of cerebellar diseases such as SCA or PCD.

Material and Methods

Generation of plasmid constructs

Plasmids were based on the pAAV-CMV-eGFP-CW3SL vector (GenBank accession number: KJ411916.2) to expand AAV packaging capacity^{46,47}. Constructs were generated using the In-Fusion cloning kit (Takara) by PCR amplifying each element with 15 bp overhangs. For generation of pAAV-CMV-mOPN4L-mChil3-CW3SL, the sequence of mCherry was integrated in the third intracellular loop of the long isoform of mouse melanopsin (mOPN4L, GenBank accession number: NM_013887.2). For OPN4-mGluR1a (pAAV-CMV-mOPN4L-mChil3-mGluR1_alpha-CW3SL) the sequence of human mGluR1 alpha C-terminus (amino acids K841-L1194) was fused to pAAV-CMV-mOPN4L-mChil3. For OPN4-mGluR1b (pAAV-CMV-mOPN4L-mChil3-mGluR1_beta-CW3SL) the sequence of human mGluR1 beta C-terminus (amino acids K841-L906) was fused to mOPN4L-mChil3. Conditional constructs were generated by inversion of the gene of interest and adding flanking pairs of Lox2272 and LoxP sites on 5' and 3' end of the gene. To generate the full-length receptor fused to a genetically encoded calcium sensor, pAAV-CMV-mGluR1-IRES-GCaMP6m-CW3SL and pAAV-CMV-mGluR1-p2A-GCaMP6m-CW3SL were generated by fusing the mGluR1 sequence from GRM1-Tango – a gift from Bryan Roth (Addgene plasmid #66387; <http://n2t.net/addgene:66387>; RRID:Addgene_66387;⁴⁸) to an internal ribosome entry site (IRES) or the short p2A sequence and GCaMP6m.

Cell culture and live cell confocal imaging

For *in vitro* imaging experiments, HEK293T cells (ATCC #1573) were cultured on 35mm glass-bottom dishes and transfected via polyethyleneimine (PEI). Cell imaging was performed using a 20X/0.7NA objective on an inverted Leica TCS SP5 confocal laser scanning microscope, 512 x 512 pixels with ~0.5 s interval between frames. Cells were detected by their membrane-localized mCherry fluorescent reporter tag using 561 nm DPSS laser. To measure Ca²⁺ responses of optogenetic tools, fluorescence change of GCaMP6m was monitored using 476 and 495 nm argon laser lines, thereby simultaneously activating the opsin-based constructs. To measure Ca²⁺ responses of the full-length receptor, 200μM (R,S)-3,5-dihydroxyphenylglycine (DHPG; Abcam) was applied to the dishes. Changes in fluorescence intensity were processed using the time series analyzer V3 function in Fiji and the responses calculated as $\frac{F(t) - \bar{F}_{baseline}}{\bar{F}_{baseline}}$ over time, where $\bar{F}_{baseline}$ is the mean fluorescence of the first two frames of each session.

Subjects

All experiments were conducted with approval of a local ethics committee (Bezirksamt Arnsberg) and the animal care committee of Nordrhein-Westfalen (LANUV; Landesamt für Umweltschutz, Naturschutz und Verbraucherschutz Nordrhein-Westfalen, Germany; AZ. 84-02.04.2014.A203 und AZ.81-02.04.2019.A228). The study was carried out in accordance with the European Communities Council Directive of 2010 (2010/63/ EU) for care of laboratory animals and supervised by the animal welfare commission of the Ruhr-University Bochum. Experiments were performed in 2- to 10-month-old male and female mice of the following lines: C57BL6/J, homozygous Pcp2-Cre. For c-Fos labeling experiments, heterozygous Fos-CreER^{T2} mice were obtained by crossing wild-type C57BL6/J x Fos-CreER^{T2} (+/-) mice. Fos-CreER^{T2} (+/-) and Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} (+/+) mice were crossed to generate Fos-CreER^{T2} (+/-) x Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J} (+/-) mice (mouse line is abbreviated as Fos-TRAP hereafter). For *in vitro* and behavioral experiments, age matched animals received viral injections with OPN4-mGluR1a, OPN4-mGluR1b (only for *in vitro* studies) or mCherry (as control). Mice were kept on a 12/12 day/night cycle with water and food *ad libitum*. To increase the dynamic range of motor learning abilities, the basic motor performance on the rotarod was decreased by application of an adapted mild stress protocol^{49,50}. Two days before and during the first two acquisition days on the rotarod, the day/night cycle was changed, and mice were kept on dark cycle for 48h.

Viral vector production

Recombinant AAV2/8 genomes were produced using the AAV helper-free system (Agilent Technologies, Santa Clara, CA) by triple transfection of HEK tsA201 cells as described previously²². Adeno-associated virus (AAV) plasmids encoding the respective gene of interest, the helper plasmid encoding AAV *rep* and *cap* genes, as well as adenoviral helper plasmid were co-transfected into HEK tsA201 cells using polyethyleneimine (PEI). Following ~72 h incubation, cells were harvested and lysed by 5-7 freeze-thaw cycles while the supernatant was incubated with 40% PEG-8000 for 2 h at 4°C on a shaker. After centrifugation at 3700 x g for 20 min at 4°C, the pellet resulting from PEG-precipitation was resuspended with the supernatant from cell lysate. To purify the AAV, the buffer was replaced with 50mM HEPES buffer using PEG-precipitation and chloroform added (1:1 ratio). The mixture was thoroughly vortexed for 2 min and centrifuged at 370 x g for 5 min. The resulting aqueous phase was collected, sterile filtered (0.22 µm syringe filter membrane), PEG-precipitated for 2 h, centrifuged at 3700 x g for 20 min and reconstituted in 0.001% PBS-Pluronic F68. Aliquots were stored at -80°C.

Surgery, virus injections and optic fibre implantation

For surgeries, mice were anesthetized with an initial dose of 5% isoflurane in 1.1 l min⁻¹ air flow. Anesthetized mice were placed in a stereotaxic frame (Narishige, Japan) on a heating pad for body temperature maintenance (1.3-2.0% Isoflurane maintenance, adjusted according to breathing rate and pinch reflex). For analgesia, carprofen (2 mg/kg) and buprenorphine

(0.1mg/kg) were administered subcutaneously. In addition, lidocaine was administered under the scalp for local anesthesia. The scalp was shaved and cleaned with 70% ethanol. After incision, the skull was cleaned using saline and 70% ethanol. For virus injections, viruses were drawn up into micropipettes generated by pulling capillaries using a Sutter puller. Three craniotomies were made over the cerebellar vermis and 1 μ l of AAV was injected using pressure injection (1st and 2nd: AP = -6.3; ML = $\pm$ 1-1.2; DV = 1-3; 3rd: AP = 6; ML = 0; DV = 1-2). For optogenetic stimulation in behavioral tests, in addition, two custom-made optical fibres (ferrule outer diameter 1.25 mm, with a fibre diameter of 200 μ m, 0.39 NA, FT200MT; Thorlabs, Dortmund, Germany) were lowered to 0.8mm with an angle of 10 degrees in the left and right craniotomy and fixed using Charisma (Heraeus Kulzer, Hanau, Germany), followed by dental cement mixed with black powder. After surgery, mice were housed individually and were allowed to recover for at least 7 days before performing electrophysiological or behavioral experiments.

Extracellular *in vivo* electrophysiological recordings in anaesthetized mice

For extracellular *in vivo* recordings, procedures were applied as previously described¹³. Briefly, anaesthetized mice were placed into a stereotaxic frame. After incision, a craniotomy above virus injection sites was made, and the dura removed. Using the Eckhorn system (Thomas Recording, Giessen, Germany) extracellular activity was recorded with up to six electrodes (impedance 2-3 MOhm at 1 kHz; Thomas Recording, Giessen, Germany), amplified and band-pass filtered at 0.1-8 kHz using a multichannel signal conditioner (CyerAmp380, Axon Instruments, Union City, CA, USA). Signals were sampled with 32 kHz (NI PCI-6259, National Instruments, Austin, TX, USA) controlled and offline analyzed using MATLAB. For optogenetic stimulation, a glass fiber was integrated in the multi-electrode system. Then, using 465 nm LED (Plexon, Dallas, USA) with 1.5-2 mW at the tip of the fibre pulses of different length were delivered. For pharmacological experiments, a micropipette was inserted in the multielectrode system (replacing the light fiber) and 200 μ M DHPG (Abcam) was locally applied by pressure injection.

***In vitro* slice recordings**

Experiments were performed in darkness. Acute cerebellar slices (250 μ m thickness) were prepared from the vermis (sagittal sectioning using a Vibratome VT1000S, Leica) of mice 7-14 days after AAV injection using a dissection solution containing (in mM): 87 NaCl, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 D-Glucose and 75 Sucrose bubbled with 95% O₂ and 5% CO₂. Slices were incubated in recording solution (in mM: 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 D-Glucose, bubbled with 95% O₂ and 5% CO₂) at 37°C for 45-60 min and then kept at room temperature. For EPSC recordings, slices were preincubated for 20 min in recording solution containing 25 μ M 9-cis-retinal, 0.025% ($\pm$)- α -tocopherol (Sigma), 0.2% essentially fatty acid free albumin from bovine serum (BSA, Sigma-Aldrich), and 100 μ M Picrotoxin (Sigma-Aldrich) to block GABA_AR-mediated inhibitory inputs.

Internal solution consisted of (in mM): 125 Potassium Gluconate, 10 HEPES, 4 NaCl, 2 MgCl₂, 0.2 EGTA, 4 Mg-ATP, 0.4 Na-GTP, and 10 Tris-Phosphocreatine and 5 lidocaine N-ethyl bromide (QX-314; Tocris Bioscience) to block Na⁺ currents (pH 7.3, 280mOsm). Patch pipette resistance was 3-5 MΩ. PCs were visually identified using 40 X objective attached to an upright microscope (BX51WI, Olympus) that was equipped with infrared illumination and a Polychrome V (TILL Photonics). Using 560nm wavelength for excitation of mCherry, flashing light pulses (light intensity 1.6mW/mm²) were utilized to identify expressing PCs. Whole-cell recordings were performed at 20 kHz and low pass-filtered at 3kHz using a EPSC10 amplifier (HEKA Elektronik). PCs were voltage clamped at -60 mV and PF stimuli were applied through a second borosilicate glass pipette filled with recording solution and placed in the molecular cell layer. The measurement consisted of three phases: (1) a baseline pre-LTD-induction recording, (2) LTD induction, and (3) post LTD-induction measurement. For (1) and (3), EPSCs were evoked by paired pulse stimulation of PF (ISI = 100ms) every 30 s for 5 min pre and 15 min post LTD-induction. To induce LTD (2), PCs 470 nm light pulses (1.8 mW/mm²) with a duration of 400 ms was combined with 75 single pulse PF stimulation at 0.5 Hz. PatchMaster software (HEKA) was used for data acquisition and MATLAB for offline analysis.

Immunohistochemistry

Mice were anesthetized and intracardially perfused using phosphate buffered saline (PBS) and 4% paraformaldehyde in phosphate buffer (PFA). Brains were removed, post-fixed overnight using PFA and cryoprotected in PBS with 30% sucrose (w/v) for at least 24 h. Sagittal slices of 35-40 μm were cut (CM3050 S, Leica), washed in 1xPBS, permeabilized 1 h with 0.3 PBS-Triton X-100 (PBST) and blocked using 10% normal donkey serum (NDS). Primary antibodies (rabbit anti mGluR1a, Santa Cruz, 1:100) were applied overnight in 3% NDS in 0.3% PBST. After washing, slices were incubated in goat anti-rabbit antibody conjugated with Alexa Fluor 488 (1:500, Invitrogen) for 3 h. Sections were mounted using Mowiol with DABCO. Images were collected using either a confocal (TC5 SP5, Leica) or 2P microscope (Ultima 2Pplus, Bruker).

Behavioral paradigms

Rotarod. Motor coordination and learning was assessed using an accelerated rotarod protocol. Rotation was set to accelerate from 4 to 40 rpm in 10 second increments. Mice were placed on a continuously rotating cylinder of 4 rpm constant speed. For Fos-TRAP experiments, training sessions consisted of 9 trials on day 1 and 2. For optogenetic experiments, mice were connected to glass fiber cables for optogenetic stimulation (PlexBright LED system, Campden Instruments, UK) and placed on the rod (at 4 rpm speed). Test and the light stimulation started as soon as a mouse was stable on the rod. Mice were trained on two consecutive days (D1 and D2) with light stimulation starting at the second trial. A third training day without stimulation followed a 7-day brake at day 8 (D8) to reassess motor skills without stimulation. The session on D1 consisted of 9 trials, sessions on day 2 and 8 consisted of 11 trials (3 min intertrial interval). As soon as the mouse fell off the rod the light stimulation was stopped and latency to

fall was determined. Offline, we analyzed (1) the learning rate of every training day (adapted from⁵¹; $((\text{mean latency to fall}_{\text{last two trials}}) - (\text{mean latency to fall}_{\text{first two trials}})) / \text{number of intertrial intervals}$), (2) performance improvement $(\text{mean latency to fall}_{\text{D2 first two trials}} - \text{mean latency to fall}_{\text{D1 first two trials}})$, (3) motor score retention index $(\text{acquisition score} - [\text{mean latency to fall}_{\text{D8 first two trials}} - \text{mean latency to fall}_{\text{D1 first two trials}}])$.

Morris watermaze. The Morris watermaze (MWM) test is an aquatic open field task, in which mice must search for a submerged platform in opaque water using external cues as orientation marks. Data acquisition was performed using a video recording system and ETHOVISION tracking software. The maze (diameter of 96 cm) was placed above the floor, and filled with water till the water level reached 1.5 cm above the escape platform (located at a fixed position – 10 cm from the edge of the pool). White milk powder was dissolved for water opacity. The temperature was kept at $26 \pm 1^\circ\text{C}$. Four symbols were placed around the maze and a white curtain surrounded the maze to limit visual distraction. Mice were tested for ten consecutive days with one training session (consisting of four trials) per day. Starting locations were assigned based on comparable swimming distances⁵² and randomly assigned on each day. The maximum time to reach the hidden platform was 90 s. If a mouse could not find a platform within 90 s, it was gently guided to the target. During a 1 min intertrial interval, mice were kept in a holding cage. For optogenetic stimulation experiments, mice were connected to glass fiber cables and stimulated (PlexBrigh LED system, Campden Instruments, UK) on each day right before the first trial. The protocol was adapted from the stimulation protocol used to induce LTD in cerebellar slices and consisted of 70 pulses with a pulse duration of 400ms, given at 0.5 Hz frequency.

Analysis of quantitative parameters was performed by ETHOVISION tracking software. Analyzed parameters were (1) total distance moved, (2) velocity, (3) time spent in periphery as well as (4) time spent in target quadrant. Furthermore, individual scores for searching strategies were determined – this parameter represents a qualitative analysis of swimming patterns based on (Brody & Holtzman, 2006). The analysis was made by two independent researchers blinded to conditions. Three-dimensional colormaps were generated using a custom-made MATLAB script.

Behavioral Labeling using 4-hydroxytamoxifen

Drug preparation. 4-hydroxytamoxifen (4-OHT) was prepared according to⁵³. 10mg of 4-OHT was dissolved in 250 μl DMSO, and 5 ml of saline with 2% Tween 80. The final solution entailed 20mg/kg 4-OHT and was administered IP to Fos-TRAP animals at indicated timepoints according to their weight. Mice were injected 30 min after the training (indicated in figure 4) and sacrificed 10 days later. After cryo-sectioning and mounting, Purkinje cells were visually identified by their location and soma diameter and counted throughout the cerebellum using THUNDER imaging system (Leica).

Data quantification and statistical analysis

Data are shown as mean $\pm$ standard error, as noted in each figure. n values are provided in the figure legends. Tests were performed with MATLAB or Sigma Plot (Systat software). For comparison between two independent groups, two-tailed Student's t-test, Mann-Whitney U test, Wilcoxon signed-rank test or Kolmogorov-Smirnov test were performed. For comparison between more than three groups, two-way analysis of variance (ANOVA) or Kruskal-Wallis tests with subsequent post hoc tests for detailed comparisons were employed. Statistical p-values of $p < 0.05$ were considered significant.

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Acknowledgments

This work was supported by DFG Project number 122679504-SFB874/B10 (to S.H.), Project number 316803389-SFB1280/A07 (to S.H.), Priority Program SPP1926 (DFG2471/18-2) (to S.H.), DFG2471/23-1 (to S.H.), DFG2471/21-1 (to S.H.), DFG Project number 316803389-SFB1280/A2 (to M.D.M.), MA 5806/2-1 (to M.D.M.), MA 5806/1-2 (to M.D.M.), JA945/3-1 (to D.J.), JA945/4-1 (to D.J.), JA945/5-1 (to D.J.), German-Israeli Project Cooperation (SL185/1-1), and DFG Project number 122679504-SFB874/A02 (to D.J.).

Author contributions

Conceptualization, T.S., S.H. and I.S.; Methodology, T.S., M.G., H.B., M.B., I.S.; Investigation, T.S., M.G., H.B., M.B., I.S.; Data analysis, T.S., Z.A., I.S.; Funding acquisition, D.J., S.H., M.D.M.; Supervision, D.J., S.H., M.D.M., I.S.; Writing, T.S., S.H., I.S.

Competing interests

Authors declare no competing interests.

Data availability

The data that support the findings to this study are available from the corresponding authors upon request.

Figures

Figure 1

Design and characterization of OPN4-mGluR1 variants. (a) Design of OPN4-mGluR1a (blue) and OPN4-mGluR1b (orange) constructs. Chimeras comprise OPN4, mCherry in the third intracellular loop and mGluR1a or mGluR1b fused c-terminally. (b) Scheme of OPN4-mGluR1 design and downstream signaling cascade following photostimulation. (c) Top, exemplary OPN4-mGluR1a expression in two HEK293 cells. The construct is localized in the cell membrane. Bottom, transient calcium signal of both cells during light stimulation. (d) Time-course of light-activated Ca^{2+} signaling of OPN4-mGluR1a (blue, $n=15$ dishes) and OPN4-mGluR1b (orange, $n=8$ dishes) over a stimulation period of 90 seconds. Shading indicates $\pm$ SEM. (e) Pharmacological activation of mGluR1a in HEK293 cells shows comparable increase and delayed peak of Ca^{2+} signaling (red, $n=13$ dishes). Pharmacological block of Gq/11 protein signaling abolishes OPN4-mGluR1a Ca^{2+} signals (green). (f) Calcium signal components τ_{on} , τ_{off} and time to peak were compared between optogenetic chimeras and showed no statistical difference. Population data of the responses shown in d are shown as boxplot (mean $\pm$ SEM).

Figure 2

Activation of OPN4-mGluR1a but not OPN4-mGluR1b induced LTD in Purkinje cell recordings *in vitro*. (a) Schematic drawing of downstream Gq/11-protein coupled signaling cascade after optogenetic stimulation of OPN4-mGluR1 variants. Optimal signaling results in phosphorylation and internalization of AMPA-receptors inducing LTD of excitatory postsynaptic currents (EPSCs) evoked by parallel fiber stimulation in Purkinje cells. (b) Scheme of experimental procedure for cerebellar slice recordings. OPN4-

mGluR1a, OPN4-mGluR1b or mCherry were expressed in Purkinje cells using AAVs. (c) Expression pattern of OPN4-mGluR1a and OPN4-mGluR1b in Purkinje cells; mGluR1a antibody (cyan), OPN4-mGluR1a and OPN4-mGluR1b (red), colocalization (white). OPN4-mGluR1b is sparsely expressed in dendrites compared to OPN4-mGluR1a. (d-f) EPSCs were evoked by double pulse stimulation of parallel fibers combined with 470 blue light stimulation of OPN4-mGluR1a (d; n = 5), OPN4-mGluR1b (e; n = 3) and mCherry-control (f; n = 9). I Design of OPN4-mGluR1a (blue), OPN4-mGluR1b (orange) and mCherry (red) constructs. Chimeras comprise OPN4, mCherry in the third intracellular loop and mGluR1a or mGluR1b fused c-terminally. II Example traces of EPSCs are shown on the top, III shows the normalized EPSC1 and IV the paired pulse ratio (PPR) over stimulation time. EPSCs were normalized to the average pre EPSC (0-5min). Response of the post EPSCs and PPRs before and after blue light stimulation are given as mean response over time (pre: 0-5 min; post: 10-30 min). Significant LTD of post EPSCs ($p = 0.031$; two-sided Wilcoxon signed-rank test) was only induced by optical stimulation of OPN4-mGluR1a. PPR showed no significant change.

Figure 3

OPN4-mGluR1a elevates Purkinje cell simple spike firing rate in vivo.

(a) Scheme of experimental procedure for single-cell recordings in cerebellum in anaesthetized mice. Recordings were made two weeks after AAV8-OPN4-mGluR1a injection, DHPG was locally applied during recordings. (b, c) Exemplary Purkinje cell recordings (identified by characteristic simple and complex spike firing pattern, marked with *) over 5 min with 60 s baseline recording and subsequent (b) pharmacological (DHPG - mGluR1 agonist) or (c) optogenetic activation using 488nm wavelength. (d) Pharmacological activation of mGluR1 (DHPG - mGluR1 agonist, pink trace) or blue light activation

OPN4-mGluR1a (488nm; blue trace) lead to a rise in simple spike firing frequency (DHPG: n=30 PCs in n=3 mice; OPN4-mGluR1a: n=12 PCs in n=4 mice). Shading indicates $\pm$ SEM. (e) Firing rate depends on pulse length and increases with light stimulation duration of 5 s (lightest blue, n=2 PCs in n = 1 mice), 10 s (blue, n = 8 PCs in n = 3 mice) or 60 s (dark blue, n=12 PCs in n = 4 mice), respectively.

Figure 4

Distribution of c-Fos activity of Purkinje cells during motor learning on the accelerated rotarod and Morris watermaze. (a) Schematic drawing showing induction of tdTomato expression by active cells in Fos-TRAP x LSL-tdTomato mouse line. Neuronal activation induces expression of immediate early gene c-Fos. CreERT2 is expressed under c-Fos promoter but is retained in the cytoplasm when 4-OHT is absent. Upon injection of 4-OHT, CreERT2 enters the nucleus, excises the STOP-cassette and enables tdTomato expression. (b) Exemply expression patterns of control (left; n = 3) and rotarod trained mice (right; n = 3). Bottom, cerebellar lobes 3, 4 and 5 are shown magnified. (c) Protocol for rotarod training and 4-OHT injection 30 min after completion of the last trial. (d) Active cerebellar areas expressed as tdTomato+ Purkinje cells in each lobe of rotarod trained mice, normalized to homecage controls (rotarod-trained, n=3; homecage, n=3).

homecage, n=4 mice). (e) Motor learning indicated by elevated latency to fall on accelerating rotarod (mean + SEM, $t_2 = -6.672$, $p = 0.022$; n = 3 trained mice). (f) Summary of active zones of the cerebellum after rotarod training. Light red stippled line corresponds to areas showing twofold increase, dark red indicates threefold increase in tdTomato positive Purkinje cells. (g) Protocol for Morris watermaze training and 4-OHT injection 30 min after last trial is completed. Mice were divided into two groups, receiving 4-OHT injections on D1 or D5, respectively. (h) Active cerebellar areas expressed as tdTomato+ Purkinje cells in each lobe of Morris watermaze trained mice injected on D1, normalized to mice injected on D5 (labeled on D1, n=2; labeled on D5, n=2). (i) Spatial learning indicated by decreasing distance moved in the Morris watermaze test (mean + SEM, $t_3 = 6.071$, $p = 0.009$, n = 4). (j) Active zones of the cerebellum after spatial navigation training. Light orange corresponds to areas showing twofold increase, bright orange indicates threefold increase in tdTomato+ Purkinje cells.

Figure 5

Enhanced motor learning performance in the accelerated rotarod induced by OPN4-mGluR1a. (a) OPN4-mGluR1a ($n = 9$) or mCherry ($n = 9$) were injected into cerebellar vermis and light guides were implanted. (b) Rotarod training protocol consisted of two days (D1 & D2) with optogenetic stimulation during rotarod sessions followed by a retention test session on day 8 (D8) without optogenetic stimulation. One training session consisted of 9 trials on D1 and 11 trials on D2. Optical stimulation started at trial 2 on both days. (c) Left, performance (measured as latency to fall) increases with training on D1 and D2 (Mean $\pm$ SEM.).

Middle, learning rates of OPN4- mGluR1a expressing mice on D2 are significantly higher after photostimulation ($p = 0.028$, Mann-Whitney U test). Right, performance did not differ between groups on starting trials of both days (without stimulation). (d) In the retention test session in absence of optogenetic stimulation, performance and learning rate did not differ between groups. (e) Performance improvement and motor skill retention (f) were not affected by tool activation or light stimulation.

Figure 6

OPN4-mGluR1a activation facilitates navigation efficiency in the Morris watermaze. (a) Training protocol to test spatial learning performance in Morris watermaze test. During training, mice had to search for a submerged platform in 4 trials over 5 consecutive days. On each day, mice were optically stimulated using the same optical stimulation protocol established for LTD induction in cerebellar slices (see figure 2; 70 pulses, 400ms duration, 0.5 Hz) before training. (b) Left, both groups learned the task within 5 days

(within subjects effect, $F_{4,68}=27.346$, $p < 0.0001$, repeated measures ANOVA). OPN4-mGluR1a expressing mice ($n = 9$) swam significantly shorter distances to find the hidden platform than mCherry expressing mice ($n = 10$) (between subjects effect, $F_{1,17} = 4.79$, $p = 0.043$, repeated measures ANOVA) while velocity (middle) and thigmotaxis (defined as time swimming in periphery) did not differ between groups (mean $\pm$ SEM). (c) Averaged swim patterns of OPN4- mGluR1a (left) and mCherry (right) expressing mice. The search radius of the mice decreases with training days. This pattern is also detected by relative time spent in the target quadrant (d; mean $\pm$ SEM). (e) Platform searching strategies were divided into three searching categories consisting of 2-3 sub strategies. Searching strategies of OPN4-mGluR1a expressing mice exhibited a shift towards enhanced spatial strategies (red) starting on D3 and show lower percentage of nonspatial (ochre) and repetitive looping strategies (grey) compared to controls.