Purkinje Neurons with Loss of STIM1 Exhibit Age-Dependent Changes in Gene Expression and Synaptic Components

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The stromal interaction molecule 1 (STIM1) is an ER- Ca^{2+} sensor and an essential component of ER- Ca^{2+} store operated Ca^{2+} entry. Loss of STIM1 affects metabotropic glutamate receptor 1 (mGluR1)-mediated synaptic transmission, neuronal Ca^{2+} homeostasis, and intrinsic plasticity in Purkinje neurons (PNs). Long-term changes of intracellular Ca^{2+} signaling in PNs led to neurodegenerative conditions, as evident in individuals with mutations of the ER- Ca^{2+} channel, the inositol 1,4,5-triphosphate receptor. Here, we asked whether changes in such intrinsic neuronal properties, because of loss of STIM1, have an age-dependent impact on PNs. Consequently, we analyzed mRNA expression profiles and cerebellar morphology in PN-specific *STIM1* KO mice (*STIM1*^{PKO}) of both sexes across ages. Our study identified a requirement for STIM1-mediated Ca^{2+} signaling in maintaining the expression of genes belonging to key biological networks of synaptic function and neurite development among others. Gene expression changes correlated with altered patterns of dendritic morphology and greater innervation of PN dendrites by climbing fibers, in aging *STIM1*^{PKO} mice. Together, our data identify STIM1 as an important regulator of Ca^{2+} homeostasis and neuronal excitability in turn required for maintaining the optimal transcriptional profile of PNs with age. Our findings are significant in the context of understanding how dysregulated calcium signals impact cellular mechanisms in multiple neurodegenerative disorders.

Key words: calcium signaling; climbing fibers; excitability; mGluR1; motor coordination; RNA-Seq

Significance Statement

In Purkinje neurons (PNs), the stromal interaction molecule 1 (STIM1) is required for mGluR1-dependent synaptic transmission, refilling of ER Ca^{2+} stores, regulation of spike frequency, and cerebellar memory consolidation. Here, we provide evidence for a novel role of STIM1 in maintaining the gene expression profile and optimal synaptic connectivity of PNs. Expression of genes related to neurite development and synaptic organization networks is altered in PNs with persistent loss of STIM1. In agreement with these findings the dendritic morphology of PNs and climbing fiber innervations on PNs also undergo significant changes with age. These findings identify a new role for dysregulated intracellular calcium signaling in neurodegenerative disorders and provide novel therapeutic insights.

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Introduction

Cerebellar Purkinje neurons (PNs) play a key role in coordination of vertebrate movements. They are GABAergic neurons that integrate synaptic inputs from other regions of the brain and cerebellum and generate the sole output of the cerebellar cortex to the deep cerebellar nuclei (Albus, 1971; Ito, 2006; Dean et al., 2010). In several vertebrates studied, such as zebrafish (Namikawa et al., 2019), mouse (Burright et al., 1995; Clark et al., 1997; Watase et al., 2002; Liu et al., 2009; Prestori et al., 2019), and humans (Koeppen, 2005; Koeppen et al., 2013; Rossi et al., 2014; Hekman and Gomez, 2015), PN malfunction leads to deficits in motor function. In humans, the deficits form a spectrum of neurodegenerative disorders called ataxias, characterized by postural instability, gait disturbances, and motor incoordination (Perkins et al.,

2016; K. J. Robinson et al., 2020). An aspect of PN function, somewhat distinct from other neurons, is their strong dependence on intracellular Ca^{2+} signals through stimulation of the metabotropic glutamate receptor 1 (mGluR1) and intracellular Ca^{2+} release through ER-resident Ca^{2+} channel, the inositol 1,4,5-trisphosphate receptor 1 (IP₃R1). The importance of intracellular Ca^{2+} signaling in PN function is well established from a number of studies. Mutants in *mGluR1*, *IP₃R1*, and a Ca^{2+} -binding protein calbindin, display motor deficits (Airaksinen et al., 1997; Ogura et al., 2001; Sugawara et al., 2013). The ER- Ca^{2+} sensor or stromal interaction molecule 1 (STIM1) is also expressed in PNs (Skibinska-Kijek et al., 2009). Previous studies have shown that STIM1 is required for mGluR1-dependent synaptic transmission and the refilling of dendritic ER Ca^{2+} in PNs (Hartmann et al., 2014). Subsequently, loss of STIM1 in PNs was also demonstrated to regulate their intrinsic excitability, plasticity, and cerebellar memory consolidation (Ryu et al., 2017). To understand how changes in intracellular Ca^{2+} signaling lead to age-dependent deficits in PN function, as found in the SCAs, here we investigated molecular and cellular changes across a longitudinal time frame in the PNs of mice with cell-specific KO of *STIM1*. Both in excitable (Mao et al., 2007; Lalonde et al., 2014; Richhariya et al., 2017) and nonexcitable cells (Feske, 2007), loss of STIM1 also affects gene expression. We hypothesized that the loss of STIM1 in PNs may affect the expression of specific genes that contribute to age-dependent deficits in PNs and cerebellar function. Analysis of gene expression profiles from PNs with cell-specific KO of the *STIM1* locus demonstrated that STIM1-dependent Ca^{2+} homeostasis and signaling help maintain the expression of multiple key components of synaptic architecture and function in aging animals. Our findings are significant in the context of finding new therapeutic means of alleviating the neurodegenerative changes associated with human SCAs.

Materials and Methods

Animals. All experimental procedures were performed in accordance with Institutional Animal Ethics Committee approved by the Control and Supervision of Experiments on Animals (New Delhi, India). All transgenic mice were bred and maintained in the NCBS Animal Facility (Bangalore, India). Conditional KO using cre-lox system was adopted to generate *STIM1* KO exclusively in the PNs. Homozygous *Stim1* floxed mice, in which exon 2 of the *Stim1* gene was flanked by loxP sites (Oh-Hora et al., 2008), were bred with mice that express the Cre gene under the control of the PCP2 promoter (B6.129-Tg(Pcp2-cre)2Mpin/J, The Jackson Laboratory, RRID:IMSR_JAX:004146) (Barski et al., 2000) and with a Cre reporter mouse strain Ai14Tdtomato (B6.Cg-Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}/J, RRID:IMSR_JAX:007914, The Jackson Laboratory). A triple transgenic mouse strain *STIM1*^{flox/flox}; *Ai14Tdtomato*^{td/+}; *PCP2*^{Cre/+} was considered to be PN type-specific STIM1 KO (*STIM1*^{PKO}) mice, and a double-transgenic strain *Ai14Tdtomato*^{td/+}; *PCP2*^{Cre/+} was taken as control. The offspring obtained were genotyped using PCR of genomic DNA extracted from the tail clippings. The WT *Stim1* gene and the floxed *Stim1* gene were detected by the following primer pairs: Stim1-forward, 5'-CGATGGTCTCACGGTCTCTAGTTTC-3'; Stim1-reverse, 5'-GGCTCTGCTGACCTGGAAGTATAGTG-3' (product length for WT Stim1: 348 bp; product length for floxed Stim1: 399 bp) (Oh-Hora et al., 2008). The presence or absence of Cre was detected using the following primers: Cre-forward, 5'-GCCGAAATTGCCAGGATCAG-3'; and Cre-reverse, 5'-AGCCACCAAGCTTGATGATC-3', respectively (product length for Cre: 421 bp). The Ai14Tdtomato mutant and WT were confirmed using the following primers: Ai14Td wild forward, 5'-AAGGGAGCTGCAGTGGAGTA-3'; Ai14Td wild reverse, 5'-CCGA AAATCTGTGGGAAGTC-3'; Ai14Td mutant forward, 5'-CTGTTCCTG

TACGGCATGG-3'; and Ai14Td mutant reverse, 5'-GGCATTAAGCAGCGTATCC-3' (product length for Ai14Td wild: 297 bp, product length for Ai14Td mutant: 196 bp). For two-photon calcium imaging, a Cre-dependent, fluorescent, calcium-indicator tool strain (*GCaMP6f*^{flox/flox} (B6;129S-Gt(ROSA)26Sor^{tm95.1(CAG-GCaMP6f)Hze}/J, RRID:IMSR_JAX:024105, The Jackson Laboratory) mice was used. Rosa26-floxed-STOP-GCaMP6f mice were crossed with PCP2Cre mice to enable Gcamp6 expression exclusively in the PNs. The double transgenic strain *GCaMP6f*^{flox/+}; *PCP2*^{Cre/+} was used as controls and triple transgenic strain *Gcamp6*^{flox/+}; *STIM1*^{flox/flox}; *PCP2*^{Cre/+} was used as *STIM1* KO mice for the two-photon calcium imaging. The *GCaMP6f* mutant and WT were confirmed using the following primers: *GCaMP6f* wild forward, 5'-AAGGGAGCTGCAGTGGAGTA-3'; *GCaMP6f* mutant forward, 5'-ACGAGTCGG ATCTCCCTTTG-3'; and *GCaMP6f* reverse, 5'-CCGAAAATCTGTGGGAAGTC-3' (product length for *GCaMP6f* wild: 297 bp, product length for *GCaMP6f* mutant: 450 bp).

Immunohistochemistry. Mice were anesthetized and transcardially perfused with 1× PBS followed by 4% PFA in 1× PBS. Brains were harvested and postfixed with 4% PFA overnight and then cryoprotected in 30% sucrose in 1× PBS. The brain tissue was embedded in 5% low melting agar and sectioned using a vibratome into 35-μm-thin sections; 100-μm-thickness sections were obtained only for quantification of dendritic arborization. Sections were washed in 1× PBS, blocked for 1 h at 4°C in 0.1% Triton X-100, 5% normal goat serum, and stained with antibodies overnight at 4°C against rabbit anti-STIM1 (1:1000; Cell Signaling Technology, catalog #5668, RRID:AB_10828699), guinea pig anti-VGLUT2 (1:1000; Synaptic Systems, catalog #135404, RRID:AB_887884), and rabbit anti-S100B (1:1000; Abcam, catalog #ab52642, RRID:AB_882426). Sections were washed in PBS-T (0.1% Triton X-100 in 1× PBS) and incubated with suitable secondary antibodies which is coupled to Alexa-488 (goat anti-rabbit AlexaFluor-488, Invitrogen, catalog #A27034, RRID:AB_2536097) and goat anti-guinea pig AlexaFluor-488, Invitrogen, catalog #A-11073, RRID:AB_2534117) for 1 h at room temperature. Slices were washed in 1× PBS, mounted in Vectashield medium (Vector Labs, catalog #H-1000), and imaged using an Olympus confocal microscope with FV10-ASW 4.2 viewer software (for Olympus FV1000) or FV31S-SW 2.1 viewer software (for Olympus FV3000).

Confocal imaging and image analysis. Confocal images were obtained using a confocal laser microscope (Olympus FV1000 or FV3000 laser scanning confocal microscope) with a 20× objective (PlanApo, NA 0.2; Nikon), a 40× objective (PlanApo, NA 1.0; Olympus oil-immersion), or a 60× objective (PlanApoN, NA 1.42; Olympus oil-immersion). For estimation of STIM1 levels in the PNs, images were acquired at 1.0-μm-thickness intervals with frame size of 512 × 512 pixels. Fluorescence intensity analysis was performed by marking the outlines of PN soma as ROIs using ImageJ. The mean intensity of STIM1 and tdTomato fluorescence was calculated from a minimum of 50–60 PNs in sections from 3 animals of each genotype. For estimation of VGLUT2 puncta along PC dendrites at both proximal and distal ends, Imaris software (Bitplane, version 9.1.2) was used (Kaneko et al., 2011). The Filament Tracer software (Auto Depth) in Imaris was used to trace each dendritic filament keeping the largest diameter threshold as 3 μm and thinnest diameter as 1.86 μm. Spot detection in Imaris software was used to quantify VGLUT2 puncta by setting spot diameter threshold as 2 μm and the total distance close to the filament as 3 μm in case of proximal dendrites and 2 μm in case of distal dendrites. For quantifying the dendritic arborization, confocal images were captured at 1 μm intervals from 100-μm-thickness sections. Images of primary and secondary dendrites of PNs were analyzed with Filament Tracer (Auto Depth) to measure the total dendritic length, dendritic area, dendritic volume, and number of intersections (Kaneko et al., 2011). Confocal sections with frame size of 1024 × 1024 pixels were captured at 0.5 μm intervals using 60× objective for quantifying distal spine density. Filament Tracer (Auto Depth) was used to trace the dendrites by setting the largest diameter as 2 μm and minimum endpoint diameter as 0.311 μm, and spine density was quantified setting the maximum spine length threshold to be 1.5 μm (De Bartolo et al., 2015).

Rotarod test. *STIM1*^{PKO}, *STIM1*^{PHet}, and control mice were habituated to the rotarod (IITC, model 775, Series 8 Software) by providing a short training session where they are subjected to a constant speed of 5 rpm for 400 s. After habituating the mice to the rotarod, mice were tested for four trials a day for 5 consecutive days. In each session, the velocity of the rotation was increased with a constant acceleration of 9 rpm/min starting from 5 rpm and finally reaching to 45 rpm with a ramp speed at 240 s (Hartmann et al., 2014). The time at which the mouse fell off the rotarod was recorded, and the mean latency on the rod was calculated for the four trials for each mouse across 5 d of sessions. The same set of mice were aged and used for the rotarod assay across different ages.

Isolation and culturing of mouse PNs. Cerebellar PNs were isolated and cultured from postnatal P1 mice (*Ai14Tdtomato*^{td/+}; *PCP2*^{Cre/+} and *STIM1*^{lox/lox}; *Ai14Tdtomato*^{td/+}; *PCP2*^{Cre/+}) as described previously (Tabata et al., 2000). Mice were killed by decapitation, and their cerebella were dissected and washed in Ca²⁺ and Mg²⁺-free HBSS containing gentamicin (10 µg/ml Invitrogen, 15750045). The tissue was dissected in 2.5 ml of HBSS containing trypsin (0.1% w/v; Sigma Millipore, T1426) at 33°C for 13–15 min. The cerebella were gently triturated in 2 ml of HBSS supplemented with MgSO₄·7H₂O (12 mM) and DNase I (5 U/ml; Sigma Millipore, 11284932001) into small aggregates; ~5 ml of HBSS was added to the cell suspension and centrifuged at 1200 rpm for 5 min at 4°C. Supernatant was removed, and the density of dissociated cells was adjusted to 5 × 10⁶ cells/ml with DMEM: Nutrient Mixture F-12 (DMEM/F12 Invitrogen, 10565018) supplemented with N₂ supplement (Invitrogen, 17502048) and FBS (10% v/v; Invitrogen, 10270106); ~100 µl of the suspension was seeded onto the poly-D-lysine-coated coverslip sealed to the plastic culture dish (diameter, 35 mm; Nunc, 153066). After 3 h of incubation in the CO₂ incubator, 2 ml of culture medium was added to each dish. Culture medium used was 1:1 mixture of DMEM/F12 and Neurobasal medium (Invitrogen, 21103-049) supplemented with N-2, B-27 nutrients, and gentamicin (10 µg/ml; Invitrogen, 15750045). The cells were maintained in the CO₂ incubator, and half of the old medium was replaced after 3 d with a fresh one supplemented additionally with BSA (100 mg/ml; A3156, Sigma Millipore) and a glial proliferation inhibitor, cytosine arabinoside (4 mM; Sigma Millipore, C1768).

Calcium imaging in PNs. Dissociated PNs were cultured for 14 DIV on poly-D-lysine-coated coverslips. For calcium measurements, the cells were loaded with Fluo-4 acetoxymethyl ester (Fluo-4 AM; Invitrogen) containing 0.002% Pluronic-F-127 (Sigma Millipore) in culture medium for 30 min in the dark (Deb et al., 2016). After incubation, cells were washed with calcium containing HBSS (contains 2 mM Ca²⁺) and placed in 0 Ca²⁺ HBSS (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 2 mM MgCl₂, pH 7.3) containing 0.5 mM EGTA and 10 mM glucose and imaged for Ca²⁺ changes. Depletion of ER store calcium was initiated by addition of thapsigargin (10 µM, Invitrogen) in 0 Ca²⁺ HBSS medium, followed by addition of 2 mM CaCl₂ for induction of store-operated Ca²⁺ entry (SOCE). Fluorescence changes associated with store Ca²⁺ release and SOCE were recorded every 15 s for 30 frames. In all experiments, 10 mM ionomycin (Calbiochem) was added finally to measure maximum fluorescence intensity on complete saturation of dye with calcium. Images were acquired using the Evolution QExi CCD camera attached to a Nikon TE2000 inverted wide-field microscope equipped with 40× objective lens, using the 488 nm excitation and 520 nm emission filter sets. The time lapse acquisition mode of the In Vivo imaging software (Media Cybernetics) was used to follow fluorescence changes over time, and images were quantified using ImageJ. Quantification of SOCE data shown in Figure 5 was from dissociated PNs marked by expression of tdTomato.

Two photon calcium imaging. Parasagittal cerebellar slices of ~250 µm were obtained from Purkinje-specific GCaMP6f-expressing mice of 17 weeks old using vibratome (Leica Microsystems, VT1200) following isoflurane anesthesia and decapitation. The slices were sectioned in ice-cold cutting solution containing the following (in mM): 87 NaCl, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 75 sucrose, and 10 glucose, bubbled with 95% O₂ and 5% CO₂. The slices were immediately placed in ACSF containing the following (in mM): 125 NaCl,

2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 20 glucose bubbled with 95% O₂ and 5% CO₂. For recovery, slices were incubated at 34°C for 45 min and then kept at room temperature (~22°C) until imaging.

For two-photon calcium imaging, acute slices were placed in a recording chamber under a custom built two-photon laser scanning microscope equipped with a laser (Coherent Chameleon Ultra II). Microscopes were equipped with 20× water-immersion objective (Olympus XLUMPLFLN20XW, 1.0 NA). PNs were stimulated with 75 mM KCl or 200 µM dihydroxy phenyl glycine (DHPG, Tocris Bioscience, catalog #0805). An mGluR1 antagonist CPCCOEt (Tocris Bioscience, catalog #1028) of 200 µM was applied to test for the specificity of mGluR1 activation (Hartmann et al., 2008). Images were recorded with frame size of 512 × 512 pixels using Scan Image 3.8 and further analyzed using ImageJ.

For quantifying the changes in fluorescence, ROI was drawn around each cell and the fluorescence intensity at each time points was determined. $\Delta F/F$ [$\Delta F/F = (F_t - F_{\text{basal}})/F_{\text{basal}}$] was measured for each time point, where F_t is fluorescence of a particular time point and F_{basal} is the fluorescence of the cell when starting the experiment. Data were plotted using the Origin 8.0 software. Peak values of $\Delta F/F$ and area under the curve were obtained for every cell, and the data were plotted as histogram. Rate of Ca²⁺ entry during KCl stimulation was calculated by computing the average rate of change in fluorescence intensity (ΔF) between the time at which F_{max} ($\Delta F/F$) occurs and 11 second time points and expressed as $\Delta F/t$.

Microdissection, RNA isolation, and real-time qPCRs. Sagittal slices of the cerebellar vermis of ~250 µm thickness were obtained from 1-year-old and 14-week-old mice. Cerebellar slices were micro-dissected into Purkinje neuronal layer (PNL) with molecular layer (ML) and granular neuronal layer (GNL) with white matter under an illuminated stereomicroscope (Ryu et al., 2017).

RNA was isolated from PNL with ML and GNL with white matter using Trizol according to the manufacturer's protocol. Tissue was homogenized in 500 µl TRIzol (Invitrogen, catalog #15596026) using micropestle homogenizer followed by vortexing the sample before proceeding with RNA isolation protocol. Purity of the isolated RNA was checked by a NanoDrop spectrophotometer (Thermo Fisher Scientific), and its integrity was detected by running it on a 1% Tris-EDTA agarose gel; ~500 ng of total RNA isolated was used per sample for cDNA synthesis. For the DNase treatment, a reaction volume of 22.1 µl containing 500 ng of isolated RNA, 0.5 U of DNase I (amplification grade), 1 mM DTT, and 20 U of RNase inhibitor (RNase OUT) was incubated at 37°C for 30 min followed by heat inactivation at 70°C for 10 min. cDNA synthesis was initiated for the DNase-treated sample to which is added 200 U of Moloney murine leukemia virus reverse transcriptase, 50 µM random hexamers, and 1 mM deoxyribonucleotide triphosphate in a final volume of 20 µl. The reaction mixture was subjected to 25°C for 10 min followed by treatment at 42°C for 60 min, and finally heat inactivation at 70°C for 10 min. All reagents used were purchased from Invitrogen.

Real-time qPCRs were performed using the KAPA SYBR FAST qPCR kit (Sigma Millipore, catalog #KK4601) on an ABI 7500 Fast machine (Applied Biosystems) operated with ABI 7500 software version 2 in a total volume of 10 µl. Primers were designed by using Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). Sequences of primers for all set of genes are listed in Table 1. The fold change of gene expression of *STIM1*^{PKO} relative to WT was normalized according to the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct = [Ct(\text{target gene}) - Ct(\text{GAPDH})] \text{ STIM1}^{\text{PKO}} - [Ct(\text{target gene}) - Ct(\text{GAPDH})] \text{ STIM1}^{\text{WT}}$.

Western blots. Microdissected PNL and ML were homogenized in 1× cell lysis buffer (RIPA buffer, Sigma Millipore, catalog #R0278) containing protease inhibitor cocktail (Sigma Millipore, catalog #P8340) and phosphatase inhibitors (Cell Signaling Technologies, catalog #58705) using micropestle homogenizer. The homogenized tissues were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was collected as the total cell lysate. Protein quantity of the cell lysate was estimated by the Bradford assay, and an equal amount of protein was loaded onto SDS-PAGE 8%–12% acrylamide gels. Proteins were transferred onto nitrocellulose membrane and blocked using 5% skim milk in TBS-T

(TBS with 0.1% Tween 20) for an hour at 37°C. Blots were then incubated overnight with primary antibodies at 4°C. The primary antibodies used were rabbit anti-STIM1 (1:1000; CST, catalog #D88E10) and mouse anti- β -actin (loading control) at 1:3000 (BD Biosciences, catalog #612656). After washing with PBS-T, blots were then incubated for 1 h with secondary antibodies. The secondary antibodies used were anti-mouse HRP (1:3000; Cell Signaling Technology, catalog #7076, RRID: AB_330924) and anti-rabbit HRP (1:3000; Thermo Fisher Scientific, catalog #32260, RRID: AB_1965959). Bands were visualized with a chemiluminescence detection kit (ECL, Thermo Fisher Scientific, catalog #32109) and captured using a chemiluminescent detection system (ImageQuant LAS 4000, GE Healthcare) with Image Quant software.

Library preparation, sequencing, and RNA-Seq data analysis. RNA isolated from the PNL together with the ML was run on a Bio-analyzer chip (Agilent Technologies, catalog #5067-1511) to ensure integrity; ~500 ng of total isolated RNA with an average RIN of 8.2 was used per sample to prepare libraries using TruSeq mRNA Library Prep Kit (Illumina, catalog #20020594) following the manufacturer's instructions. The prepared libraries were run on a DNA chip (Agilent Technologies, catalog #5067-4626) in the bio-analyzer to check for their size followed by its quantification using qPCR. The libraries were then run on an Illumina HiSeq 2500 platform using single end and 50 bp read protocol (NGS Facility, NCBS). Six samples were run in a single lane, and biological triplicates were performed for each sample consisting of RNA isolated from WT PNs (with ML) and *STIM1* KO PNs (with ML).

More than 60 million reads were obtained per sample with a uniform distribution of reads across samples. FASTQ sequencing reads obtained were aligned to the annotated UCSC *Mus musculus* mm10 (GRCm38/mm10) using Tophat (version 2.0.13) (Trapnell et al., 2009). Read counts for each transcript or exon were calculated using the Python-based package HTSeq (version 0.9.1) (Anders et al., 2015). Differential expression on *STIM1* KO was estimated by using two R-based Bioconductor software packages: DESeq (RRID:SCR_000154) (Anders and Huber, 2012) and EdgeR (RRID:SCR_000154, empirical analysis of digital gene expression in R) (Anders and Huber, 2010; M. D. Robinson et al., 2010) that analyze the read counts per transcript per sample and normalize them before using an exact test to identify differentially expressed genes using a negative binomial model (Anders and Huber, 2010; Seyednasrollah et al., 2015). DESeq package uses a Relative Log Expression normalization strategy (Anders and Huber, 2010; Anders et al., 2013; Love et al., 2014), and EdgeR package uses Trimmed Mean of M values normalization (M. D. Robinson and Oshlack, 2010; M. D. Robinson et al., 2010). In both the differential analysis methods, $p < 0.05$ was set as cutoff. Genes that are found to be significantly altered by both DESeq and EdgeR analysis methods were considered further. FunRich (Functional enrichment analysis tool) (Pathan et al., 2015) was used to compare significantly altered gene lists from DESeq and EdgeR, and to generate Venn diagrams keeping mouse genome as the background gene set. Volcano plot was plotted using R package (version 3.3.3), and heat maps were generated using HemI (Heatmap Illustrator, version 1.0.3.7) (Deng et al., 2014). Gene enrichment analysis and visualization of the Gene Ontology (GO) network were performed using Metascape (<http://metascape.org>) using parameters specific for *Mus musculus* with p value cutoff as 0.01, count threshold at 3, and minimum enrichment as 1.5. Significantly enriched GO terms for biological process, molecular, cellular component, KEGG pathways, and Reactome have been plotted as bar graphs.

Experimental design and statistical analysis. Mice of both sexes were used for experiments. Statistical analysis was performed using Origin 8.0 software, GraphPad Prism 7.0, or R package. The statistical methods used in each experiment are described in the figure legends. All bar graphs and dot plots show mean $\pm$ SEM, and differences were considered as significant for $p < 0.05$, highly significant for $p < 0.01$ and $p < 0.001$ as determined using the paired Student's t test or a two-way ANOVA for multiple measurements. For rotarod test, two-way ANOVA, followed by Sidak's or Tukey's multiple comparison test, was used for comparisons between groups, and $p < 0.05$, $p < 0.001$, and $p < 0.0001$ were considered. Tukey's multiple comparison test was used for comparing the significance between the mean latency on the rod for

Table 1. Primers used for real-time qPCR^a

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Pcp2</i>	CCAGGCCAGAACCCAGAAAG	CCCAGGTCGTTCTGCATTC
<i>Stim1</i>	ACAACCTGGACTGTGGATGAGG	TGGTACTGCTAGCCTTGGC
<i>Gabra6</i>	TGCTGGAAGGCTATGACAACC	GTCTGGCGGAAGAAACATCC
<i>Itpr1</i>	TGAAGGGGAACAGAACGAGC	AGGCCGATCTTTGTTCTGC
<i>Pvalb</i>	ATGGGGACGGCAAGATTGG	GCGAGAAGGACTGAGATGGG
<i>Calm1</i>	CGTCTCTCTCTCTGCTCG	TTCTTGTGTGTGATGGTCC
<i>Orai3</i>	CCTGTGGCTGGTTTTATC	GTGCCCGGTGTAGAGAATG
<i>Casq2</i>	AGCCCAACGTCATCCCTAAC	AGTCGCTCTCTCTGTAGTCC
<i>S100b</i>	GATGCTCTCCACAGTATCCG	AGCGTCTCTCTGTGTGTC
<i>Cacng5</i>	CTTCTGTGATGTGAGGGCG	CAAAAGTGGAGTCGAGCGC
<i>Atp1a3</i>	CTGCCGACATGATTCTGCTGG	AGGAAGGGTGTGATCTCAGGG
<i>Kctd17</i>	GGCTCTCTACAACTATGGG	GGAGGGAGAAAGGTTAGCGG
<i>Syt11</i>	CAAGAGGAACATTCAGAAGTGC	CCTGAGAGACCGGTGATATCC
<i>Vamp1</i>	CCCGTCTCGTTGCATTCTCC	GTGATGTTGGGAGGAGGAGG
<i>Dlg4</i>	ACCAAGATGAAGACACGCC	TTCCGTTACATATCTGGGG
<i>Robo2</i>	CTGCCATCTAGACCTGACTCC	ACGAGATCTTGACCTTGCC
<i>Gigyl2</i>	GGACCGCAGTGTTAAAGACG	TCTGCTGCCATCTCTCCG
<i>Map4</i>	AGCCAGGTTGAAGGTATCCC	TGGTCTGCTGATAATCCGG
<i>Tfap2b</i>	TCAATGCATCTCTCTGGGG	TGAACAGCTTCCCTCTACC
<i>Setd6</i>	TGGTTTGTGAGCCCTATCC	CCCTACCATCTCTCTGTTGC
<i>Bad</i>	GGGGAGCAACATTCATCAGC	CATCCCTCATCTCTCTCCG
<i>Insr</i>	TTCTAAAGATCCGCCGCTCC	GTTGTGTTGTCTCAGTCCC
<i>Gapdh</i>	CTTGGCATTGTGAAGGGC	TGCAGGATGATGTTCTGGG

^aSequences of primers for all set of genes for validation using qRT-PCR. Primers were designed using Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). *Pcp2*, Purkinje cell protein 2; *Stim1*, stromal interaction molecule 1; *Gabra6*, GABA type A receptor subunit alpha6; *Itpr1*, inositol 1,4,5-trisphosphate receptor 1; *Pvalb*, parvalbumin; *Calm1*, calmodulin 1; *Casq2*, calsequestrin 2; *Cacng5*, calcium voltage-gated channel auxiliary subunit γ 5; *Atp1a3*, ATPase Na⁺/K⁺ transporting subunit α 3; *Kctd17*, potassium channel tetramerization domain containing 17; *Syt11*, synaptotagmin 11; *Vamp1*, vesicle-associated membrane protein 1; *Dlg4*, discs large homolog 4; *Robo2*, roundabout guidance receptor 2; *Gigyl2*, GRB10-interacting GYF protein 2; *Map4*, microtubule-associated protein 4; *Tfap2b*, transcription factor AP-2 β ; *Setd6*, SET domain containing 6; *Bad*, Bcl-2 associated agonist of cell death; *Insr*, insulin receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

Table 2. Fold changes in mRNA on *STIM1* KO in PNs^a

Gene	14 weeks			1 year		
	<i>STIM1</i> ^{WT}	<i>STIM1</i> ^{PKO}	p	<i>STIM1</i> ^{WT}	<i>STIM1</i> ^{PKO}	p
<i>Itpr1</i>	1.02 $\pm$ 0.09	0.92 $\pm$ 0.05	0.38765	1.01 $\pm$ 0.08	0.71 $\pm$ 0.09*	0.03393
<i>Pvalb</i>	1.01 $\pm$ 0.06	0.91 $\pm$ 0.05	0.22470	1.01 $\pm$ 0.05	0.68 $\pm$ 0.05***	0.00098
<i>Calm1</i>	1.04 $\pm$ 0.14	1.03 $\pm$ 0.14	0.98871	1.02 $\pm$ 0.08	0.64 $\pm$ 0.14*	0.04656
<i>Orai3</i>	1.03 $\pm$ 0.11	0.81 $\pm$ 0.11	0.19959	1.00 $\pm$ 0.02	0.80 $\pm$ 0.04**	0.00596
<i>Casq2</i>	1.03 $\pm$ 0.11	1.21 $\pm$ 0.13	0.29986	1.02 $\pm$ 0.09	0.73 $\pm$ 0.06*	0.02960
<i>S100b</i>	1.02 $\pm$ 0.09	0.78 $\pm$ 0.10	0.10524	1.01 $\pm$ 0.06	0.70 $\pm$ 0.11*	0.04868
<i>Cacng5</i>	1.04 $\pm$ 0.12	1.16 $\pm$ 0.15	0.54134	1.01 $\pm$ 0.06	0.81 $\pm$ 0.05*	0.03345
<i>Atp1a3</i>	1.01 $\pm$ 0.04	0.92 $\pm$ 0.06	0.30635	1.00 $\pm$ 0.04	0.77 $\pm$ 0.09*	0.04507
<i>Kctd17</i>	1.04 $\pm$ 0.13	1.15 $\pm$ 0.12	0.56564	1.01 $\pm$ 0.09	0.77 $\pm$ 0.05*	0.03983
<i>Syt11</i>	1.02 $\pm$ 0.09	0.89 $\pm$ 0.06	0.28322	1.01 $\pm$ 0.08	0.80 $\pm$ 0.05*	0.04942
<i>Vamp1</i>	1.06 $\pm$ 0.19	0.87 $\pm$ 0.09	0.37641	1.02 $\pm$ 0.06	0.65 $\pm$ 0.13*	0.04647
<i>Dlg4</i>	1.04 $\pm$ 0.12	0.79 $\pm$ 0.07	0.12287	1.01 $\pm$ 0.08	0.77 $\pm$ 0.06*	0.03421
<i>Robo2</i>	1.05 $\pm$ 0.14	0.79 $\pm$ 0.15	0.24889	1.01 $\pm$ 0.08	0.75 $\pm$ 0.08*	0.04005
<i>Gigyl2</i>	1.01 $\pm$ 0.07	1.24 $\pm$ 0.07	0.04737	1.00 $\pm$ 0.06	0.83 $\pm$ 0.05*	0.04587
<i>Map4</i>	1.04 $\pm$ 0.13	1.06 $\pm$ 0.15	0.94084	1.01 $\pm$ 0.07	0.83 $\pm$ 0.03*	0.04470
<i>Tfap2b</i>	1.09 $\pm$ 0.22	1.06 $\pm$ 0.16	0.88493	1.01 $\pm$ 0.06	0.79 $\pm$ 0.07*	0.04835
<i>Setd6</i>	1.02 $\pm$ 0.08	0.90 $\pm$ 0.07	0.32807	1.01 $\pm$ 0.09	0.59 $\pm$ 0.08**	0.00651
<i>Ddx17</i>	1.03 $\pm$ 0.11	0.87 $\pm$ 0.04	0.21657	1.01 $\pm$ 0.07	0.79 $\pm$ 0.06*	0.04358
<i>Bad</i>	1.04 $\pm$ 0.13	0.91 $\pm$ 0.09	0.47290	1.00 $\pm$ 0.04	1.25 $\pm$ 0.09*	0.04716
<i>Insr</i>	1.03 $\pm$ 0.10	1.19 $\pm$ 0.06	0.22536	1.00 $\pm$ 0.05	1.16 $\pm$ 0.04*	0.03543

^aThe fold changes of the indicated genes from biologically significant pathways in WT and *STIM1* KO conditions of 14-week- and 1-year-old mice. All measurements are done by qRT-PCR. Fold changes are normalized to WT levels (reference gene, *GAPDH*) ($n = 6$). Data are mean $\pm$ SEM.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-tailed Student's t test.

STIM1^{WT}, *STIM1*^{PHet}, and *STIM1*^{PKO} mice, and Sidak's multiple comparison test was used for comparison between *STIM1*^{WT} and *STIM1*^{PKO} mice.

Data availability. The RNA-Seq data associated with this manuscript has been submitted to GEO with accession number GSE158513.

Figure 1. Genotyping of control and *STIM1* KO transgenic mice. **A**, Top, Schematic diagram showing exon 2 of *STIM1* gene flanked by *loxP* recombination sites (yellow triangles). Deletion of this exon following Cre-mediated recombination is expected to result in a frame shift that generates a premature stop codon in the next exon. Middle, Schematic diagram showing Cre reporter cassette inserted into the intron between endogenous exons 1 and 2 of the *Rosa26* locus. Bottom, Schematic diagram showing insertion of Cre-recombinase cDNA into the exon 4 of *PCP2* gene. Primers are indicated by arrows (FP, forward primer; RP, reverse primer). **B**, Top, Reporter mouse line with tdTomato-expressing Purkinje cells was generated by cross breeding floxed stop *tdTomato* mice (*Ai14-tdTomato*) and *PCP2-Cre*. Bottom, *STIM1*^{PKO} mouse line of tdTomato-expressing Purkinje cells was generated by cross breeding homozygous double transgenic *STIM1*^{flox/flox}; *Ai14-tdTomato*^{td/td} with *STIM1*^{flox/+}; *PCP2-Cre*^{Cre/+}. **C**, Agarose gel with genotyping of *STIM1* KO mice. PCRs of genomic DNA from *Stim1*^{flox/flox}; *Ai14*^{td/+}; *PCP2*^{Cre/+} (*STIM1*^{PKO}) mice are given on lanes 1–3 and 4–6. A single band at 399 bp is for homozygous *STIM1* flox (lanes 1, 4), two bands at 297 bp (*Rosa*^{WT}) and 196 bp (*Ai14tdT* insert in the *Rosa* locus) (lanes 2, 5), and 421 bp for *PCP2Cre* (lanes 3, 6). PCRs with genomic DNA from *Stim1*^{flox/+}; *Ai14*^{td/+}; *PCP2*^{Cre/+} (*STIM1*^{PHet}) mice are shown in lanes 7–9. **D**, Genotyping of control mice. Lanes 1–4 with *Ai14*^{td/+}; *PCP2*^{Cre/+} (*STIM1*^{WT}), sizes of DNA bands are as described above for **C**. L, DNA ladder for both **C** and **D**.

Results

Characterization of STIM1 protein in cerebellar PNs across ages

STIM1 is a key component of SOCE (Liou et al., 2005; Roos et al., 2005), and previous studies have shown that loss of STIM1 in PNs leads to changes in intracellular calcium homeostasis and reduced neuronal excitability. At the organismal level, loss of STIM1 in PNs affects motor learning and memory consolidation (Hartmann et al., 2014; Ryu et al., 2017). To understand the molecular basis of these deficits, we generated PN-specific *STIM1* KO (*STIM1*^{PKO}) mice using the conditional Cre-lox system (see Materials and Methods). Exon 2, which encodes the EF hand of

STIM1 (Oh-Hora et al., 2008), was deleted using a *PCP2* (L7)-*Cre* transgenic mouse strain that exhibits near-specific expression in PNs postembryonic day 19 (Barski et al., 2000). To mark *STIM1* KO neurons, a triple transgenic mouse strain was generated, where expression of the red fluorescent protein tdTomato was based on Cre activity (*STIM1*^{flox/flox}; *Ai14Tdtomato*^{td/+}; *PCP2-Cre*^{Cre/+}, subsequently referred to as *STIM1*^{PKO}) (Fig. 1A–C). A double transgenic strain *Ai14Tdtomato*^{td/+}; *PCP2-Cre*^{Cre/+} (*STIM1*^{WT}) was taken as the control (Fig. 1A,B,D).

It has been reported that Cre-mediated recombination by *PCP2-Cre* is fully established by postnatal day 21 (Barski et al., 2000). Therefore, we tested for loss of STIM1 from PNs at 6

Figure 2. Loss of STIM1 occurs slowly after *STIM1* KO in PNs. Cerebellar sections with PNs from *STIM1*^{WT} and *STIM1*^{PKO} mice, immunostained as indicated. Sagittal sections prepared from *STIM1*^{WT} and *STIM1*^{PKO} mice at 6 weeks (**A**), 12 weeks (**B**), and 1 year (**C**) were stained with anti-STIM1 antibody and imaged at 20× (left) and 40×. PNs (white arrows), GL (red asterisks), PN dendrites (black arrows), and PN axons (white asterisks) are marked. Scale bars, 50 μm. Quantification of STIM1 levels in the soma of PNs across different ages is shown in Figure 3.

weeks by immunohistochemistry. Despite expression of tdTomato in PNs at 6 weeks, indicating PCP2-Cre activity, a substantial level of STIM1 remained in the PNs (Fig. 2*A*, white arrowheads). Expression of STIM1 in the soma of PNs was not detected at 12 weeks, as evident by loss of colocalization between anti-STIM1 (green) and tdTomato (red; Fig. 2*B*, white arrowheads). In general, we observed that STIM1 expression was low in axonal processes

(Fig. 2, white asterisks) compared with PN soma and dendrites (Fig. 2, black arrows on PN dendrites). STIM1 expression in the granule layer (GL) of both control and *STIM1*^{PKO} appears unaltered in the immunostained sections (GL, red asterisks). Quantification of STIM1 in PN soma, obtained by fluorescence intensity measurements, also identified residual STIM1 in *STIM1*^{PKO} PNs from mice 6 weeks of age, that was significantly reduced by 12 weeks (Fig. 3*A*,

Figure 3. Quantification of STIM1 and characterization of PN and Bergmann glia in cerebellar sections. **A**, Bar graph with quantification of STIM1 in the soma of PN compared with the expression of tdTomato seen at 6 weeks ($STIM1^{WT}$, $n = 53$ PN; $STIM1^{PKO}$, $n = 68$ PN), 12 weeks ($STIM1^{WT}$, $n = 64$ PN; $STIM1^{PKO}$, $n = 74$ PN), and 1 year ($STIM1^{WT}$, $n = 65$ PN; $STIM1^{PKO}$, $n = 77$ PN). Immunofluorescence intensities of STIM1 and tdTomato were quantified from PN soma by placing identical hand-drawn ROIs on the respective images, using ImageJ software. The total PN analyzed are from 3 mice per group. Data are mean $\pm$ SEM. $*p < 0.001$ (two-tailed Student's t test). **B**, Representative images of confocal sections from the indicated genotypes and ages showing the STIM1 (green) and tdTomato (red) in PN soma (white arrowheads). **C**, Immunostained cerebellar sections from $STIM1^{WT}$ and $STIM1^{PKO}$ mice 1.5 years of age with intact PN. Red represents tdTomato. Green represents STIM1. **D**, Characterization of Bergmann glia marked by S100B in cerebellar sections from control mice 1 year of age. Top, Sagittal sections were imaged at $40\times$ magnification. Bottom, An enlarged image is shown with the location of the Bergmann glial cell body. A PN and a Bergmann glial cell body are marked with white asterisks and white arrowheads, respectively. Cyan arrowheads indicate non-Purkinje cells expressing tdTomato and not marked by S100B. Scale bars: **B–D**, 50 μ m.

B). Loss of STIM1 did not affect the viability of PN until 1 and 1.5 years, as evident in Figures 2C and 3C. The time lag between Cre expression (3 weeks) and loss of STIM1 protein (12 weeks) might be because of slow protein turnover of STIM1. Alternately, there might be a difference between recombination efficiency of the *STIM1* flox allele and the *tdTomato* flox allele. A few interneurons in the ML also express tdTomato indicating PCP2-Cre expression in cells other than PN. Subsequent studies for understanding STIM1 function in PN were performed in mice 12 weeks of age or older.

Loss of STIM1 in PN leads to deficits in motor learning and coordination

Previous studies have reported the importance of STIM1 in regulating cerebellar motor behavior (Hartmann et al., 2014) and memory consolidation (Ryu et al., 2017). To identify the exact stage at which the motor learning phenotype develops and to investigate its progression with age, control and $STIM1^{PKO}$ mice were subjected to a standard rotarod assay over 5 d (Hartmann et al., 2014) (see Materials and Methods). During each test session, animals were placed on a rotating rod that was accelerated continuously from 5 to 45 rpm, and the time at which the animal

fell off the accelerating rotarod was noted. The ability to stay longer on the rotarod indicates better motor learning and coordination. Whereas both control and $STIM1^{PKO}$ mice performed comparably at either 9 or 14 weeks (Fig. 4A,B), a significant decrease in time spent by $STIM1^{PKO}$ mice on the accelerating rotarod was evident from 17 weeks onward, particularly after 4 d of training (Fig. 4C). Comparable rotarod performance at 9 and 14 weeks is not surprising given that STIM1 immunoreactivity from $STIM1^{PKO}$ PN is lost at 12 weeks. In control mice, average latency to fall improved from 152.4 ± 7.2 s (day 1) to 232 ± 12.7 s over 5 d (Fig. 4C) in case of mice 17 weeks of age. In contrast, $STIM1^{PKO}$ mice fell off the rotarod with a similar latency as controls on day 1 (146.4 ± 10.1 s) but over 5 d did not exhibit improvement to the same extent as controls (day 5, 163.1 s; Fig. 4C). The same trend was observed at 29 weeks (Fig. 4D) and at 1 year of age (Fig. 4E). To our surprise, we also observed a decrease in the latency on the rotarod in case of $STIM1^{PHet}$ animals compared with that of control mice at all ages tested, indicating an effect of the loss of one copy of STIM1 in PN on motor learning. Progression of the deficit in motor learning with age appeared similar in control, $STIM1^{PHet}$, and $STIM1^{PKO}$ mice, as evident by comparing motor performance across ages on the fifth day of training (Fig. 4F).

Reduced membrane depolarization and loss of mGluR1 stimulated Ca^{2+} signals in *STIM1* KO PNs

It is likely that altered calcium homeostasis in *STIM1*^{PKO} PNs underlies the motor learning deficits observed in *STIM1*^{PKO} mice. Because STIM1 is an integral component of SOCE in both nonexcitable (Liou et al., 2005; Roos et al., 2005; Oh-Hora et al., 2008; H. Cheng et al., 2016) and excitable cells (Venkiteswaran and Hasan, 2009; Gruszczynska-Biegala et al., 2011; Hartmann et al., 2014; Ryu et al., 2017), as a first step we investigated SOCE in cultured PNs obtained from postnatal day 1 mice and cultured for 14 d (Fig. 5). In comparison with control PNs (*Ai14Tdtomato*^{td/+}; *PCP2-Cre*^{cre/+}), SOCE from *STIM1*^{PKO} PNs was muted but not abolished (Fig. 5A,B). The continued presence of reduced levels of STIM1 protein even after gene KO, as evident in Figures 2 and 3A, B, probably contributes to the SOCE in *STIM1*^{PKO} PNs. Our efforts to culture PNs either beyond 14 d or from cerebella of mice older than postnatal day 7 were not successful, hindering attempts to measure SOCE from PNs in which the STIM1 protein was abolished. Instead, calcium transients were measured in acute cerebellar slices from PNs expressing the genetically encoded calcium sensor, GCaMP6f using two-photon calcium imaging (Fig. 6). Changes in excitability between control PNs and *STIM1*^{PKO} PNs were measured from cerebellar slices of mice 17 weeks of age and stimulated with 75 mM KCl. WT PNs show robust cytosolic calcium elevations on addition of KCl (Fig. 6A–E, blue trace), whereas calcium responses from *STIM1*^{PKO} PNs were significantly reduced compared with that of controls (Fig. 6A–E, red trace). The significant reduction in calcium responses of *STIM1*^{PKO} PNs, on depolarization with KCl, agrees with the motor deficit observed in mice 17 weeks of age (Fig. 4C). These data are also in agreement with a range of electrophysiological measurements, including current clamps undertaken in-depth in a previous study (Ryu et al., 2017). The authors found reduced firing frequency and reduced excitability in *STIM1*^{PKO} PNs, which they attribute to the dysregulation of ionic currents through multiple membrane channels by changes in intracellular calcium dynamics (Ryu et al., 2017).

The mGluR1 is abundantly expressed in PNs (Lein et al., 2007; Ohtani et al., 2014). Mice with genomic deletion of *mGluR1* exhibit severe ataxic symptoms (Aiba et al., 1994) that can be rescued by expression of mGluR1 exclusively in PNs (Ichise et al., 2000). Moreover, autoantibodies against mGluR1 in humans with paraneoplastic cerebellar ataxia (Smitt et al., 2000; Coesmans et al., 2003) impair cerebellar motor coordination. Thus, mGluR1 signaling in PNs is required for cerebellar function, including motor learning and coordination. We assessed mGluR1 activation in the PNs of both control and *STIM1*^{PKO} mice by stimulating cerebellar slices with the mGluR1 agonist DHPG (200 μM , Fig. 6F–H). In control PNs, DHPG stimulation evoked large calcium transients in most PNs (Fig. 6F,H),

Figure 4. Motor coordination deficits in *STIM1* KO mice. **A**, Mean latency on the rotarod for *STIM1*^{WT} and *STIM1*^{PKO} mice at 9 weeks (*STIM1*^{WT}, *n* = 5 and *STIM1*^{PKO}, *n* = 5) and **B** at 14 weeks (*STIM1*^{WT}, *n* = 7 and *STIM1*^{PKO}, *n* = 5). Two-way ANOVA, *post hoc* test, and Sidak's multiple comparison test were used for comparisons. **C–E**, Mean latency on the rotarod for *STIM1*^{WT} (*n* = 23), *STIM1*^{het} (*n* = 13), and *STIM1*^{PKO} (*n* = 24) mice. Significant changes in latencies were estimated using two-way ANOVA, *post hoc* test, followed by Tukey's multiple comparisons test. **p* < 0.05; ***p* < 0.01; ****p* < 0.0001; comparison of *STIM1*^{WT} and *STIM1*^{PKO} mice. **F**, Latencies to fall from the accelerated rotarod on day 5 for *STIM1*^{WT}, *STIM1*^{het}, and *STIM1*^{PKO} mice across different ages, shown as box plots. Horizontal line indicates the median. Black solid diamond represents the mean. Colored diamonds represent individual data points. In every box plot, the limits extend from 25th to 75th percentile.

although initiation of the calcium responses by DHPG occurred at varying time points in different PNs (Fig. 6F). This variation in the response time could be because of differences in the time taken for DHPG to reach the area focused under the microscope. Interestingly, in *STIM1*^{PKO} PNs, DHPG application failed to evoke measurable calcium transients (Fig. 6G,H). The specificity of mGluR1 activation was tested by addition of CPCCOEt, an mGluR1 antagonist (Hartmann et al., 2008). Application of CPCCOEt (200 μM) abolished the DHPG-induced calcium responses otherwise observed in control PNs (Fig. 6I). Notably, loss of mGluR1 activation observed in *STIM1*^{PKO} PNs from mice 17 weeks of age (Fig. 6G,H) occurs within a few weeks of loss of STIM1 protein (12 weeks, Fig. 2B) and also correlates with the onset of motor dysfunction noticed in *STIM1*^{PKO} mice (17 weeks, Fig. 4C). These data demonstrate that STIM1 is required for a significant proportion of mGluR1-activated calcium transients that appear in the soma and dendrites of PNs. A previous study demonstrated significant reduction in DHPG-evoked

inward currents and local Ca^{2+} transients in the dendrites of $\text{STIM1}^{\text{PKO}}$ PN, which they attribute to reduced levels of Ca^{2+} in dendritic ER stores that in turn are insufficient to stimulate DHPG-evoked slow EPSC Ca^{2+} currents (sEPSCs) (Hartmann et al., 2014). Because DHPG-evoked ER- Ca^{2+} release followed by Ca^{2+} entry resulting from the overlap in time of sEPSCs (Hartmann et al., 2014), these cannot be easily distinguished from each other, but it is likely that the larger amplitude and longer time scale of the sEPSCs contribute primarily to the Ca^{2+} signals seen in Figure 6F. Thus, our findings further augment the role of STIM1 in regulating neuronal excitability and synaptic transmission.

KO of *STIM1* alters the profile of gene expression in PNs

A key aim of this study was to understand long-term molecular and cellular changes that occur in fully differentiated PNs because of dysregulated intracellular calcium signaling and SOCE by loss of STIM1. Changes in gene expression have been reported on reduced SOCE in nonexcitable immune cells (Feske, 2007), the developing mouse brain (Somasundaram et al., 2014), *Drosophila* pupal neurons (Richhariya et al., 2017), and human neural precursor cells (Gopurappilly et al., 2018). Gene expression profiles of mature differentiated neurons with loss of STIM1/nSOC have not been published to date. To test for gene expression changes in $\text{STIM1}^{\text{PKO}}$ PNs, we micro-dissected PNs from 1-year-old $\text{STIM1}^{\text{PKO}}$ and control animals. RNA was isolated from the PNL+ML (Fig. 7A) and from the GL. Specificity of tissue dissection was tested by measuring levels of RNA of a Purkinje neuronal marker, the Purkinje cell protein-2 (PCP2), and a granule cell marker, the GABA(A) receptor $\alpha 6$ subunit (GABRA6) (Boyden et al., 2006). Micro-dissected PNL and ML from control and $\text{STIM1}^{\text{PKO}}$ cerebella showed high expression of PCP2 compared with the granular layer (Fig. 7B, left) and a low level of the granule cell marker GABRA6 (Fig. 7B, right), suggesting minimal contamination from granule neurons in the PN-ML sample. Importantly, a significant reduction of STIM1 RNA was observed in PN-ML isolated from the STIM1 KO mice compared with control mice (Fig. 7B, middle). STIM1 expression levels in the GL were not significantly altered (Fig. 7B, middle) as observed in the immunostained sections in Figure 2. Western blot data also showed that the STIM1 protein was significantly reduced in $\text{STIM1}^{\text{PKO}}$ PN-ML (Fig. 7C).

RNA expression studies were performed with RNA isolated from the PN-ML of $\text{STIM1}^{\text{PKO}}$ and control animals. Analysis of the RNA sequencing data demonstrated significant changes in gene expression in micro-dissected PN-ML from $\text{STIM1}^{\text{PKO}}$ cerebella compared with controls. Differentially expressed genes are shown as a volcano plot where red dots and green dots represent sets of transcripts expressed at significantly lower and higher levels in $\text{STIM1}^{\text{PKO}}$ PN-ML, respectively (Fig. 7D; EdgeR analysis). The expression of 285 genes was significantly different between control and $\text{STIM1}^{\text{PKO}}$ PN-ML based on differential analysis by both EdgeR and DeSeq and $p < 0.05$. Of these, 168 genes were downregulated and 117 were upregulated (Fig. 7E).

Interestingly, calcium signaling genes, such as *IP3R1*, *IP3R2*, and *Orai3*, and genes encoding calcium-binding proteins, such as parvalbumin (*Pvalb*), calmodulin1 (*Calml1*), calsequestrin 2 (*Casq2*), and S100B (*S100β*), were significantly downregulated in $\text{STIM1}^{\text{PKO}}$ PN-ML (Fig. 7F). Expression of *Orai1* appears very low in PNs of both STIM1^{WT} (biological replicate read counts: 0, 0, 3) and $\text{STIM1}^{\text{PKO}}$ (biological replicate read counts: 0, 2, 0) mice. Expression of *Orai2* is slightly higher than *Orai1* in both genotypes (STIM1^{WT} biological replicate read counts: 7, 11, and

Figure 5. SOCE in cultured PNs. **A**, Changes in cytosolic Ca^{2+} on ER store depletion by treatment with thapsigargin (TG) followed by SOCE on addition of extracellular Ca^{2+} . PNs were obtained from P1 mice, cultured 14 DIV, and loaded with the Ca^{2+} -sensitive dye, Fluo-4 AM. Each trace represents the mean response curve of 31 PNs (STIM1^{WT}) and 14 PNs ($\text{STIM1}^{\text{PKO}}$). **B**, Representative images of cultured PNs from the indicated genotypes showing tdTomato fluorescence, basal cytosolic Ca^{2+} (0 Ca^{2+}) followed by changes in cytosolic Ca^{2+} levels on ER- Ca^{2+} store release (TG), SOCE (2 mM Ca^{2+}), and measurement of dye saturation (ionomycin). Scale bars, $20 \mu\text{m}$.

12 and $\text{STIM1}^{\text{PKO}}$ biological replicate read counts: 11, 8, and 7; from data in GEO: GSE158513). Neither *Orai1* nor *Orai2* expression appears affected in PN+ML from $\text{STIM1}^{\text{PKO}}$ mice. Change in *S100β* levels were intriguing because this protein is expressed in Bergmann glial cells (Fig. 3D, marked with white arrowheads) that are unipolar astrocytes located around PNs (Fig. 3D, marked with white asterisks) and Bergmann fibers enwrap synapses on Purkinje cell dendrites (Landry et al., 1989; Yamada and Watanabe, 2002; Hachem et al., 2007). Altered expression of *S100β* in $\text{STIM1}^{\text{PKO}}$ suggests that loss of mGluR1 signals and reduced excitability in PNs also affect gene expression of closely associated glial cells (see Discussion).

Abolishing STIM1 protein in PNs also reduced the expression of various ion channels and pumps, such as calcium voltage-gated channel auxiliary subunit $\gamma 5$ (*Cacng5*), ATPase Na^+/K^+ transporting subunit $\alpha 3$ (*Atp1a3*), and synaptic signaling genes, such as Synaptotagmin 11 (*Syt11*) and vesicle-associated membrane protein 1 (*Vamp1*) (Fig. 7F). Thus, loss of STIM1 affects the expression of key components of intracellular Ca^{2+} signaling and homeostasis as well as some components of membrane excitability. Genes involved in regulation of cell cycle process, such as *Plk4* (Polo-like kinase 4), *Ovol1* (Ovo-like transcriptional

Figure 6. Reduced membrane depolarization and mGluR1 activation in *STIM1* KO PNs. **A**, Line plot of the mean traces ($\pm$ SEM) of normalized GCaMP6f fluorescence responses ($\Delta F/F$) in PN soma on 75 mM KCl stimulation (*STIM1*^{WT}, 84 PNs, 8 mice; *STIM1*^{PKO}, 44 PNs, 5 mice). **B**, Representative images of changes in GCaMP6f fluorescence from PN soma at indicated time points following KCl stimulation. Red arrowheads indicate PN soma. Scale bars, 20 μ m. **C–E**, Bar graphs with peak $\Delta F/F$ (*STIM1*^{WT}, 12.41 ± 0.57 ; *STIM1*^{PKO}, 7.08 ± 0.40 ; $p = 4.10 \times 10^{-12}$), area under the curve (*STIM1*^{WT}, 360.21 ± 27.82 ; *STIM1*^{PKO}, 273.59 ± 31.92 , $p = 0.04338$), and rate of calcium entry $\Delta F/\text{Sec}$ (*STIM1*^{WT}, 2.54 ± 0.27 ; *STIM1*^{PKO}, 0.56 ± 0.04 , $p = 2.11 \times 10^{-10}$) quantified from **A**, respectively. Each bar is compared with control shown in blue. * $p < 0.05$; ** $p < 0.0001$; two-tailed Student's *t* test. Line plots of GCaMP6f fluorescence responses ($\Delta F/F$) in PN soma of (**F**) control (30 PNs, 4 mice) and (**G**) *STIM1*^{PKO} (55 PNs, 4 mice) on addition of the mGluR1 agonist, 200 μ M DHPG. **H**, Snapshots of GCaMP6f responses from PN soma at indicated time points on DHPG stimulation. Red arrowheads indicate the PN soma. Scale bars, 20 μ m. **I**, Mean traces ($\pm$ SEM) of normalized GCaMP6f fluorescence responses ($\Delta F/F$) in PN soma on DHPG stimulation in slices incubated with 200 μ M CPCCOEt, an mGluR1 antagonist (*STIM1*^{WT}, 39 PNs, 4 mice; *STIM1*^{PKO}, 43 PNs, 4 mice).

repressor 1), and *Map6d1* (MAP6 domain containing 1), and those involved in regulation of cellular carbohydrate metabolic process, such as *Bad* (BclII Associated Agonist of Cell Death), *InsR* (Insulin Receptor), and *Kat2b* (Lysine Acetyltransferase 2B), are significantly upregulated (Fig. 7G). Together, these data suggest that signaling through STIM1 has an important role in regulating gene expression in mature differentiated PNs.

Biological pathways affected by absence of STIM1 in PNs

To further understand the nature of signaling mechanisms regulated by STIM1 in murine PNs, we used Metascape to predict the

GO of differentially regulated genes (Figs. 8, 9). Endocytic recycling, neuron projection development, protein transport, ER calcium ion homeostasis, postsynapse organization, and glutamate receptor signaling pathway were among significantly downregulated GO biological processes (Fig. 8A). A network plot of GO biological processes for the downregulated gene set was constructed using Metascape (Fig. 8B) to help understand intercluster gene similarities and intracluster gene redundancies among the enriched GO terms. Regulation of neuron projection development (blue) and postsynapse organization (gray) share several genes, and the interconnecting edges indicate the relative

relatedness of these two processes. As might be expected, endocytic recycling (red), receptor-mediated endocytosis (green), lysosomal transport (pink), and membrane organization (light blue) form a tight network. Interestingly, genes in the ER calcium ion homeostasis cluster (brown) form a distinct cluster that is interlinked through the glutamate receptor-signaling pathway (light blue) with postsynapse organization and receptor-mediated endocytosis (Fig. 8B; Extended Data Fig. 8-1). This analysis suggests that loss of STIM1-mediated Ca^{2+} signaling in PN further affects the expression of genes that influence ER calcium ion homeostasis, glutamate receptor signaling, neuron excitability, and synaptic transmission.

Biological pathways associated with regulation of cell cycle process, meiotic cell cycle, signal release, cell development, cellular carbohydrate metabolic process, and transmembrane transport are significantly upregulated in the *STIM1*^{PKO} PNs (Fig. 8C). Clustering of upregulated genes from “GO biological process” shows that regulation of cell cycle processes is the most enriched pathway with gene sets being shared among its various subclusters; no intercluster connections with other GO terms were observed (Fig. 8D; Extended Data Fig. 8-2).

A similar GO analysis of cellular components identified three interrelated clusters of “neuron to neuron synapse,” “axon,” and “presynapse” among the downregulated genes in *STIM1*^{PKO}, supporting an effect of STIM1-mediated Ca^{2+} signaling on multiple synaptic components, including postsynaptic densities, synaptic membranes, and glutamatergic synapses (Fig. 9A,B; Extended Data Fig. 9-1). A second set of interrelated clusters, “early endosome,” “cytoplasmic vesicle part,” and “membrane coat,” suggest an effect on vesicular transport. Interestingly, the KEGG pathway and Reactome analysis identified an interconnected network that links to Alzheimer’s disease and includes several nodes belonging to ion homeostasis (Fig. 9C,D; Extended Data Fig. 9-2). The functional relevance of single nodes in the network plots is hard to assess from this analysis. Together, these findings indicate that regulation of gene expression by STIM1 impacts various biological, cellular, and molecular processes that could in turn alter synaptic morphology and function as well as neuronal excitability.

Gene expression changes because of loss of STIM1 are age-dependent

Next, we investigated the age at which gene expression changes occur after *STIM1* KO in PNs. Accordingly, candidate genes from various GO-enriched categories (Fig. 8) were chosen, including calcium signaling, voltage-gated ion channels, synaptic signaling, neuron projection development, transcription factors, and carbohydrate metabolic process, and their expression was measured by qRT-PCR in RNA isolated from the dissected cerebella of control and *STIM1*^{PKO} mice at 14 weeks and 1 year. Interestingly, expression level of genes encoding IP₃R1, Orai3, and calcium-binding proteins, such as Pvalb, Calm1, Casq2, and S100B, were significantly downregulated at 1 year (Fig. 10A, red vs blue bars) but not at 14 weeks of age (Fig. 10A, dark pink vs light purple bars). A similar pattern of age-dependent changes in

gene expression was observed for all other gene classes tested by qRT-PCR (Fig. 10B–F). Results of the qRT-PCR experiments from *STIM1*^{PKO} animals 1 year of age helped to validate the change in gene expression of various ion channels and pumps, such as *Cacng5*, *Atp1a3*, and *Kctd17* (Fig. 10B), and synaptic signaling genes, such as *Syt11* and *Vamp1* (Fig. 10C). In addition, we confirmed the downregulation of genes involved in neuron projection development, such as *Dlg4* (discs large homolog 4), *Robo2* (Roundabout guidance receptor 2), *Gigyl2* (GRB10 Interacting GYF Protein 2), and *Map4* (Microtubule-associated protein 4) (Fig. 10D) and those involved in regulating gene expression, such as *Tfap2b* (Transcription factor AP-2 β) and *Setd6* (SET domain containing 6) (Fig. 10E) in *STIM1*^{PKO} PNs. Two upregulated genes, *Bad* and *Insr*, both related to carbohydrate metabolic process, were also validated (Fig. 10F).

Possibly the altered gene expression observed in 1-year-old *STIM1*^{PKO} PNs arises from a combinatorial effect of loss of mGluR1 signals through STIM1 (Fig. 6G,H), and other mechanisms that are dysregulated because of prolonged loss of STIM1-dependent signaling in PNs, such as reduced excitability (Fig. 6A–E). Alternatively, in the initial phase of *STIM1* KO, certain compensatory mechanisms might exist that are abrogated with age. These findings suggest that changes in neuronal activity (Fig. 6) and onset of motor dysfunction (Fig. 4), because of loss of STIM1 in PNs, precede changes in gene expression (Figs. 7–10).

Dendritic morphology of PNs undergoes subtle changes in *STIM1* KO animals

PNs integrate afferent inputs received on an elaborate dendritic tree and their function is largely influenced by development and maintenance of dendritic morphology and spine density (K. J. Lee et al., 2005; McKay and Turner, 2005). Based on our findings that genes for neuron projection development, such as *Dlg4*, *Robo2*, *Gigyl2*, and *Map4* (Figs. 8, 10), were significantly downregulated in PNs of *STIM1*^{PKO} mice, and that changes in the dendritic pattern might also occur because of altered regulation of genes for aspects of metabolic regulation (Figs. 8, 9) (Bauernfeind et al., 2014; Steiner, 2019; Maffezzini et al., 2020); we investigated the dendritic morphology of PNs. Dendritic arborization of the primary and secondary dendritic branches of PNs were traced using the Filament Tracer (Auto Depth) tool of Imaris software (Fig. 11A; see Materials and Methods). The number of intersections observed at specific distances from the soma were quantified to obtain a measure of the dendritic branch complexity (Fig. 11B). A significant reduction in the number of intersections was observed at a distance of 120–140 μm from the Purkinje soma ($p < 0.0001$ at 120 μm , $p < 0.05$ at 130 μm , and $p < 0.01$ at 140 μm , two-way ANOVA, Sidak’s multiple comparisons test; Fig. 11B) as indicated in Figure 11A (white arrowheads, at 120 μm), suggesting reduced inputs from parallel fibers (Ichikawa et al., 2002, 2016) and climbing fibers (CFs) (Sugawara et al., 2013). Interestingly, overall dendritic volume of *STIM1*^{PKO} PNs was reduced to a significant extent from $2322.23 \pm 69.48 \mu\text{m}^3$ (WT) to $1840.56 \pm 71.48 \mu\text{m}^3$ (*STIM1*^{PKO}; $p < 0.00005$; Fig. 11C). A modest effect was also observed on the dendritic area (Fig. 11D). Total dendritic length remained similar between WT and *STIM1*^{PKO} PNs (Fig. 11E). Because this method does not allow reliable tracing of finer dendritic branches, it is possible that changes, beyond those seen in the primary and secondary dendrites, have not been detected in our study. Previous studies have demonstrated the importance of STIM2-mediated neuronal SOCE in maintenance of mushroom spines in hippocampal neurons (Sun et al., 2014). However, spine

(EdgeR analysis). RNA-Seq experiments were performed with three biological replicates from 1-year-old mice. **E**, Venn diagrams representing the number of genes identified as differentially expressed by DEseq and EdgeR analysis. **F**, **G**, Heat maps depicting read counts of selected downregulated (**F**) and upregulated (**G**) genes, in order of maximal average fold change (right panels), from WT and *STIM1* KO conditions, are shown for three biological replicates (left panels).

Figure 8. Biological processes affected by the absence of STIM1 in the PNs. **A**, Biological processes enriched among genes that are downregulated in *STIM1* KO PNs. **B**, Network analysis of significant biological processes from **A**. **C**, Biological processes enriched among genes that are upregulated in *STIM1* KO PNs. **D**, Network analysis of significant biological processes of upregulated genes. In the network plots, each circle node represents GO terms where its size is proportional to the number of input genes included in the term, and its color represents its cluster identity. Nodes that share common genes are connected by edges. Edge width corresponds to the number of genes that are shared between nodes, and edge length represents the similarity coefficient between nodes. GO analysis was performed using Metascape and parameters specific for *Mus musculus* with *p* value cutoff as 0.01, count threshold at 3, and minimum enrichment as 1.5. Details of GO terms are provided in Extended Data Figures 8-1 and 8-2.

density at the distal end of the *STIM1*^{PKO} PNs appeared no different from WT (Fig. 11F,G). An estimation of different subtypes of spines in *STIM1*^{PKO} PNs would have been informative. However, the resolution of confocal images obtained was insufficient to

identify spine subtypes. Moreover, the spine density observed appeared higher than from published data with Golgi staining (Sugawara et al., 2013; Xiao et al., 2020). This discrepancy may be either because of the different methods used or the fact that we

Figure 9. GO analysis of enriched cellular components and KEGG pathway among downregulated genes. **A**, Analysis of enriched cellular components among genes that are downregulated in *STIM1* KO PNs. **B**, Network analysis of significant cellular components from **A**. **C**, Enriched KEGG pathway and Reactome analysis of downregulated genes in *STIM1* KO PNs. **D**, Network analysis of significant KEGG and Reactome pathway from **C**. GO analysis was performed using Metascape and parameters specific for *Mus musculus* with *p* value cutoff as 0.01, count threshold at 3, and minimum enrichment as 1.5. Details of GO terms are provided in Extended Data Figures 9-1 and 9-2.

have used older mice for our studies. Our data demonstrate that signaling through STIM1 helps maintain the dendritic arborization and dendritic morphology of PNs, and suggest that altered expression of genes because of loss of STIM1 could be responsible in part for the changes in dendritic morphology.

Loss of STIM1 in PNs enhances climbing fiber innervation of PN dendrites

Appropriate CF-PN synaptic wiring influences information processing and integration essential for motor learning and coordination (Ichikawa et al., 2016). Multiple CFs innervate PNs at birth and subsequently undergo pruning so that a single CF innervates a PN in mature adults. This extensive pruning of CF-PN synapses continues until postnatal week 3 (Crepel, 1982; Hashimoto et al., 2009; Watanabe and Kano, 2011) and depends on mGluR1 activation during the late phases (Kano et al., 1995, 1997, 1998; Offermanns et al., 1997; Hashimoto et al., 2001). Attenuation of mGluR1 signaling and reduced excitability were observed in PNs of *STIM1*^{PKO} animals (Fig. 6). Consequently, we examined the density and distribution of CF-PN synapses by counting VGLUT2

(vesicular glutamate transporter type 2) puncta along the dendrites of PNs from animals 14 weeks and 1 year of age (Fig. 12). A significant increase in VGLUT2 puncta was observed along the proximal dendrites of *STIM1*^{PKO} PNs in mice 14 weeks of age (Fig. 12A,B), whereas the density of VGLUT2 puncta at the distal dendrites appeared comparable between control and *STIM1*^{PKO} cerebellum (Fig. 12C,D). Interestingly, in animals 1 year of age, the density of VGLUT2 puncta along both the proximal (Fig. 12E,F) and distal dendrites of *STIM1*^{PKO} PNs (Fig. 12G,H) increased significantly, suggesting that loss of STIM1 leads to long-term changes in synapse formation extending from 14 weeks to 1 year. Transcriptional profiling data from animals 1 year of age show that genes for endocytic recycling, postsynapse organization, and glutamate receptor signaling, all of which are likely to impact synapse function, are significantly downregulated in *STIM1*^{PKO} PNs (Fig. 8A,B). Reduced expression of synaptic components in *STIM1*^{PKO} PNs may thus be a compensatory mechanism for appropriate loss of synapse elimination, as suggested by excess VGLUT2 puncta in 1-year-old animals.

Figure 10. Age-dependent gene expression changes in *STIM1*^{PKO} PNs. Bar graphs of fold changes in expression levels of the indicated genes from biologically significant pathways, such as (A) calcium signaling, (B) voltage-gated ion channels, (C) synaptic signaling, (D) neuron projection development, (E) transcription factor, and (F) carbohydrate metabolic process. Fold changes were normalized to *GAPDH*. All measurements are by qRT-PCR of cDNA prepared from RNA isolated from micro-dissected PNL+ML ($n = 6$). Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; two-tailed Student's t test. *Pcp2*, Purkinje cell protein 2; *Stim1*, stromal interaction molecule 1; *Gabra6*, GABA type A receptor subunit $\alpha 6$; *Itpr1*, inositol 1,4,5-trisphosphate receptor 1; *Pvalb*, parvalbumin; *Calm1*, calmodulin 1; *Casq2*, calsequestrin 2; *Cacng5*, calcium voltage-gated channel auxiliary subunit $\gamma 5$; *Atp1a3*, ATPase Na^+/K^+ transporting subunit $\alpha 3$; *Kctd17*, potassium channel tetramerization domain containing 17; *Syt11*, synaptotagmin 11; *Vamp1*, vesicle-associated membrane protein 1; *Dlg4*, discs large homolog 4; *Robo2*, roundabout guidance receptor 2; *Gigf2*, GRB10-interacting GYF protein 2; *Map4*, microtubule-associated protein 4; *Tfap2b*, transcription factor AP-2 β ; *Setd6*, SET domain containing 6; *Bad*, BclII-associated agonist of cell death; *Insr*, insulin receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase. Details of fold change and exact p value are provided in Table 2.

Figure 11. Alteration in dendritic morphology observed in *STIM1* KO PNs. **A**, Confocal analysis of dendritic arborization in PNs of control and *STIM1* KO mice using Filament Tracer (Auto Depth) in Imaris software. Confocal images of PN dendrites (left) and their projection image, traced using Imaris (right) are shown. White arrowheads indicate 120 μm. Scale bar, 20 μm. **B**, Sholl analysis with number of intersections (*y* axis) and distance from the soma (in μm; *x* axis) for PN dendrites from control and *STIM1*^{PKO} animals. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; two-way ANOVA with Sidak's multiple comparison test. **C**, Bar graph with quantification of the total dendritic volume (*STIM1*^{WT}, 2322.24 ± 69.48 μm³; *STIM1*^{PKO}, 1840.56 ± 71.49 μm³; *p* = 5.82 × 10^{−6}) (**D**) dendritic area (*STIM1*^{WT}, 2979.99 ± 90.96 μm²; *STIM1*^{PKO}, 2658.20 ± 100.96 μm²; *p* = 0.0202) and (**E**) dendritic length (*STIM1*^{WT}, 361.74 ± 12.36 μm; *STIM1*^{PKO}, 351.35 ± 14.18 μm; *p* = 0.5821) of control and *STIM1*^{PKO} dendrites [*STIM1*^{WT}, *n* = 54; *STIM1*^{PKO}, *n* = 38 PNs]. **F**, Spine morphology on distal dendrites of PNs from *STIM1*^{WT} and *STIM1*^{PKO}. Confocal images of distal PN dendrites expressing tdTomato (from left; scale bar, 4 μm) followed by enlarged images of the marked regions (scale bar, 2 μm). The enlarged confocal image is followed by an overlay with the corresponding projection image traced using Imaris and the projection image from Imaris analysis (extreme right). White arrowheads indicate some of the spines present on a distal dendrite. **G**, Bar graph represents the quantitative analysis of spine density (spines/10 μm) along the distal dendrites of PN for the indicated genotype [*STIM1*^{WT}, 31.28 ± 0.38 (*n* = 16 PNs); *STIM1*^{PKO}, 31.08 ± 0.43 (*n* = 14 PNs); *p* = 0.7324]. Three mice (age 1 year) were analyzed per group. Data are mean ± SEM. **p* < 0.05; ***p* < 0.01; two-tailed Student's *t* test; n.s., not significant.

Discussion

In this study, we have identified molecular and cellular changes that occur in murine PNs with cell-specific loss of STIM1. Excitability and Ca²⁺ signaling in response to mGluR1 stimulation are affected early and are concomitant with loss of STIM1 protein (after 12 weeks), whereas changes in gene expression appear to be age-dependent and are evident in older animals.

Older *STIM1*^{PKO} mice also exhibit an age-dependent increase in VGLUT2 puncta, which correspond to CF-PN synapses (Fremau et al., 2001) accompanied by subtle changes in dendritic morphology (Fig. 13). Altered dendritic morphology and excessive CF-PN inputs imply that error encoding during motor learning tasks is likely to be impaired in aged *STIM1*^{PKO} mice (Nguyen-Vu et al., 2013). This idea requires further testing. The

Figure 12. Defects in synaptogenesis in the PNs of $STIM1$ KO mutants *in vivo*. Immunohistochemical analysis of CF innervation of PN dendrites in cerebella from $STIM1^{WT}$ and $STIM1^{PKO}$ 14-week- and 1-year-old mice. **A, C, E, G.** Immunostaining of PNs with VGLUT2 puncta (green) and Td tomato expression (red; left), overlay of projection images from Imaris analysis with dendritic filaments marked in blue on the image from the left (middle) and VGLUT2 puncta (green) along with projection images of dendritic filaments from Imaris analysis marked in yellow (right) in mice of the indicated ages and genotypes. Scale bars, 10 μm . **B, D, F, H.** Bar graph represents the density of VGLUT2 puncta (count per $10^3 \mu m^2$) at the indicated dendritic regions in young (14 week) and old (1 year) mice. Proximal 14 weeks: $STIM1^{WT}$, 42.70 ± 1.16 ; $STIM1^{PKO}$, 46.13 ± 1.11 ; $p = 0.0363$; distal 14 weeks: $STIM1^{WT}$, 14.57 ± 1.55 ; $STIM1^{PKO}$, 17.13 ± 1.56 ; $p = 0.2569$; proximal 1 year: $STIM1^{WT}$, 31.02 ± 2.41 ; $STIM1^{PKO}$, 52.39 ± 2.80 ; $p = 4.06 \times 10^{-7}$; distal 1 year: $STIM1^{WT}$, 12.78 ± 2.32 ; $STIM1^{PKO}$, 30.09 ± 2.61 ; $p = 3.87 \times 10^{-5}$. Puncta density quantifications were from 3 mice of each age and genotype and 12 or more PNs from each genotype. Data are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.0001$; two-tailed Student's *t* test; n.s., not significant.

loss of mGluR1 Ca^{2+} signals in $STIM1^{PKO}$ PNs through adult life might result in the increase of CF-PN puncta with age. Based on the observation that parallel changes in gene expression occur and affect multiple synapse components, it is likely that reduced mGluR1 signaling in the PN dendrites sets up a negative feedback loop between reduced synaptic activity, ER-

Ca^{2+} homeostasis, and changes in gene expression, thus further affecting PN synaptic function and CF-PN synapses with age.

STIM1 and Ca^{2+} signaling in Purkinje neurons

Interestingly, within a few weeks of loss of STIM1 from PNs, mGluR1 responses reduce significantly as also the ability to learn

and perform a motor coordination task (Figs. 4, 6). An important physiological output of mGluR1/IP₃R-mediated Ca²⁺ signaling is LTD of PF-PN synapses. Loss of either mGluR1 or the IP₃R1 abrogates LTD (Aiba et al., 1994; Inoue et al., 1998; Ichise et al., 2000). LTD requires that the PNs receive within a 100–500 ms window both repetitive PF inputs for stimulating mGluR1/IP₃-mediated Ca²⁺ release and CF-activated action potentials (Sarkisov and Wang, 2008). Attenuated Ca²⁺ entry in *STIM1*^{PKO} mice affects refilling of ER stores in PNs, which in turn suppresses mGluR1-stimulated Ca²⁺ signals and therefore the ability to respond to repetitive PF inputs (Hartmann et al., 2014). Based on the reduced KCl responses observed in PNs of *STIM1*^{PKO} mice, it is likely that PN response to CF inputs is also attenuated. The status of LTD in *STIM1*^{PKO} thus needs further investigation.

Unlike *IP₃R1* (Sugawara et al., 2013) or *mGluR1* mutant mice (Aiba et al., 1994), loss of STIM1 in PNs does not lead to either neurodegeneration or ataxia. *STIM1*^{PKO} mice appeared normal under standard cage conditions, and motor learning deficits appear only when they are challenged with a more complex motor task, such as walking on an accelerated rotarod. With age, their ability to perform the motor coordination task deteriorates, but no more than the deterioration found in aging WT mice (Fig. 4F). We did not observe loss of PNs at either 1 year (Fig. 2C) or 1.5 years (Fig. 3C) of age. Thus, cellular and physiological phenotypes of *STIM1*^{PKO} mice appear in a milder version of phenotypes in *mGluR1* mutant mice and mice with PN specific KO of the *IP₃R1* (Sugawara et al., 2013).

Both neuronal and glial calcium homeostasis may be affected in the *STIM1*^{PKO} cerebellum

Transcriptomic analysis of PNs from *STIM1*^{PKO} mice identified STIM1-dependent mechanisms as essential for maintaining the expression of multiple genes that impact Ca²⁺ signaling and Ca²⁺ homeostasis with age (Figs. 7F,G, 8–10). PNs express a range of Ca²⁺ channels, Ca²⁺-binding proteins, Ca²⁺-dependent kinases, and phosphatases that together presumably help maintain cellular Ca²⁺ homeostasis, regulate various Ca²⁺-dependent processes, and modulate the multiple inputs received by PNs (Prestori et al., 2019). Among such downregulated genes, the *IP₃R1* is significant because previous studies have reported downregulation of *IP₃R1* expression in mouse models for SCAs (Lin et al., 2000; Serra et al., 2004; Crespo-Barreto et al., 2010; Ingram et al., 2016; Stoyas et al., 2020). *STIM1*^{PKO} PNs also show reduced expression of a range of Ca²⁺-binding proteins, including parvalbumin, which is expressed abundantly in PNs (Caillard et al., 2000; Schwaller et al., 2002). PNs from SCA1 patients (Vig et al., 1996) and transgenic mice expressing the human SCA1 causing gene (Vig et al., 1998) also show significant reduction in parvalbumin expression. Also downregulated in *STIM1*^{PKO} PNs is calmodulin 1, another the EF hand-containing calcium-binding protein that regulates activity of several Ca²⁺-regulated enzymes, such as α CaMKII and β CaMKII required for

Figure 13. Schematic representation of age-dependent STIM1 functions in cerebellar PNs. The ER-Ca²⁺ sensor STIM1 lies at the heart of intracellular Ca²⁺ signaling in PNs. Loss of STIM1 attenuates Ca²⁺ entry and affects refilling of ER stores, which suppresses mGluR1-stimulated Ca²⁺ signals and PN excitability. Over time, these changes affect gene expression and optimal synaptic connectivity of cerebellar PNs. Phrases in blue represent changes in *STIM1*^{PKO} PNs that occur over 17 weeks. Phrases in red represent longer-term changes observed at 1 year. The schematic encapsulates novel findings from this work and from previous studies (Hartmann et al., 2014; Ryu et al., 2017). Model created using Biorender (www.BioRender.com).

cerebellar LTD and motor learning (Hansel et al., 2006; Van Woerden et al., 2009). Calmodulin levels are also reduced in the cerebellar vermis of a spontaneously ataxic mouse (*Pogo*) (K. Y. Lee et al., 2011).

Downregulated genes for Ca²⁺-binding proteins include S100β, which is not expressed to a detectable extent in PNs but is expressed abundantly in the Bergmann glia (Fig. 3D), whose processes enwrap synapses of PN dendrites (Yamada and Watanabe, 2002). Our data demonstrate that changes in Ca²⁺ homeostasis because of loss of STIM1 in PNs can impact glial gene expression by mechanism(s) that need identification. In the hippocampus, loss of S100B from glial cells affects neuronal plasticity, indicating the significance of reduced S100B on neuronal function and the importance of glia-neuron interactions (Nishiyama et al., 2002).

Gene expression changes observed in PNs from *STIM1*^{PKO} mice suggest the existence of specific transcription factors whose activity is affected by altered cellular Ca²⁺ homeostasis. Our study does not identify such factors; but interestingly, we observed that expression of *Setd6* was significantly downregulated in *STIM1*^{PKO} PNs from mice 1 year of age (Figs. 7F, 10E). SETD6 is an N-lysine methyltransferase that directly methylates RelA (Chang et al., 2011; Levy et al., 2011) and PAK4 (p21-activated kinase 4) (Verzhinin et al., 2016). A recent study demonstrated that knockdown of *Setd6* interferes with memory consolidation, gene expression patterns, and spine morphology in the hippocampus of rat (Webb et al., 2020). Reduced expression of *Setd6* and another transcription factor *Tfap2b* (Transcription factor AP-2 β) in PNs from *STIM1*^{PKO} mice (Figs. 7F, 10E) might thus be responsible for some of the altered gene expression patterns observed in *STIM1*^{PKO} PNs.

STIM1 in Purkinje neurons impacts expression of multiple genes in common with cerebella from ataxic individuals

A common feature of a number of downregulated genes, corresponding to different biological processes, is their known effect on maintenance of normal gait and motor coordination. These include *Itpr1* (Lin et al., 2000; Serra et al., 2004; Crespo-Barreto et al., 2010; Ingram et al., 2016; Stoyas et al., 2020), *Dlg4* (Feyder et al., 2010), *Robo2* (Gibson et al., 2014), and *Gigf2* (Giovannone et al., 2009). *IP₃R1* mutant mice exhibit ataxia and dystonia (Matsumoto et al., 1996; Hisatsune et al., 2013), and human patients with loss or mutation of one copy of the *IP₃R1* exhibit SCA15, SCA29, or Gillespie's syndrome (Hasan and Sharma, 2020). *Dlg4* encodes PSD-95 (postsynaptic density protein 95), a membrane-associated guanylate kinase and a major scaffolding protein in the excitatory postsynaptic density that regulates synaptic strength (Kim and Sheng, 2004; Funke et al., 2005; D. Cheng et al., 2006). *Dlg4* KO mice show impaired motor coordination (Feyder et al., 2010). *Robo2* is a transmembrane receptor for the secreted molecule Slit2 (Slit homolog 2 protein) and functions during axon guidance and cell migration (Ma and Tessier-Lavigne, 2007; Giovannone et al., 2012). PN-specific *Robo2*-deficient mice exhibit gait alterations (Gibson et al., 2014). Mice heterozygous for *Gigf2* exhibit motor dysfunction (Giovannone et al., 2009). GO analysis indicates that these genes belong to independent biological processes (Fig. 8A; Extended Data Fig. 8-1). Network analysis (Fig. 8B) suggests that genes, such as *Itpr1*, *Dlg4*, *Robo2*, and *Gigf2*, impact postsynapse organization of PNs and could thus affect synaptic plasticity required for motor learning. In this context, the reduction in dendritic volume in PNs of *STIM1^{PKO}* mice (Fig. 11C) is also significant because reduced dendritic volume is observed in mice with KO of the SCA5 causing gene *β-III spectrin* (Gao et al., 2011). Reduced expression of ion channels and ion pumps (*Cacng5*, *Atp1a3*, *Kctd17*) as well as synaptic transmission-related genes, such as *Syt11* and *Vamp1* in *STIM1^{PKO}* mice (Fig. 10B,C), indicates an effect of *STIM1* KO on the density and composition of membrane channels and pumps in PNs.

Disruption of intracellular Ca^{2+} signaling in PNs has been proposed as a key mechanism in the pathogenesis of SCAs (Liu et al., 2009; Schorge et al., 2010; Kasumu and Bezprozvanny, 2012; Shimobayashi and Kapfhammer, 2017). Previous studies on SCA models identified several major biological pathways in common with *STIM1^{PKO}* analysis, such as calcium signaling, glutamate receptor signaling, and synaptic LTD (Serra et al., 2004; Gatchel et al., 2008; Crespo-Barreto et al., 2010; Ingram et al., 2016). Defective intracellular Ca^{2+} signaling is also associated with several other neurodegenerative disorders that affect motor coordination, such as Parkinson's disease (Zhou et al., 2016) and Huntington's disease (Wu et al., 2011, 2016; Nekrasov et al., 2016). Pharmaceutical agents that modulate *STIM1* function and potentially restore Ca^{2+} homeostasis could thus function as therapeutics for multiple neurodegenerative disorders associated with dysregulated intracellular Ca^{2+} signaling.

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