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PKCγ-A24E knock-in mouse model related to SCA14

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Conflicts of interest/Competing interests

The authors declare that they have no conflict interests.

Note about the use of the term mutation in this manuscript:

In this manuscript we use the term “mutation” in the cell biological sense referring to a change in the amino acid sequence of a known gene. It shall not imply that this change was found or identified in human SCA14 patients. In human genetics, the term mutation is only used for amino acid sequence changes which cause a disease, other changes are called variants. As the A24E sequence was not identified in human patients, it is not an SCA14 mutation in human genetics.

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Abstract
Spinocerebellar ataxias (SCAs) are diseases characterized by cerebellar atrophy and loss of Purkinje neurons caused by mutations in diverse genes. In SCA14, the disease is caused by point mutations or small deletions in protein kinase C gamma (PKCγ), a crucial signaling protein in Purkinje cells. It is still unclear whether increased or decreased PKCγ activity may be involved in the SCA14 pathogenesis.

In this study we present a new knock-in mouse model related to SCA14 with a point mutation in the pseudosubstrate domain, PKCγ-A24E, known to induce a constitutive PKCγ activation. In this protein conformation, the kinase domain of PKCγ is activated, but at the same time the protein is subject to dephosphorylation and protein degradation. As a result, we find a dramatic reduction of PKCγ protein expression in PKCγ-A24E mice of either sex. Despite this reduction there is clear evidence for an increased PKC activity in Purkinje cells from PKCγ-A24E mice. Purkinje cells derived from PKCγ-A24E have short thickened dendrites typical for PKC activation. These mice also develop a marked ataxia and signs of Purkinje cell dysfunction making them an interesting new mouse model related to SCA. Recently, a similar mutation in a human patient was discovered and found to be associated with overt SCA14. RNA profiling of PKCγ-A24E mice showed a dysregulation of related signaling pathways like mGluR1 or mTOR. Our results show that the induction of PKCγ activation in Purkinje cells results in the SCA-like phenotype indicating PKC activation as one pathogenetic avenue leading to a SCA.

Significance Statement
Spinocerebellar ataxias (SCAs) are hereditary diseases affecting cerebellar Purkinje cells and are a one of neurodegenerative diseases. While mutation in several genes
have been identified as causing SCAs, it is unclear how these mutations cause the disease phenotype. Mutations in PKCγ cause one subtype of SCAs, SCA14. In this study we have generated a knock-in mouse with a mutation in the pseudosubstrate domain of PKCγ, which keeps PKCγ in the constitutive active open conformation. We show that this mutation leading to a constant activation of PKCγ results in a SCA-like phenotype in these mice. Our findings establish the constant activation of PKC signaling as one pathogenetic avenue leading to an SCA phenotype and a mechanism causing a neurodegenerative disease.
Introduction

Spinocerebellar ataxia 14 (SCA14) (OMIM 605361) is a rare autosomal dominant neurodegenerative disease caused by protein kinase C gamma gene (PRKCG) mutations which incidence is 1% to 4% of all autosomal dominant cerebellar ataxias (Verbeek et al., 2005; Chelban et al., 2018).

PKCγ is a serine/threonine kinase dominantly expressed in cerebellar Purkinje cells and playing an important role for Purkinje cell functions (Chopra et al., 2018; Hirai, 2018). Increased PKC activity has a strong negative impact on Purkinje cell dendritic outgrowth and development in organotypic slice cultures (Metzger and Kapfhammer, 2000) while Purkinje cells from PKCγ-deficient mice show no gross morphological abnormalities (Chen, 2005). SCA14 is a dominantly inherited disease, therefore gain of function or a dominant negative function rather than a loss of function of PKCγ might cause SCA14.

To date, more than 40 missense mutations or deletions in PRKCG have been reported and many mutations have been found in the cysteine-rich regulatory domain (C1A and C1B domain) while some other mutations have been found in the pseudosubstrate domain, the calcium binding C2 domain or the kinase domain (Adachi et al., 2008). The question is how all these mutations in PRKCG are linked to the disease phenotype. For some mutations an increased PKCγ kinase activity was shown pointing towards a gain of function phenotype (Verbeek et al., 2005; Adachi et al., 2008). In contrast, other SCA14 mutations especially in the C1 domain are functionally defective due to decreased binding to Diacylglycerol (DAG) pointing towards a loss of function phenotype (Verbeek et al., 2008). These findings suggest that pathology in SCA14 is not simply due to one single mechanism but rather the result of complex
mechanisms involving dysregulation of PKCγ (Shimobayashi and Kapfhammer, 2018; Wong et al., 2018).

We have previously created a transgenic mouse model expressing a kinase domain mutant PKCγ with a constitutive activation. In this mouse model, we found subtle changes of the Purkinje cell dendritic tree and a mild ataxia (Ji et al., 2014; Trzesniewski et al., 2019). In these mice, there are still two normal alleles of PKCγ present making it difficult to compare the model to human disease. We have now created a new knock-in mouse model with a mutation in the pseudosubstrate domain. This autoinhibitory domain is crucial for regulating PKCγ activity by preventing access of substrates to the kinase domain (Newton, 2018). The pseudosubstrate domain is only dissociated from the kinase domain after binding of DAG (Baffi et al., 2019) allowing PKCγ substrates to access the kinase domain and become phosphorylated (Newton, 2018). This “open-active” conformation of PKCγ is then subject to dephosphorylation and degradation. This activation cycle of PKCγ is well controlled in Purkinje cells and critical for the regulation of dendritic development, synaptic function in long-term depression (LTD), and synapse formation (Kano et al., 1995; Saito and Shirai, 2002). Mutations in the autoinhibitory pseudosubstrate domain reduce its affinity to the kinase domain and will increase kinase activity but also induce PKCγ dephosphorylation and degradation (Pears et al., 1990).

In this study we introduced a mutation in the pseudosubstrate sequence, which keeps PKCγ in the constitutive active open conformation and we generated a knock-in mouse carrying this mutation. Another pseudosubstrate domain mutation at the same A24 position (A24T) was recently identified in a human SCA14 patient (Chelban et al., 2018). We found that the A24E mutation indeed induced increased PKC activity but at the same time made PKCγ very prone to degradation. Purkinje cells expressing the mutated PKCγ showed compromised dendritic development and
in the corresponding knock-in mouse model we observed a marked ataxia, altered Purkinje cell morphology and abnormal climbing fiber (CF) termination. Gene expression profiling revealed alterations in related signaling pathways like mGluR1 or mTOR. Our results support the concept that the regulation of PKC activity is crucial for Purkinje cell function and one important contributor to the pathogenesis of SCA14 and other SCAs.
Materials and Methods:

Plasmid Construction

Mutated PRKCG genes were generated as described before (Shimobayashi et al., 2016), using the following mutagenic primers.

A24E forward primer: 5’ TTTGCAGAAAGGGGAGCTGAGGCAGAAGGTGGT 3’,
A24E reverse primer: 5’ ACCACCTTCTGCCTCAGCTCCCCCTTTCTGCAAA 3’,
A24T forward primer: 5’ TTTGCAGAAAGGGGACTCTGAGGCAGAAGGTGGT 3’,
A24T reverse primer: 5’ ACCACCTTCTGCCTCAGAGTCCCCTTTCTGCAAA 3’.

The PCR products sequence were confirmed by DNA sequencing (Microsynth).

PKCγ-A24E overexpression in Hela cells and HEK293T cells

Human PRKCG gene was obtained from Origene in pCMV6-XL4 (pCMV6-XL4-PRKCG). 5 μg of pCMV-GFP control, pCMV-PKCγ-Wt or pCMV-PKCγ-A24E were transfected into Hela cells (ATCC, RRID: CVCL_0030) or HEK293T cells (ATCC, RRID: CVCL_0063) using X-fect Transfection Reagent (Takara). After 24 hr or 48 hr, cells were fixed with 4% paraformaldehyde (PFA) and stained with following antibodies: mouse anti-GFP (1:1000, Abcam; ab290), rabbit anti-PKCγ (1:1000, Santa Cruz Biotechnology; sc-211) and DAPI. The staining was visualized with Alexa Fluor-568 goat anti-rabbit (1:500, Molecular Probes; A11011) and Alexa Fluor-488 goat anti-mouse (1:500, Molecular Probes; A11001). Images were acquired with Confocal microscopy (Zeiss LSM700) equipped with solid state lasers using a Plan-Apochromat 100x / 1.3 Oil DIC M27 objective (Zeiss). To monitor protein half-life by cycloheximide-chase, HEK293T cells were treated with 35 μg/ml cycloheximide (Sigma-Aldrich) in DMSO at 48 hr after transfection, cells were collected at multiple time points (0 min, 30 min, 90 min, 240 min and 24 hr) after treatment. To inhibit protein
degradation via ubiquitin proteasome pathway 5 μM MG132 (Sigma-Aldrich) was added to cells at 48 hr after transfection and samples were collected at 24 hr after MG132 treatment. Each sample was homogenized on ice using an ultrasound probe in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.25% Deoxycholic acid/sodium deoxycholate, 1% NP-40, 1 mM EDTA) added protease- and phosphatase-inhibitors (Roche) and then centrifuged at 7500 g for 15 minutes. Protein concentration were determined using the BCA kit (Bio-Rad) and 50 μg of each sample was subjected to SDS-PAGE for analyzing protein expression.

**Generation of transgenic mice**

Animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were reviewed and permitted by Swiss authorities. All experiments were done for both male and female mice. The point mutations (c. 71C > A; p. Ala 24 Glu, c. 78G > A; p. Arg 26 Arg) in the *PRKCG* gene (Chromosome 7, 1.93 cM) was introduced into FVB background mice. Protospacer Adjacent Motif (PAM) sequence, 78G > A mutation which doesn’t change amino acid was introduced in order to prevent the donor DNA from being a suitable target for Cas9 cleavage. The knock-in mice using Cas9/CRISPR engineering system was generated at the Center of Transgenic Models, University of Basel, with the rapid oocyte injection method. Alt-R® CRISPR-Cas9 crRNA was designed specific to exon 1 of *PRKCG* (T TGC AGA AAG GGG GCG CTG) upstream of PAM sequence, Alt-R® CRISPR-Cas9 tracrRNA and donor DNA (CAC CAG ATG AAG TCG GTA CAG TGA CTG CAG AAG GTT GGC TGC TTG AAG AAA CGA GCG GTG AAC TTG TGG CTC TTC ACC TCG TGG ACC ACC TTC TGT CTC AGC TCC CCC TTT CTG CAA AAC AGG GGT CGG GGT CCC CCC TCT GAG TCG CCT CCG CCA GGG CCC AGA CCC GCC ATG) were obtained from Integrated DNA Technologies (IDT). Crispr
RNA, Cas9 and donor DNA were injected into FVB zygotes at the pronuclear site and surviving embryos were transferred into pseudo pregnant mothers. To identify founders, genotyping with genomic DNA samples from biopsies was performed by PCR. The primers for genotyping were: forward primer: 5’ TCC TTC CTA TCT CAG AGT CTG CG 3’ and reverse primer: 5’ GTT CCC AAG TCC CCT CCT TTT CC 3’ (Micro-synth). Then the mutations at 71C > A and 78G > A in the PRKCG was confirmed by DNA sequencing. The fragment for sequencing was obtained by PCR with genomic DNA samples and primers as mentioned above. The confirmed point mutation knock-in founders were crossed with FVB (Jackson Laboratory) mice to obtain wildtype (Wt), heterozygous (Het) and homozygous (Homo) PKCy-A24E mice.

**Real time quantitative PCR**

RNA was purified from the cerebellum of control and PKCy-A24E mice at different ages using the RNeasy Mini-kit (Qiagen) following the instructions of the manufacturer. 1 μg of total RNA was used for reverse transcription reaction with SuperScript IV Reverse Transcriptase (Takara). Real time quantitative PCR was performed on a StepOne real-time PCR system (Applied Biosystems) using the Syber green master mix (Applied Biosystems). The following primers and reaction conditions were used:

Mouse **PRKCG** forward primer: 5’ CAAAAACAGAAGACAAGACC 3’,

Mouse **PRKCG** reverse primer: 5’ GGCCTTGAGTAGCTCTGAGACA 3’,

**GAPDH** forward primer: 5’ AACTTTGGCATTGTGGAAGG 3’,

**GAPDH** reverse primer: 5’ ACACATTGGGGGTAGGAACA 3’.

Reaction conditions: 1 cycle of (10 min at 95 °C), 40 cycles of (15 s at 95 °C and 60 s at 65 °C), and 1 cycle of (15 s at 95 °C, 30 s at 72 °C and 15 s at 95 °C).
Reactions were quantified by the relative standard curve system and the cycle threshold method using the SDS2.2 software (Applied Biosystems). A relative quantitation value (RQ) for each sample from the triplicates of that sample was calculated for each gene. The data were analyzed with GraphPad Prism software.

Western blot analysis

Wt and PKCγ-A24E mice at different ages were sacrificed by an overdose of pentobarbital, then the cerebellum was quickly dissected and frozen in liquid nitrogen. Samples were homogenized as mentioned above. Organotypic slice culture samples were harvested and transferred to RIPA buffer added protease and phosphatase inhibitors (Roche), 25nM okadaic acid (TOCRIS; 1136) was added for protein phosphatase 1 and 2A inhibition. Protein concentrations were determined using the BCA kit (Bio-Rad) and 50 μg of each sample were subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane using a semidry blotting machine (Bio-Rad). After blotting, membranes were blocked with 5% BSA (Sigma-Aldrich) in TBS for 1 hr and incubated with appropriate primary antibodies, rabbit anti-PKCγ (1:1000, Santa Cruz Biotechnology; sc-211), rabbit anti-PKCα (1:1000, Invitrogen; PA5-17551), rabbit anti-Phospho-(Ser) PKC Substrate (1:1000, cell signaling; #2261), rabbit anti-Ubiquitin (1:1000, cell signaling; #43124), rabbit anti-NMDA Receptor 1 (GluN1; 1:500, cell signaling; #5704), rabbit anti-MARCKS (1:1000, Invitrogen; PA 5-105296), rabbit anti-Phospho-NMDA Receptor 1 (GluN1-Ser890; 1:1000, cell signaling; #3381), rabbit anti-Phospho-(Ser152, Ser156)-MARCKS (1:1000, Invitrogen; PA1-4629), rabbit anti-Homer3 (1:1000, Invitrogen; PA5-59383), rabbit anti-Rmdn3 (1:1000, ABclonal; A5820) or mouse anti-Actinβ (1:2000, Sigma-Aldrich; A5441). After washing with TBS plus 0.5% Triton X-100 (TBS-T), membranes were incubated with secondary antibodies. Secondary antibodies were: IRDye® 800CW
Donkey anti-mouse (1:10000, Li-Cor; 926-32212) and IRDye® 680LT Donkey anti-rabbit (1:10000, Li-Cor; 926-68023). After washing, signal was detected using a LI-COR Odyssey instrument and software (LI-COR Biosciences).

Organotypic Slice Cultures

Slice cultures were prepared as described previously (Gugger et al., 2012). Briefly, mice were decapitated at postnatal day 8 (P8), their brains were aseptically removed and the cerebellum was dissected in ice-cold preparation medium (minimal essential medium (MEM), 1% glutamax (Gibco, pH 7.3)). Sagittal sections (350 μm thickness) were cut on a McIlwain tissue chopper under aseptic conditions. Slices were separated, transferred onto permeable membranes (Millicell-CM, Millipore) and incubated on a layer of neurobasal medium (Neurobasal A medium (Life Technologies) supplemented with B27 supplement (Life Technologies) and glutamax (Gibco, pH 7.3)) in a humidified atmosphere with 5% CO₂ at 37°C. The medium was changed every 2-3 days. 300 nM Phorbol 12-myristate 13-acetate (PMA) (Tocris) was added for PKC activation 24 hr before slices were fixed. 10 μM Gö6983 (Tocris) was added to the medium at each medium change for PKC inhibition, starting at 3 days in vitro (DIV3). Slices were kept in culture for a total of 7 days and analyzed with western blot and immunohistochemical staining. To monitor protein degradation, slices were treated with 50 μg/ml cycloheximide (Sigma-Aldrich) or 10 μM MG132 (Sigma-Aldrich) or both at DIV7 and protein was extracted at DIV8.

Histology and Immunohistochemistry

Immunohistochemistry was performed as described previously (Shimobayashi et al., 2016). For the analysis of the cerebellar sections, Wt and PKCγ-A24E mice were sacrificed by perfusion with 4% PFA then the cerebellum was removed, fixed with 4%
PFA for 1 day at 4°C and cryoprotected in 30% sucrose for 1 day at 4°C. Cerebella were frozen in isopentane on dry ice and embedded in optimal cutting temperature compound (O.C.T.) and parasagittal cryosections of 20 μm thickness were cut on a Leica CM1900 cryostat and used for immunohistochemistry. Organotypic slice cultures were fixed at DIV7 in 4% PFA overnight at 4°C. All reagents were diluted in 100 mM phosphate buffer (PB), pH 7.3. Cryosections or organotypic slices were incubated in blocking solution (0.5% Triton X-100, 3% normal goat serum (GIBCO)) for 1 hr in order to permeabilize the tissue and block non-specific antigen binding. Primary antibodies were added in blocking solution and incubated overnight at 4°C with the following antibodies: mouse anti-calbindin D28K (1:1000, Swant; No. 300), guinea pig anti-vGlut2 (1:1000, Millipore; AB2251-I), rabbit anti-PKCγ (1:1000, Santa Cruz Biotechnology; sc-211). The staining was visualized with Alexa Fluor-568 goat anti-rabbit (1:500, Molecular Probes; A11011) and Alexa Fluor-488 goat anti-mouse (1:500, Molecular Probes; A11001). Stained slices or sections were mounted on glass slides using Mowiol. Cryosections or organotypic slices were viewed on an Olympus AX-70 microscope equipped with a Spot digital camera. Recorded images were adjusted for brightness and contrast with Photoshop image processing software.

**Dissociated cerebellar cultures**

Dissociated cerebellar cultures were prepared from mice essentially as described (Shimobayashi and Kapfhammer, 2018). After starting the culture, half of medium were changed twice a week. For PKC activation or inhibition assay, 15 nM PMA (Tocris) for PKC activation or 5 μM Gö6983 (Tocris) for PKC inhibition was added to the medium at each change starting at DIV7 or DIV4. For plasmid transfection, L7 based expression vectors were constructed using the primers mentioned above.
Transfections were performed as described before (Shimobayashi et al., 2016) using the 4D-Nucleofector™ System (Lonza) according to the manufacturer's instructions.

Immunohistochemistry of dissociated cerebellar cells

After 14-18 days, cells were fixed in 4% PFA for 1 hr at 4°C. All reagents were diluted in 100 mM PB, pH 7.3. Cells were incubated in blocking solution (0.5% Triton X-100, 3% normal goat serum (GIBCO)) for 30 min at room temperature. Two different primary antibodies were simultaneously added to the cells in fresh blocking solution and incubated for 30 min at room temperature. After washing in PB, secondary antibodies were added to the slices in PB containing 0.1% Triton X-100 for 30 min at room temperature. For the analysis of vector expression in Purkinje cells, mouse anti-Calbindin D-28K (1:1000, Swant; No. 300) and polyclonal rabbit anti-GFP (1:1000, Abcam; ab6556) were used as primary antibodies and Alexa Fluor-568 goat anti-rabbit (1:1000, Molecular Probes; A11011) and Alexa Fluor-488 goat anti-mouse (1:1000, Molecular Probes; A11001) were used as secondary antibodies to visualize Purkinje cells (Ji et al., 2014). Stained cells were viewed on an Olympus AX-70 microscope equipped with a Spot digital camera or were acquired with Confocal microscopy (Zeiss LSM710) equipped with solid state lasers using a LD Plan-Neofluar 40x objective (Zeiss). Recorded images were adjusted for brightness and contrast with Photoshop image processing software.

Golgi-cox Staining

Mice from P14 to one year old were used for the Golgi-cox study. The FD Rapid GolgiStain™ Kit (FD Neuro Technologies) was used for Golgi staining. Mice were euthanized and perfused with 4% PFA. The cerebellum was collected in 4 ml of Impregnation solution A and B according to the instructions. After 3 weeks of impregnation, the cerebellum was fixed in 4% PFA for 3 days at 4°C. The cerebellum was embedded in paraffin, sectioned, and stained with Bismarck Brown according to the manufacturer's instructions.
tion, the cerebellum was placed in solution C from the FD Rapid GolgiStain™ kit and stored at room temperature for 3 days. Then, cerebella were frozen in isopentane on dry ice and kept in −80°C until use. Sections were cut at 100 μm using a Leica CM1900 cryostat, collected and mounted on gelatin coated-slides using solution C and dried at room temperature overnight in the dark place. The next day, the staining was developed using solutions D, E and distilled water at a 1:1:2 concentrations. The sections were then dehydrated in increasing alcohol concentrations and coverslipped using Eukitt mounting medium (Sigma-Aldrich). Slides were viewed on bright field microscope and images were taken using a Spot digital camera.

**Experimental design and statistical analysis**

The age of the mice for each experiment is shown in Table 1. Statistical comparisons were made using the GraphPad Prism 8.3.1 software package (GraphPad Software). The quantification of Purkinje cell dendritic tree size was done as previously described (Gugger et al., 2012). Purkinje cells which had a dendritic tree isolated from its surroundings were selected for analysis. Cells were photographed with a digital camera. An image analysis program (ImageJ, https://imagej.nih.gov/ij/) was used to trace the outline of the Purkinje cell dendritic trees yielding the area covered by the dendritic tree. More than 20 cells were acquired from at least three independent experiments were analyzed. The statistical significance was assessed by non-parametric Mann-Whitney’s test. Confidence intervals were 95%, statistical significance when p < 0.05. Graphical data are represented as the mean ± SEM.

To quantify the protein expression in western blots, the immunoreactivity of each sample was normalized to the actinβ signal and the ratio was evaluated and then normalized to wildtype control using LI-COR software (LI-COR Biosciences). Protein
or phospho-protein expression differences between each sample were analyzed using the Two-tailed Mann Whitney test. Statistical significance of the Footprint pattern test, the Rotarod test and the Walking beam test were analyzed using ANOVA models and Wt and PKCy-A24E mice groups were analyzed by two-way ANOVA test with Bonferroni correction.

**Behavioral testing**

Rotarod: To measure motor function, mice were placed on an accelerating rotarod (Rotamex-5, Columbus Instruments) and the speed of rotation was increased from 2 to 52 revolutions per minute over 4 min. The latency to fall from the rotarod was recorded. Data were collected for 5 trials per day after a training period of 5 trials per day for 4-5 days. The two-way ANOVA test with Bonferroni correction was conducted to examine the main effect of genotype on each day.

Walking beam test: In this test, the mice had to traverse an 80 cm long and 8 mm wide wooden bar. Every slip of a hind paw was recorded and counted. After a one day training period, data were collected for 10 trials on 4 or 5 different days. The statistical significance was assessed by two-way ANOVA test with Bonferroni correction.

Footprint pattern test: The footprint patterns were evaluated at 9-month-old Wt and PKCy-A24E mice groups. Mouse paws were painted with non-toxic ink and mice were placed at one end of a dark tunnel. Mice walked through the tunnel, and their footprints were analyzed for the width length of each step. The statistical significance was assessed by two-way ANOVA test with Bonferroni correction.

All data were analyzed using GraphPad Prism software. Statistical significance was assumed when *p < 0.05, **p < 0.01, ***p < 0.001 or ****p < 0.0001.

**Total RNA extraction and total RNA-sequence**
The cerebellum was isolated and quickly slices of 500 μm thickness were made. Purkinje layer and molecular layer were microdissected and harvested in RNAlater. Cerebellum lysates from three mice in each group were collected for total RNA isolation, following the instructions of the manufacturers of the Trizol and RNeasy Lipid Tissue Mini Kit (Qiagen). Samples were eluted in in 15 μl of RNase-free H₂O, quantified using a Nanodrop ND-1000 (Thermo Fisher Scientific) spectrophotometer. RNA qualities were checked using Agarose Gel Electrophoresis and Bioanalyzer 2100 (Agilent Technologies Inc). All samples were adjusted as 100 ng/μl and only used when RNA integrity number (RIN) was ≥ 7.1. Library preparation and sequencing were performed at the Quantitative Genomics Facility of the University of Basel and the ETH Zurich at Basel.

**Phospho proteomics**

The cerebella were isolated and quickly frozen with liquid nitrogen. Protein was extracted with lysis buffer including PhosSTOP (Roche), then protein concentration was measured with the BCA Protein Assay kit (Invitrogen). 1 mg of mouse tissue was lysed in 80 μl of 8 M Urea, 0.1M ammonium bicarbonate, phosphatase inhibitors (Sigma P5726 & P0044) by sonication (Bioruptor, 10 cycles, 30 seconds on/off, Diagenode, Belgium) and proteins were digested as described previously (PMID:27345528). Peptide samples were enriched for phosphorylated peptides using Fe(III)-IMAC cartridges on an AssayMAP Bravo platform as recently described (PMID: 28107008). Phospho-enriched peptides were resuspended in 0.1% aqueous formic acid and subjected to LC–MS/MS analysis using an Orbitrap Fusion Lumos Mass Spectrometer fitted with an EASY-nLC 1200 (Thermo Fisher Scientific). LC-MS was performed by Dr. Alexander Schmidt in the Biocenter of the University of Basel, Switzerland.
Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) is a web-based biological analysis tool for omics data, and genomic data. We applied RNA sequence data and phosphoproteomics data into QIAGEN’s IPA software (IPA®, QIAGEN, Redwood City, CA, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/) and performed a core analysis that indicates not only direct but also indirect relationships between genes and proteins. The IPA software shows the overlapping canonical pathways, upstream regulators, and affected genes/proteins networks.
Results

*A24E mutant PKCγ is unstable, aggregate and partially degradation by proteasome*

There are now more than 40 different missense mutations or deletions which have been found in human SCA14 patients (Chelban et al., 2018; Wong et al., 2018) (Fig. 1A). We have previously reported that within these mutations PKCγ-S361G has increased kinase activity resulting in abnormal Purkinje cell development (Ji et al., 2014; Shimobayashi and Kapfhammer, 2017). PKCγ-S361G mutation is located in the kinase domain and thus not subject to regulation of PKC activity within the cell. We now aimed to generate a constitutive active form of PKCγ by a mutation in the regulatory parts of the molecule. From PKCα it is known that the substitution of the conserved alanine residue in the pseudosubstrate site of PKCα with a charged glutamic acid residue (E25) causes a reduction in the affinity of this sequence on the catalytic domain leading to activated conformation of the PKCα protein (Pears et al., 1990). The pseudosubstrate domain is well conserved and exactly the same amino acid sequence from amino acid 22 - 29 from PKCα is found in PKCγ with the alanine residue being in position 24. We first introduced the single point mutation on Alanine 24 changing it to Glutamic acid, called PKCγ-A24E. Then GFP-PKCγ-A24E was transfected to Hela cells or HEK293T cells to observe the protein expression. As shown in Fig. 1B, GFP-PKCγ-A24E had a strong tendency for aggregation compared to GFP-PKCγ-Wt and the amount of protein expression was markedly reduced (GFP-PKCγ-Wt = 100% vs GFP-PKCγ-A24E = 29.7%; Fig. 1C, at 0 min (Starting point)). In order to see the protein half-life and degradation, we applied the translation inhibitor cycloheximide. 24 hours after cycloheximide treatment, GFP-PKCγ-Wt protein was still present in normal amounts (96.68% of the original GFP-PKCγ-Wt expression)
while GFP-PKCγ-A24E was reduced down to 41.27% of the original GFP-PKCγ-A24E expression (Fig. 1C) indicating that the reduced amounts of GFP-PKCγ-A24E protein are due to increased degradation. As it is known that PKC protein family is mainly degraded via ubiquitin proteasome pathway (Wang et al., 2016), we used the proteasome inhibitor MG132 in order to block the ubiquitin proteasome degradation. 24 hours after MG132 treatment, reduced GFP-PKCγ-A24E protein level was rescued (PKCγ-Wt=100.0%; PKCγ-Wt + MG132 = 192.9%; PKCγ-A24E = 32.26%; PKCγ-A24E + MG132 = 114.6%; n = 6) indicating GFP-PKCγ-A24E is unstable in cells and at least partially targeted for degradation via the proteasome pathway (Fig. 1D).

**A24E overexpression in Purkinje cells induces a strong reduction of dendritic development**

We previously reported that PKC activation negatively regulates Purkinje cell dendritic development (Ji et al., 2014). PKC activator treated Purkinje cells show compromised dendrites while PKC inhibitor treated Purkinje cells show dendrites with increased branching (Shimobayashi and Kapfhammer, 2017) (Fig. 2A, B). Therefore, Purkinje cell dendritic development is an indicator of biological PKC kinase activity. We then tested whether mutant PKCγ-A24E would affect Purkinje cell dendritic development in dissociated cerebellar culture. Purkinje cells were transfected PKCγ with the A24E mutation or the A24T mutation which was found in human SCA14 patients (Chelban et al., 2018). The L7 based plasmids yielded a Purkinje cell specific expression of the mutated proteins. In order to confirm the transfection and expression of Purkinje cells easily, Wt and mutant PKCγ-GFP fusion constructs were generated and Purkinje cells expressed fusion proteins with PKCγ-Wt, PKCγ-A24E or PKCγ-A24T fused to GFP. Purkinje cells transfected with GFP-PKCγ-A24E or GFP-
PKC\(\gamma\)-A24T only developed small dendritic trees with few side branches (Fig. 2C). This morphology is identical to that of PKC activator treated Purkinje cells (Fig. 2A). Statistical analysis showed a significant reduction of the gross area of A24E or A24T mutant PKC\(\gamma\) transfected Purkinje cells compared to that of GFP transfected Purkinje cells or PKC\(\gamma\)-Wt transfected Purkinje cells (GFP-PKC\(\gamma\)-Wt = 100.0% ± 6.92, n = 20; GFP-PKC\(\gamma\)-A24E = 62.16% ± 4.68, n = 20, p = 0.0002; GFP-PKC\(\gamma\)-A24T = 60.50% ± 5.35, n = 20, p < 0.0001) (Fig. 2D). These results indicate that two pseudosubstrate domain mutations negatively regulate dendritic growth.

**PKC\(\gamma\)-A24E mice show less PKC\(\gamma\) protein compared to control**

We generated a PKC\(\gamma\)-A24E knock-in mouse with CRISPR/Cas9-mediated genome editing methods (Menke, 2013) to observe the mutated PKC\(\gamma\) function in Purkinje cells. We introduced the point mutations (c. 71C > A; p. Ala 24 Glu, c. 78G > A; p. Arg 26 Arg) in the *PRKCG* gene (Fig. 3A-C). A R26R mutation in the PAM sequence which doesn’t change the amino acids was also introduced in order to prevent the donor DNA from being a suitable target for Cas9 cleavage. After identification of mutant founders with PCR and sequencing, we characterized the new knock-in mouse called PKC\(\gamma\)-A24E mouse.

The heterozygous (Het) and homozygous (Homo) PKC\(\gamma\)-A24E mice had normal survival and growth as compared to Wt littermates (data not shown). The PKC\(\gamma\) protein expression level in cerebellum in Homo PKC\(\gamma\)-A24E mouse was drastically reduced (Fig. 3E) at all developmental stages (4-week-old: Wt = 100.0% ± 8.34, n = 4; Het = 49.12% ± 6.80, n = 4; Homo = 7.519% ± 2.48, p = 0.0286, n = 4; 6-week-old: Wt = 100.0% ± 14.56, n = 4; Het = 63.09% ± 6.39, n = 4; Homo = 4.674% ± 0.40, p = 0.0286, n = 4). PKC\(\gamma\) protein in the PKC\(\gamma\)-A24E mice was strongly reduced but still present (Fig. 3F) as compared to that of the PKC\(\gamma\) knockout mouse. The expression...
of PKCα, another classical PKC isoform expressed in Purkinje cells, was unchanged in all genotypes (Fig. 3E). mRNA expression in Het and Homo was comparable to that of Wt littermates (Fig. 3D) indicating that translation of A24E mutant PKCγ is normal. Cycloheximide trace assay showed increased protein degradation in Homo PKCγ-A24E mice while proteasome inhibitor MG132 treatment for 24 hours partially rescued the reduced amount of PKCγ expression (Fig. 3G). These data agree with the findings in cell transfection assays (Fig. 1) and confirm that mutant PKCγ-A24E protein is rapidly targeted for degradation. In Purkinje cells from PKCγ-A24E mice we didn’t observe aggregation of mutant PKCγ-A24E protein as seen with overexpression in cell lines.

*Increased PKC kinase activity in PKCγ-A24E mice despite reduced protein levels*

As the mutation in the pseudosubstrate domain is supposed to keep the protein in a constantly active open conformation we studied whether the overall PKC activity in cerebellar slice cultures of PKCγ-A24E mice was reduced or increased. We used a general anti-phospho-serine PKC substrate antibody to see whether the phosphorylation of PKC substrates was increased or not and found that both Het and Homo PKCγ-A24E mice had increased PKC kinase activity compared to Wt littermate controls (Fig. 4A). We also studied the phosphorylation of a known PKCγ target protein, the N-methyl-D-aspartate receptor (NMDAR) which is composed of a heterodimer of at least one NR1 and one NR2A-D subunit. The NR1 subunit can be phosphorylated by PKCγ at Ser890 (Sánchez-Pérez and Felipo, 2005). Using a phospho-specific antibody we found that phosphorylation at this site was markedly increased both in Het and Homo PKCγ-A24E mice (Fig. 4B). Myristoylated alanine-rich C kinase substrate (MARCKS) is also a known phosphorylation target of PKC in many cells. Surprising-
ly, phospho-MARCKS protein expression compared to total MARCKS expression was strongly decreased in the PKCγ-A24E mouse. A similar reduction was found in Wt slice cultures treated with PMA (Wt = 100.4% ± 4.37, n = 4; Het = 61.92% ± 15.25, n = 4; Homo = 33.98% ± 5.83, p = 0.0009, n = 4; Wt + PMA = 42.54% ± 15.4, p = 0.0033, n = 4; Het + PMA = 34.58% ± 9.39, p = 0.0010, n = 4; Homo + PMA = 16.89% ± 3.557, p < 0.0001, n = 4) suggesting that phospho-MARCKS protein is more dephosphorylated by phosphatases under the PKCγ activation condition. This dephosphorylation could be mostly prevented by the use of the phosphatase inhibitor okadaic acid (Fig. 4C).

Altered morphology of Purkinje cells from PKCγ-A24E mice in vitro

We studied the dendritic morphology of Purkinje cells from PKCγ-A24E mice. In organotypic slice culture the dendritic expansion of Purkinje cells from PKCγ-A24E mice was severely impaired and the cells developed only short thickened dendrites similar to those found after PMA treatment (Ji et al., 2014). This was most evident in Homo PKCγ-A24E mice, but also noticeable in Het PKCγ-A24E mice. (Fig. 5A). Statistical analysis showed that the size of Purkinje cells from Homo PKCγ-A24E mice (62.1% ± 5.23, p < 0.0001) is significantly smaller than that of Wt (100% ± 5.64). Purkinje cell size of Het is also smaller than that of Wt (Het, 84.2% ± 5.40, p = 0.0718) but the difference did not reach statistical significance (Values are shown as percentage of Wt (100%) and are the mean ± SEM of 20 Purkinje cells).

As this change in morphology is supposed to be due to increased PKC activity we checked whether a PKC inhibitor might rescue the dendritic morphology in PKCγ-A24E mice. We applied the PKC inhibitor Gö6983 to cerebellar slice cultures during the culture period (Fig. 5B). Purkinje cells from Homo PKCγ-A24E mice had longer dendrites with more branches after PKC inhibitor treatment, showing a significant
rescue (Purkinje cells area Wt = 100.0% ± 5.00, n = 20; Homo PKCγ-A24E = 47.50% ± 3.42, n = 20), indicating that the reduction in dendritic expansion of Purkinje cells from PKCγ-A24E mice is caused by increased PKC activity (Wt vs Homo PKCγ-A24E, p < 0.0001; Homo PKCγ-A24E vs Homo PKCγ-A24E + Gö6983, p < 0.0001).

**Altered climbing fiber innervation and Purkinje cell morphology in PKCγ-A24E mice**

Synapse formation on Purkinje cells from parallel fibers (PFs) and climbing fibers (CFs) is well controlled during postnatal developmental and is crucial for cerebellar function (Kano et al., 1995). It is known that activation of the type 1 metabotropic glutamate receptor (mGluR1)-PLCβ-PKCγ signaling pathway is involved in the regulation of this synapses formation: the initially overlapping CFs become reduced by CF-elimination during the first three postnatal weeks resulting in a one CF to one Purkinje cell relationship (Kano et al., 2018) which is essential for the establishment of the precise neuronal circuit in the cerebellum (De Zeeuw et al., 1998; Shuvaev et al., 2011; Ichikawa et al., 2016). In PKCγ deficient mice, multiple CFs innervation is disturbed (Kano et al., 1995) while mutant PKCγ transgenic mice showed a reduced innervation of Purkinje cells by CFs terminals (Trzesniewski et al., 2019). PKCγ is also involved in the formation of appropriate PFs/CFs territories (Ichikawa et al., 2016). Taken together, these findings show that PKCγ signaling is required for proper PFs/CFs formation and innervation. We studied the CF terminals on Purkinje cells in PKCγ-A24E mice with the CF marker vesicular glutamate transporter 2 (vGlut2) and compared them with Wt mice. Purkinje cells from Wt mice are innervated by CF on their proximal dendrites covering 70% to 90% of the molecular layer. In contrast, CF terminals in PKCγ-A24E mice have a reduced density in particular on the distal part
of the dendrites (Fig. 6A). We quantified the difference by counting the number of terminal puncta and found significantly less CF terminals in the molecular layer on the distal part of the dendrites from 40-week-old PKCy-A24E mice (Fig. 6B). This finding indicates that proper CF innervation is disturbed by the increased PKC activity in PKCy-A24E mice. We further studied the morphology of Purkinje cells from PKCy-A24E mice in vivo using the Golgi-Cox impregnation method because only few Purkinje cells become labeled with this method which avoids problems with overlapping Purkinje cell dendrites. We found that in Homo PKCy-A24E mice Purkinje cells had less extended, condensed dendritic trees covering a reduced area (Fig. 6C) both in 4-week-old (Purkinje cells area Wt = 100.0% ± 5.36; Het = 91.85% ± 6.81; Homo = 69.74% ± 6.07, p = 0.0013; n = 20 for each genotype) and 1-year-old mice (Purkinje cells area Wt = 100.0% ± 8.17; Het = 99.85% ± 6.51; Homo = 74.91% ± 5.14, p = 0.0122; n = 20 for each genotype). This finding is similar to that previously observed in PKCy-S361G transgenic mice (Trzesniewski et al., 2019). We also investigated the thickness of the molecular layer where Purkinje cell dendrites extend. The thickness of the molecular layer measured in lobule VII was slightly reduced in 40-week-old Homo PKCy-A24E (Fig. 6D; Wt, 155.5 ± 2.832 μm; Homo, 143.4 ± 3.137 μm, p = 0.0027; n = 40 for each genotype). These data indicate PKCy-A24E mice have a reduction of Purkinje cell dendritic tree size and structure in vivo. We did not notice an obvious Purkinje cell loss in PKCy-A24E mice aged from 4-weeks to 40-weeks.

**PKCy-A24E mice are ataxic**

Ataxia is a prominent phenotype in human SCA14 patients, so we studied the motor coordination of PKCy-A24E mice. Subtle motor coordination and balance was assessed with the balance beam test in 3-month-old to 6-month-old mice. After training, mice walked along the 80 cm long square wooden beam of 8mm width and lateral
slips were counted in two complete and consecutive crossings per day, on five con-
secutive days. The mean number of slips per 80 cm travelled was calculated. Some
PKCγ-A24E mice showed poor performance and couldn’t cross the beam because
they fell down or were unable to perform the task. For these mice, we assigned a
maximum value of slips = 20. Wt mice could walk the balance beam without or with
very few slips (1.235 ± 0.271, n = 28) while both Het (9.265 ± 1.079, n = 28, p <
0.0001) and Homo PKCγ-A24E (17.50 ± 0.056, n = 28, p < 0.0001) showed marked
ataxia both at 3 and 6 months of age (Fig. 7A and Movie. 1-3). In the rotarod test, the la-
tency to fall off the accelerating rod was assessed at 4-5 consecutive days. A sig-
ificant deficit was found in 3-month-old PKCγ-A24E mice (Fig. 7B) on day 4 (Latency to
fall: Wt = 17.00 rpm ± 2.000, n = 11; Het = 18.00 rpm ± 1.528, n = 14; Homo = 4.333
rpm ± 1.212, p < 0.0001, n = 14) and on day 5 (Latency to fall: Wt = 24.33 rpm ±
1.202, n = 11; Het = 14.67 rpm ± 1.202, p = 0.0317, n = 14; Homo = 6.67 rpm ±
0.667, p < 0.0001, n = 14). In 6-month-old mice (Fig. 7C), a deficit was found on day
3 (Latency to fall: Wt = 13.60 rpm ± 2.227; Het = 8.200 rpm ± 1.241, p = 0.0127; Ho-
mo = 5.200 rpm ± 0.374, p < 0.0001; n = 5 for each genotype) and on day 4 (Latency
to fall: Wt = 15.80 rpm ± 2.200; Het = 12.60 rpm ± 1.749; Homo = 7.80 rpm ± 0.735,
p = 0.0002; n = 5 for each genotype).

With footprint gait analysis, we calculated the step width. A representative trace im-
age of the walking pattern of 9-month-old Wt, Het and Homo PKCγ-A24E mice is
shown in Fig. 7D showing the ataxic gait with increased step width of Het and Homo
PKCγ-A24E mice. The difference in the step width was significant for Het and Homo
PKCγ-A24E mice (Wt = 1.30 cm ± 0.058, n = 12; Het = 2.60 cm ± 0.154, n = 12; Ho-
mo = 2.95 cm ± 0.047, n = 12; Fig. 7D, E). The footprint pattern in both Het and Ho-
mo PKCγ-A24E mice showed clear signs of an ataxic gait.
Molecular characterization of PKCγ-A24E mice

The molecular mechanisms underlying pathogenesis in SCA14 are still unclear. In order to identify changes in gene expression which might contribute to altered Purkinje cell development and to pathogenesis in mutant PKCγ, we did RNA profiling among Wt, Het and Homo PKCγ-A24E mice (n = 3 per genotype). For sample preparation, we dissected the molecular layer and Purkinje cell layer of the cerebellum from 5-week-old mice. Total RNA was extracted and subjected to RNA sequence analysis. Interestingly, we found many mitochondrial genes involved in oxidative metabolism to be upregulated in Het PKCγ-A24E mice according to Ingenuity Pathway Analysis (IPA, Qiagen) (Fig. 8A, B and Extended data Fig. 8-1A) indicating that mitochondrial function is altered in Het PKCγ-A24E mice. On the other hand, molecules which are related to synaptogenesis and glutamate receptor signaling are downregulated, e.g. HOMER2↓, CAMK4↓, GRIN2A↓, GRIA2↓, and GRM5↓ (Extended data Fig. 8-1B). Many genes affecting Purkinje cell development are also downregulated including ATM↓, ATAXIN↓, HTT↓ and signaling molecule CAMK4↓ (Fig. 8C). Based on upstream pathway analysis in IPA we observed a significant change in the mTOR pathway, with CAB39↓, RICTOR↓ and STK11↑, the expression level of many targets of RICTOR were altered indicating that the mTOR pathway might one of the targets affected by mutant PKCγ (Extended data Fig. 8-1C). Notably, we identified less significant changes in the expression profile of Homo PKCγ-A24E mice (Het, 3703 genes; Homo, 206 genes; p < 0.01), probably due to compensatory mechanisms. Homo PKCγ-A24E mice exhibited more upregulated genes related to ephrin receptors (Extended data Fig. 8-2). It was suggested that activation of the Eph receptors in Purkinje cells may restrict Purkinje cell dendritic spine formation (Cesa et al., 2011, Heintz et al., 2016) and Eph receptors might also be involved in Alzheimer's disease and Amyotrophic lateral sclerosis (ALS) (Yang et al., 2018). The expression of some...
ionotropic glutamate receptor subunits was also altered (GRIN2A↓, GRID1↑, GRIK4↑, GRIA1↑, GRIK3↑) in Homo PKCγ-A24E mice (Extended data Fig. 8-2) while in Het PKCγ-A24E mice glutamate receptor signaling was downregulated (GRIN2A↓, GRIA2↓, GRM5↓). In both Het and Homo PKCγ-A24E mice GRIN2A was significantly downregulated. We have also profiled protein phosphorylation by using phospho-proteomics which might provide valuable insight into the mutant PKCγ signaling. The abundance of 105 phosphopeptides was significantly upregulated while 69 phosphopeptides were significantly downregulated in Homo PKCγ-A24E mice (p < 0.05) (Table 2 and Extended data Fig. 8-3). The volcano plot data show proteins with increased phosphorylation in Homo PKCγ-A24E mice to the right side and with less phosphorylation to the left side (Fig. 8D). The data show that Rmdn3 (also known as PTPIP51) and Homer3 are highly phosphorylated in Homo PKCγ-A24E mice (Fig. 8D and Extended data Fig. 8-3). Rmdn3 was shown to be expressed in the Purkinje cell soma and dendrites (Koch et al., 2009) and is implied in calcium handling and interactions between mitochondria and the endoplasmic reticulum (Fecher et al., 2019). In the RNA sequence data both Rmdn3 and Homer3 genes are also upregulated in PKCγ-A24E mice (Fig. 8E). Western blotting confirmed the abundant expression of Rmdn3 and Homer3 in the cerebellum, with a slight upregulation in PKCγ-A24E (Fig. 8F). IPA network analysis shows that Dlgap1-Homer3-Shank1/3-Syne1-Pclo signaling is more phosphorylated in PKCγ-A24E mice suggesting that postsynaptic scaffolding proteins might also be targets of PKCγ phosphorylation.
Discussion

In this study we present a new knock-in mouse model related to SCA14 with a constitutive activation by a mutated pseudosubstrate domain which no longer can bind to the kinase domain and keeps the PKCγ protein in the open active conformation. This leads to a dramatic increase of dephosphorylation and protein degradation and to a drastic reduction of protein expression in cerebellar Purkinje cells. Despite this reduction in protein levels there is clear evidence for an increased PKC activity in Purkinje cells from PKCγ-A24E mice with the typical morphology of short thickened dendrites, a marked ataxia and signs of Purkinje cell dysfunction. A similar mutation in a human patient was associated with overt SCA14. Our results show that the introduction of a new mutation leading to a constant activation of PKCγ results in a SCA-like phenotype in these mice establishing PKC activation as one pathogenetic avenue leading to an SCA phenotype.

Increased PKC activity despite reduced protein levels of PKCγ-A24E due to increased degradation

We introduced mutations in the pseudosubstrate domain for abolishing self-inhibitory effect result in a constantly activated PKCγ enzyme (Pears et al., 1990; Newton, 2018). This concept was confirmed by transfecting pseudosubstrate domain mutated PKCγ in Purkinje cells, which showed a severe reduction of their dendritic tree consistent with a constitutive activation of PKCγ. Together with the increased PKC activation we found a reduced amount of mutated PKCγ-A24E protein. Treatment with cycloheximide and qPCR studies confirmed that this reduction was due to increased degradation as expected for the protein in the “open active” conformation (Newton, 2018). In this conformation the kinase is subject to dephosphorylation by protein phosphatases and to ubiquitination and degradation via the proteasomal pathway.
(Hansra et al., 1999). Our in vitro studies thus agree with the known regulation of PKC activation and degradation and show that the increased degradation of the open active protein in transfected cells did result in reduced protein levels. These findings are similar to those reported for the original A25E construct in PKCα (Pears et al., 1990).

**Dramatic reduction of PKCγ-A24E protein in the PKCγ-A24E mouse model is compatible with increased PKC activity**

While the effect of pseudosubstrate mutations have been studied extensively with diverse PKC variants in cell culture assays, the PKCγ-A24E mouse is to our knowledge the first mouse model of such a pseudosubstrate mutation. We were surprised to find an almost complete absence of PKCγ-A24E protein in the cerebellum but qPCR confirmed that mRNA levels were similar in all genotypes suggesting that the reduction was due to increased degradation (Fig. 3). Despite this dramatic reduction of PKCγ-A24E protein there was clear evidence for increased PKC activity. An antibody against an epitope recognizing PKC-mediated phosphorylation showed increased phosphorylation of target proteins both in Het and Homo PKCγ-A24E mice and phosphorylation of the known target protein P890-NMDAR was also increased. The mice demonstrate that only a very small amount of active PKCγ is required for inducing increased PKC activity in a cell.

**Increased constitutive PKC activity interferes with Purkinje cell development and function**

The increased constitutive activity of PKCγ-A24E has negative effects on Purkinje cells both in cell culture and in vivo. This is particularly evident in organotypic slice cultures and dissociated cerebellar cultures. In both cases Purkinje cells only develop
a small abnormal dendritic tree with thickened dendrites. In slice cultures we could show that this effect is due to increased PKC activity because it can be rescued by the application of PKC inhibitor like Gö6983. When analyzed by Golgi staining, a mild reduction of the Purkinje cell dendritic tree size becomes evident. A similar difference has already been found in the PKCy-S361G transgenic mouse (Ji et al., 2014; Trzesniewski et al., 2019). Importantly, also CF innervation is reduced particularly in the distal parts of the Purkinje cell dendrites. This finding goes together with the known importance of Purkinje cell PKCy activity for CF innervation (Chen et al., 1995) and corresponds to similar findings in the PKCy-S361G transgenic mouse (Trzesniewski et al., 2019). The PKCy-A24E mice have a marked ataxia which is most evident with testing on the walking beam which some Homo PKCy-A24E can barely cross and also Het PKCy-A24E mice have a clearly increased number of slips at both ages tested. The presence of the ataxia reflects a dysfunction of the neuronal cerebellar circuits controlling precision of movements and integration of vestibular information.

The PKCy-A24E mouse is a novel mouse model related to SCA14

The PKCy-A24E mouse shows an ataxic phenotype but no extensive loss of Purkinje cells (data not shown). A similar situation applies to the PKCy-S361G mouse (Ji et al., 2014). The presence of the ataxia in the absence of major Purkinje cell loss points to an important aspect of SCAs. While it is generally assumed that Purkinje cell loss is the major cause of the patients’ problems, the evidence for this assumption is weak. In fact, in mice a loss of 90% of Purkinje cells is required for the manifestation of overt motor behavioral deficits (Martin et al., 2003). In a recent study of SCA14 families, most patients had only mild to moderate atrophy of the cerebellum making it doubtful that their ataxia can be explained exclusively by Purkinje cell loss.
In the patient carrying the A24T mutation corresponding to the PKCγ-A24E mouse only a mild cerebellar atrophy was found. In an SCA1 mouse model, the development of ataxia and Purkinje cell loss could be dissociated (Duvick et al., 2010) suggesting that Purkinje cell dysfunction is a crucial aspect for the development of the ataxic phenotype together with Purkinje cell loss. The increased PKC activity, the impairment of dendritic development and the behavioral deficits make the Het PKCγ-A24E mice a valid mouse model related to SCA1.

Changes in gene expression and phosphorylation in PKCγ-A24E mice

The mRNA profiling surprisingly yielded more results from the Het mice compared to the Homo mice. Many molecules in the oxidative phosphorylation pathway and mitochondrial molecules were upregulated in Het PKCγ-A24E mice (Fig. 8A, B and Extended data Fig. 8-1A). Mitochondria trafficking into dendrites is essential for Purkinje cell dendritic outgrowth and proper oxidative phosphorylation for energy production in mitochondria is very important for neuron activity. Indeed, mitochondrial dysfunction has been found in several neurodegenerative diseases such as Alzheimer’s Disease (Friedland-Leuner et al., 2014), Parkinson’s Disease, Huntington’s Disease and SCA1 (Stucki et al., 2016). In addition, the Rictor signaling pathway is strongly affected (Extended data Fig. 8-1C). This pathway is well known to be of outstanding importance for Purkinje cell development and function (Angliker et al., 2015). In the Homo PKCγ-A24E mice we find dysregulated genes involved in outgrowth and pruning of neuronal processes like the Eph receptors and changes in glutamate receptors and calcium homeostasis. This fits well with the idea that in the PKCγ-A24E mice the constitutive activation of PKCγ mimics a state of very strong synaptic activation making it crucial for the Purkinje cells to handle the calcium release associated with this activation and limit the “natural” receptor activation through glutamate. With these
compensatory mechanisms the Purkinje cell would be stable and can survive but it would be functionally compromised resulting in the ataxic phenotype. This concept is also supported by the outcome of the phosphoproteomics analysis. Some of the most strongly phosphorylated proteins like Homer3 and Rmdn3 are involved in the control of receptor signaling and calcium handling, another one, Dpysl3, might control process outgrowth. Further studies will be required to further elucidate the exact role of these dysregulated proteins.

**PKCγ signaling and SCAs**

In this manuscript we show that the constitutive activation of PKCγ in the PKCγ-A24E mouse model is sufficient to induce a pathology related to SCA14, and this finding is supported by the recent identification of a human patient with a mutation at the same position (Chelban et al., 2018). However, other mutations causing SCA14 in humans affect the regulation of PKCγ differently or even are kinase dead (Shirafuji et al., 2019) and of course most SCAs are caused by mutations in different genes. Nevertheless, slowly a picture is emerging that many of these mutations affect the mGluR1-PKCγ-Inositol1,4,5-trisphosphate receptor type1 (Ip3r1) -calcium release pathway and can cause disease irrespective of stimulation or inhibition of this pathway (Shimobayashi and Kapfhammer, 2018), meaning that it is not so important in which direction the activity if this pathway is pushed, but rather that the dynamic regulation of the activity of this pathway is disturbed. Although their overall activity pattern may look rather normal at first glance, Purkinje cells in these cases would be dysfunctional and the ataxic phenotype will become the more evident the more challenging the task is and can be present without noticeable Purkinje cell loss. Of course, in other cases, the loss of Purkinje cells rather than their dysfunction may be the key to SCA pathology. In the cases in which Purkinje cell dysfunction rather than
death is at the base of the deficits a pharmacological correction of the regulation of
the affected pathway may be a valid therapeutic option.

Declarations

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Ethics approval and consent to participate

All experiments were carried out in accordance with the EU Directive 2010/63/EU for
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Figure 1 Pseudosubstrate mutant PKCγ protein is unstable and shows aggregation

(A) Illustrations of PKCγ protein domain mutations and deletions found in SCA14 families. Most mutations are found in the C1B domain. (B) 5 μg of GFP-Control plasmid, GFP-PKCγ-Wt or GFP-PKCγ-A24E were transfected to the Hela cells and after 24 hr cells were fixed with PFA following the immunostaining. GFP-PKCγ-A24E showed aggregation and accumulated in HEtLA cells. Images were acquired with Confocal microscopy (Zeiss LSM700) using a Plan-Apochromat 100x / 1.3 Oil DIC M27 objective (Zeiss). Scale bar in H = 10 μm. (C) Pseudosubstrate domain mutant PKCγ is unstable and is degraded after 35 μg/ml cycloheximide treatment. After 48 hr transfection, cycloheximide was applied to the cells. Samples were collected at 0 min, 30 min, 90 min, 240 min and 24 hr. 24 hr after cycloheximide treatment, the PKCγ protein expression level is GFP-PKCγ-Wt = 96.68% and GFP-PKCγ-A24E = 41.27% compared to each starting point, respectively. (D) Degradation of pseudosubstrate domain mutant PKCγ occurs via the proteasome pathway. 24 hr after 5 μM of proteasome inhibitor MG132 treatment, HEK293T cells show more ubiquitinated proteins and this treatment rescued GFP-PKCγ-A24E protein levels (GFP-PKCγ-Wt = 100.0%; GFP-PKCγ-Wt + MG132 = 192.90% ± 25.35; GFP-PKCγ-A24E = 32.26% ± 6.75; GFP-PKCγ-A24E + MG132 = 114.60% ± 28.54; n = 6). Protein expression was analyzed using the two-tailed Mann Whitney test (GFP-PKCγ-Wt vs GFP-PKCγ-A24E, p = 0.0022, GFP-PKCγ-Wt vs GFP-PKCγ-Wt + MG132, p = 0.0022; GFP-PKCγ-A24E vs GFP-PKCγ-A24E + MG132, p = 0.0087).
Figure 2 Immunofluorescence staining of Purkinje cells transfected with PKCγ pseudosubstrate domain mutants

(A) The morphology of Purkinje cells was analyzed after 2 weeks in dissociated cerebellar culture. Purkinje cells treated with 15 nM PMA for one week show small dendrites. Scale bar in H = 50 μm. (B) Differences between control and PMA treated Purkinje cell size were analyzed using the two-tailed Mann Whitney test (Wt + DMSO = 100.0% ± 7.15; Wt + PMA = 42.21% ± 3.92; Wt + Gö6983 = 131.5% ± 7.98; for DMSO vs PMA, p < 0.0001). Data were shown as the mean ± SEM of 20 Purkinje cells. (C) Purkinje cells transfected with pseudosubstrate domain mutations show inhibition of dendritic growth with anti-GFP staining. Scale bar in H = 20 μm. Images were acquired with a confocal microscope (Zeiss LSM710) equipped with solid state lasers using a LD Plan-Neofluar objective (Zeiss). (D) Quantification of the Purkinje cell area. The dendritic tree size of Purkinje transfected with PKCγ pseudosubstrate domain mutations GFP-PKCy-A24E or GFP-PKCy-A24T was strongly reduced compared to that of GFP-PKCy-Wt (GFP-PKCy-Wt = 100.0% ± 6.92; GFP-PKCy-A24E = 62.16% ± 4.68; GFP-PKCy-A24T = 60.53% ± 5.35). Each Purkinje cell area was measured with image J and analyzed with Graphpad prism. Differences between GFP-PKCy-Wt and GFP-PKCy-A24E or GFP-PKCy-Wt and GFP-PKCy-A24T were analyzed using the two-tailed Mann Whitney test. (GFP-PKCy-A24E, p = 0.0002 and GFP-PKCy-A24T, p < 0.0001). Data are shown as the mean ± SEM of 20 Purkinje cells.

Figure 3 Generation and characterization of the PKCy-A24E mouse

(A) Target sequence in the PRKCG gene (Chromosome 7, 1.93 cM) for producing point mutated (c. 71C > A; p. Ala 24 Glu) PRKCG with Cas9/CRISPR engineering
system. To prevent donor DNA cleavage, the PAM sequence mutation was also introduced (78G > A) which doesn’t give a change in amino acids. (B) Mutations were validated with sequencing. The figure shows Het PKCγ-A24E sequence. (C) To identify the genotypes, PCR was performed followed by Alu1 (BioLabs, R0137S) digestion for 1 hr which digests only Ala 24 Glu mutated DNA. Wt shows a single band (250 bp), Het PKCγ-A24E shows a Wt band and two digested bands (250 bp, 150 bp and 100 bp) and Homo PKCγ-A24E shows only two digested bands (150 bp and 100 bp). (D) qPCR using 5 week old cerebellar samples from each genotype. Data show that all three genotypes express PKCγ mRNA. (E) Western blot analysis of total PKCγ protein in the cerebellum from the three genotypes (n = 4). PKCγ protein degradation in PKCγ-A24E mice was observed (4-week-old: Wt = 100.0% ± 8.34, n = 4; Het = 49.12% ± 6.80, n = 4; Homo = 7.519% ± 2.48, p = 0.0286, n = 4; 6-week-old: Wt = 100.0% ± 14.56, n = 4; Het = 63.09% ± 6.39, n = 4; Homo = 4.674% ± 0.40, p = 0.0286, n = 4) while PKCα protein expression didn’t change in all genotypes (4-week-old: Wt = 100.0% ± 9.30, n = 4; Het = 83.13% ± 2.09, n = 4; Homo = 87.67% ± 14.1, n = 4; 6-week-old: Wt = 100.0% ± 8.94, n = 4; Het = 117.4% ± 19.3, n = 4; Homo = 106.8% ± 21.1, n = 4). The total expression levels of PKCγ and PKCα were normalized to the Actinβ expression level and the expression levels of PKCγ and PKCα in Wt are shown as 100%. Data represent means ± SEM (n = 4, the statistical analysis showed no significance). Differences between Wt and Homo PKCγ-A24E were analyzed using the Two-tailed Mann Whitney test. (F) Western blot analysis of total PKCγ protein in cerebellum from 7-week-old Wt, PKCγ-A24E and PKCγ knockout mice. PKCγ protein expression in Homo PKCγ-A24E mouse is confirmed via western blot with long exposure while PKCγ knockout mouse shows no PKCγ protein expression (Wt = 100.0%, n = 4; Homo PKCγ-A24E = 2.088%, n = 4; PKCγ knockout = 0.067%, n = 4). (G) Organotypic slice culture from Wt, Het and
Homo PKCγ-A24E mice treated with 50 μg/ml cycloheximide or 10 μM MG132 or both at DIV7, protein was extracted at DIV8. Western blot data show that PKCγ protein expression is strongly reduced in PKCγ-A24E mice after cycloheximide treatment while MG132 treatment rescued protein degradation in PKCγ-A24E mice. The total expression level of PKCγ was normalized to Actinβ expression level and DMSO treated Wt is shown as 100.0%. (Wt + DMSO = 100.0%; Wt + CHX = 124.9%; Wt + MG132 = 159.0%; Wt + MG132 + CHX = 114.2%; Het + DMSO = 107.8%; Het + CHX = 71.3%; Het + MG132 = 91.7%; Het + MG132 + CHX = 73.0%; Homo + DMSO = 3.1%; Homo + CHX = 0.03%; Homo + MG132 = 3.8%; Homo + MG132 + CHX = 1.2%, n = 2).

Figure 4 PKCγ-A24E mouse shows highly PKC kinase activity

(A) Western blot analysis of total PKCγ protein and phospho-PKC substrates from organotypic slice cultures. Phosphorylation of PKC substrates were increased in Het and Homo PKCγ-A24E mice. (B) Western blot analysis normalized to actin shows PKCγ protein reduction in PKCγ-A24E mice but phospho-PKC substrate is upregulated in PKCγ-A24E mice. NMDAR-S890 is known to be phosphorylated by PKCγ. Phospho-NMDAR-S890 protein expression is normalized to total NMDAR-N1 protein expression. This phosphorylation is increased in PKCγ-A24E mice. The data were normalized to Wt as 100% from three independent experiments. (C) Phospho-MARCKS is a known substrate of PKC. Phospho-MARCKS S152/S156 compare to total MARCK was decreased in PKCγ-A24E mice and PMA treated organotypic slice cultures from Wt mice (Wt = 100.4% ± 4.37, n = 4; Het = 61.92% ± 15.25, n = 4; Homo = 33.98% ± 5.83, p = 0.0009, n = 4; Wt + PMA = 42.54% ± 15.4, p = 0.0033, n = 4; Het + PMA = 34.58% ± 9.39, p = 0.0010, n = 4; Homo + PMA = 16.89% ± 3.557, p < 0.0001, n = 4). With added phosphatase inhibitor, the phospho-MARCKS reduction
was partially rescued (Wt = 116.5% ± 11.8, n = 3; Het = 64.68% ± 13.8, n = 3; Homo = 97.15% ± 10.3, n = 3). The two-tailed Mann Whitney test was used to analyze the difference between each group and Wt without any treatment is shown as 100%. Data represent means ± SEM.

Figure 5 Altered morphology of Purkinje cells from PKCγ-A24E mice in organotypic cerebellar slice culture which is rescued by PKC inhibitor

(A) Purkinje cells in organotypic slice cultures from each genotypes are shown. Anti-calbindin staining showing all Purkinje cells and PKCγ expression was strongly reduced in PKCγ-A24E mice with anti-PKCγ immunostaining. Scale bar in H = 50 μm. Each Purkinje cell area was measured with image J and analyzed with Graphpad prism. Differences between PKCγ-Wt and PKCγ-A24E were analyzed using the two-tailed Mann Whitney test. Wt = 100.0% ± 5.64; Het = 84.29% ± 5.40, p = 0.0718 and Homo = 62.14% ± 5.23, p < 0.0001. Data are shown as the mean ± SEM of 20 Purkinje cells. (B) Purkinje cells from organotypic slice cultures at DIV 7 with or without PKC inhibitor (Gö6983) treatment. For control, the same volume of DMSO was added and 10 μM Gö6983 was added at DIV3. Control Purkinje cells show elaborated branched dendrites while Purkinje cells from PKCγ-A24E mice have reduced dendritic growth, which was rescued by PKC inhibitor (Gö6983) treatment. The dendritic area of Purkinje cells was measured with image J and the analysis shows that PKC inhibitor treatment could rescue the dendritic tree size (Purkinje cells area Wt = 100.0% ± 5.00; Homo PKCγ-A24E = 47.50% ± 3.42; Homo PKCγ-A24E + Gö6983 = 74.11% ± 4.40). Differences between PKCγ-A24E and Gö6983 treated PKCγ-A24E were analyzed using the two-tailed Mann Whitney test. (p < 0.0001). The number of measured cells was 20 for all experiments.
Figure 6 Altered climbing fiber innervation and Purkinje cell morphology in vivo in PKCγ-A24E mice

(A) Anti-Calbindin D28K antibody staining shows Purkinje cells and anti-vGlut2 staining shows CF terminals on Purkinje cell dendrites from 40-week-old Wt and Homo PKCγ-A24E mice. White arrowheads represent CF terminals. Scale bar in H = 10 μm.

(B) The CF terminals on Purkinje cells were analyzed with anti-vGlut2 immunoreactivity. Scale bar in H = 50 μm. The CF terminals in the molecular layer were counted by ImageJ. Quantification of the number of terminal puncta on Purkinje cell branchlets in distal molecular layer in a 284 μm x 75 μm rectangle (vGlut2 positive puncta)

Wt = 394.4 ± 18.14; Het = 345.1 ± 10.61, p = 0.0344; Homo = 177.6 ± 26.62, p = 0.0002). Data are shown as the mean ± SEM of 8 samples each. Scale bar in H = 10 μm.

(C) Bright field microscopy analysis of Golgi-cox staining from 4-week-old and 1-year-old Wt, Het and Homo PKCγ-A24E mice shows significantly reduced size of Purkinje cells in Homo PKCγ-A24E mice. Purkinje cells size from 4-week-old (Wt = 100.0% ± 5.36; Het PKCγ-A24E = 91.85% ± 6.81; Homo PKCγ-A24E = 69.74% ± 6.07) and Purkinje cells size from 1-year-old (Wt = 100.0% ± 8.17; Het PKCγ-A24E = 99.85% ± 6.51; Homo PKCγ-A24E = 74.91% ± 5.14) were analyzed using the two-tailed Mann Whitney test. (4-week-old, p = 0.0013, 1-year-old, p = 0.0122). Scale bar in H = 50 μm. The number of measured cells was 20 for all experiments. (D)

Calbindin D-28 immunohistochemistry staining of cerebellar cryosections (20 μm) from 4-week-old to 40-week-old Wt, Het PKCγ-A24E and Homo PKCγ-A24E mice. ML, Molecular layer; PCL, Purkinje cell layer; GL, Granule cell layer. Scale bar in H = 50 μm. The width of the molecular layer is slightly decreased in Lobule VII of 40-week-old Homo PKCγ-A24E mice (p = 0.0027).
Figure 7 Behaviour analysis shows motor deficit and ataxic phenotype in PKCy-A24E mice

(A) The beam test was done in 3-month-old and 6-month-old mice. Slip count of Wt (1.235 ± 0.271, n = 28), Het (9.265 ± 1.079, n = 28, p < 0.0001) and Homo PKCy-A24E mice (17.50 ± 0.056, n = 28, p < 0.0001). Data are shown as means ± SEM. Statistical significance of the values of Wt and PKCy-A24E mice groups were analyzed two-way ANOVA test with Bonferroni correction, **** p < 0.0001. (B, C) Rotarod performance test was evaluated in 3-month-old mice (B) and 6-month-old mice (C). (B) Latency to fall on day 4: Wt = 17.00 rpm ± 2.00, n = 11; Het = 18.00 rpm ± 1.528, n = 14; Homo= 4.333 rpm ± 1.212, p < 0.0001, n = 14 and on day 5: Wt = 24.33 rpm ± 1.202, n = 11; Het = 14.67 rpm ± 1.202, p = 0.0317, n = 14; Homo= 6.67 rpm ± 0.667, p < 0.0001, n = 14. (C) Latency to fall on day 3: Wt = 13.60 rpm ± 2.227; Het = 8.200 rpm ± 1.241, p = 0.0127; Homo = 5.200 rpm ± 0.374, p < 0.0001; n = 5 for each genotype and on day 4: Wt = 15.80 rpm ± 1.749; Homo = 7.80 rpm ± 0.735, p = 0.0002; n = 5 for each genotype. Data are shown as means ± SEM. Statistical significance of the values of Wt and PKCy-A24E mice groups were analyzed by two-way ANOVA test with Bonferroni correction, * p < 0.05, *** p < 0.001, or **** p < 0.0001. (D) Footprints of 9-month-old Wt and PKCy-A24E mice were evaluated for step width (E) Wt = 1.30 cm ± 0.058, n = 12; Het = 2.60 cm ± 0.154, n = 12; Homo = 2.95 cm ± 0.047, n = 12. Data are shown as means ± SEM. Statistical significance of the values of Wt and Het or Homo PKCy-A24E mice groups were analyzed by two-way ANOVA test with Bonferroni correction, **** p < 0.0001.
Figure 8 RNA sequence analysis and phosphoproteomics analysis

(A-B) Many oxidative phosphorylation related genes in Complex I, Complex III, Complex IV and Chemiosmosis are upregulated in Het PKCy-A24E mice in RNA sequencing. (A) IPA analysis showed that many ubiquinone oxidoreductase subunits and cytochrome c oxidase subunits are significantly upregulated in Het PKCy-A24E mice. Y-axis is log2 fold change. (B) Complexes with red colour are upregulated in Het PKCy-A24E mice, which locate in the mitochondrial membrane. (C) Network analysis was performed by IPA analysis of gene sets up- or down-regulated in Het PKCy-A24E mice compared to Wt littermates. Genes colored green display decreases, while genes colored red display increases. Many genes related to Purkinje cell morphology are downregulated in Het PKCy-A24E mice. (D) Volcano plot of phospho-proteomics analysis of Wt vs Homo PKCy-A24E mice (n = 3). Lysates from 7 week-old Wt and PKCy-A24E mice were subjected to phosphoproteomic analysis by mass spectrometry. Differentially enriched phosphopeptides are shown in the volcano plot. X-axis is log2 fold change and Y-axis is p-value. Rnmd3 and Homer3 are among the proteins with a significantly increased phosphorylation in PKCy-A24E mice. (E) RNA sequence data show that Rnmd3 and Homer3 are upregulated in PKCy-A24E mice. (F) Western blot analysis of Rnmd3 and Homer3 from organotypic slice cultures. Homer3 protein expression (Wt = 100.0%, n = 4; Het = 149.7%, p = 0.0286, n = 4; Homo = 184.2%, p = 0.0286, n = 4) and Rnmd3 protein expression (Wt = 100.0%, n = 4; Het = 167.0%, p = 0.0286, n = 4; Homo = 206.5%, p = 0.0286, n = 4) are increased in PKCy-A24E mice. Statistical analysis using the two-tailed Mann Whitney test showing increased protein expression in PKCy-A24E mice.
Movie legends

Figure 7- movie 1 The beam test of Wt
Wt mouse at 25-week-old walking on the 80 cm long and 8 mm wide wooden bar.

Figure 7- movie 2 The beam test of Het PKCγ-A24E
Het PKCγ-A24E mouse at 25-week-old walking on the 80 cm long and 8 mm wide wooden bar.

Figure 7- movie 3 The beam test of Homo PKCγ-A24E
Homo PKCγ-A24E mouse at 25-week-old walking on the 80 cm long and 8 mm wide wooden bar.

Table legends
Table 1 Summary of the ages of mice used for each experiment.
Table 2 Cellular localizations of proteins with significantly increased phosphorylation in Homo PKCγ-A24E mice

Extended data Figure legends
Extended data Figure 8-1 Summary of the significantly changed genes in Het PKCγ-A24E mice (Symbol, gene name, fold changes, p-values and locations) (A)
Many mitochondrial function related genes are upregulated in Het PKCγ-A24E mice. (B) Summary of the significantly changed genes related to glutamate receptor signal-
ling pathway in Het PKCy-A24E mice. (C) Summary of the significantly changed genes related to RICTOR signaling pathway in Het PKCy-A24E mice in RNA sequencing.

Extended data Figure 8-2 Summary of the significantly changed genes in Homo PKCy-A24E mice (Symbol, gene name, fold changes, p-values and locations). (A) Summary of the significantly changed genes related to Ehprin receptor signaling pathways in Homo PKCy-A24E mice. (B) Summary of the significantly changed genes related to glutamate receptor signalling pathway in Homo PKCy-A24E mice in RNA sequencing.

Extended data Figure 8-3 Summary of phosphoproteomics analysis (Symbol, gene name, fold changes, p-values and locations).

(A) 174 protein phosphorylations are significantly changed in Homo PKCy-A24E mice. 105 out of 174 protein phosphorylations are significantly increased while 69 out of 174 protein phosphorylations are significantly decreased in Homo PKCy-A24E mice.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
### Table. 1

| Figure                  | Age of Mice                |
|-------------------------|----------------------------|
| Fig. 3E                 | 4 and 6-week-old           |
| Fig. 3F                 | 7-week-old                 |
| Fig. 6A                 | 40-week-old                |
| Fig. 6B                 | 40-week-old                |
| Fig. 6C                 | 4 and 53-week-old          |
| Fig. 6D                 | 4 and 40-week-old          |
| Fig. 7 Beam walk        | 3-month and 6-month-old    |
| Fig. 7 Rotarod          | 3-month and 6-month-old    |
| Fig. 7 Foot print       | 9-month-old                |
| Fig. 8                  | 5-week-old and 7-week-old |
Table 2. Analysis of the phosphoprotein localization

| Location             | Total phosphoprotein | Up- or Down-regulated in A24E (P<0.05) | Up-regulated phosphoprotein out of total phosphoprotein (%) |
|----------------------|----------------------|----------------------------------------|------------------------------------------------------------|
| Cytoplasm            | 1200                 | 73                                     | 44                                                         | 3.667%                                                     |
| Plasma membrane      | 532                  | 44                                     | 33                                                         | 6.203%                                                     |
| Nucleus              | 795                  | 46                                     | 24                                                         | 3.019%                                                     |
| Extracellular space  | 85                   | 6                                      | 2                                                          | 2.353%                                                     |
| Other                | 193                  | 5                                      | 2                                                          | 1.036%                                                     |
| Total                | 2805                 | 174                                    | 109                                                        | 3.886%                                                     |