

Published in final edited form as:

*Biochem Biophys Res Commun.* 2009 January 16; 378(3): 524–528. doi:10.1016/j.bbrc.2008.11.082.

## Loss of Purkinje Cells in the PKC $\gamma$ H101Y Transgenic Mouse

Yunong Zhang<sup>1,\*</sup>, Adam Snider<sup>2,\*</sup>, Lloyd Willard<sup>3</sup>, Dolores J Takemoto<sup>2</sup>, and Dingbo Lin<sup>1</sup>

<sup>1</sup>Department of Human Nutrition, Kansas State University, Manhattan, KS 66506

<sup>2</sup>Department of Biochemistry, Kansas State University, Manhattan, KS 66506

<sup>3</sup>Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS 66506

### Abstract

Spinocerebellar ataxia type 14 (SCA14) is an autosomal, dominant neurodegenerative disorder caused by mutations in PKC $\gamma$ . The objective of this study was to determine effects of PKC $\gamma$  H101Y SCA14 mutation on Purkinje cells in the transgenic mouse. Results demonstrated that wild type PKC $\gamma$ -like Purkinje cell localization of HA-tagged PKC $\gamma$  H101Y mutant proteins, altered morphology and loss of Purkinje cells were observed in the PKC $\gamma$  H101Y SCA14 transgenic mouse at four weeks of age. Failure of stereotypical clasping responses in the hind limbs of transgenic mice was also observed. Further, PKC $\gamma$  H101Y SCA14 mutation caused lack of total cellular PKC $\gamma$  enzyme activity, loss of connexin 57 phosphorylation on serines, and activation of caspase-12 in the PKC $\gamma$  H101Y SCA14 transgenic mouse. Results clearly demonstrate a need for PKC $\gamma$  control of gap junctions for maintenance of Purkinje cells. This is the first transgenic mouse to our knowledge which models a human SCA14 mutation.

### Introduction

Spinocerebellar ataxias (SCAs) are autosomal, heterogeneous, dominant neurodegenerative disorders. SCAs are classified at least into 27 types. Fourteen out of 27 SCAs are linked to particular gene mutations [1]. Spinocerebellar ataxia type 14 (SCA14) is caused by mutations in the PKC $\gamma$  gene with onset age as early as three years [2–27]. SCA14 is a newly identified neurodegenerative disorder, the mechanistic aspects of this disease remain unknown.

PKC is a family of phospholipid-dependent serine/threonine kinases that participates in many cellular functions. PKC $\gamma$  is a classical PKC, primarily found in the central and peripheral nervous systems. It is particularly abundant in cerebellar Purkinje cells and in hippocampal pyramidal cells [28]. SCA14 mutations occur throughout the PKC $\gamma$  gene from the regulatory to catalytic domains and also include a six-pair in-frame deletion and a splice site mutation in the C1B region, where most mutations occur [2–27]. Verbeek et al. [10] reported that PKC $\gamma$  SCA14 mutations have increased kinase activity and altered membrane

Corresponding author: Dingbo Lin, PhD, Department of Human Nutrition, Kansas State University, 212 Justin Hall, Manhattan, KS 66506, 785-532-0656, 785-532-3132 (FAX), dingbo@ksu.edu.

\*Yunong Zhang and Adam Snider equally contributed to this work

targeting in kidney COS-7 cells with overexpression of SCA14 mutants. No significant mutant protein aggregation is observed. However, Seki et al. reported that SCA14 mutants are susceptible to aggregation in the Chinese hamster ovary CHO-K1 [7]. These contradictory conclusions, by two separate groups, indicates that irrelevant cell lines, such as kidney COS-7 and Chinese hamster ovary CHO-K1, may not be appropriate in vitro cell systems to study the molecular mechanism of a neurodegenerative disorder, such as SCA14.

Since SCA14 is rare and not fatal in most cases, it is difficult to investigate the pathogenesis due to lacking of human brain tissue [2]. Thus, it is critical to generate an animal model with SCA14 phenotype. In our lab, we have previously used well-characterized, hippocampal neuronal HT22 cells [6,29]. We have demonstrated that these mutant PKC $\gamma$ 's cause a caspase-3 linked apoptosis in culture and PKC $\gamma$  SCA14 mutants are not activated by an oxidative signal such as H<sub>2</sub>O<sub>2</sub> when expressed in neuronal HT22 cells in culture [6]. Endogenous wild type PKC $\gamma$  is negatively affected by the presence of the mutations (ie., a dominant effect). Here we reported the generation of the PKC $\gamma$  H101Y SCA14 transgenic mouse which expresses loss of Purkinje cells and neurological phenotype.

## Materials and Methods

### Generation of the transgenic mouse

The HA-tagged PKC $\gamma$  H101Y mice were generated in a C57BL/6J background by the standard transgenic strategy. The experiments were done at the University of Missouri-Columbia Transgenic Animal Facility using the pronuclear injection method. Injected DNA fragments in a length of 3.14 kb consisted of the CMV promoter, PKC $\gamma$  H101Y SCA14 mutant with N-terminal HA tags, and a SV40 poly A terminus. Positive pups were confirmed by PCR using tail genomic DNA. Primers used for PCR screen were:

Primer 1. HAFOR, T<sub>m</sub>= 68 C, 35 mers:

5' CC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT CTT 3'

Primer 2. SCAREV, T<sub>m</sub>=70 C, 28 mers:

5' GGT CGC AGA AGG TGG GAC TGC TGT AGC T 3'

Predicted PCR products were 403 bp in length containing the HA tag and a partial N-terminal portion of PKC $\gamma$  H101Y SCA14 mutants.

Internal control primer pairs (ZP3-1 and ZP3-2) were used as a DNA quality control to amplify genomic DNA:

ZP3-1 CAG CTC TAC ATC ACC TGC CA

Zp3-2 CAC TGG GAA GAG ACA CTC AG

The predicted products were 500 bp.

Western blot was performed to further confirm the positive generations using anti-HA antibody. Founders were mated with wild type C57BL/6J mice to create the next generation. After the 6th generation, mice from same littermates were used for studies.

### Mouse cerebellar slice culture

Cerebellar slice culture was modified from the previous reports [28, 30]. Briefly, after euthanization, sagittal cerebellar slices were obtained immediately from the wild type and/or PKC $\gamma$  H101Y transgenic mice at age of 4 weeks. The cerebellar slices were sectioned at 250  $\mu$ m and cultured in serum-free BME medium supplemented with 2.5 mM glutamine, 5 mM glucose and 5 ng/mL nerve growth factor at 5 % CO<sub>2</sub> in an incubator at 37 °C for up to 1 hour. H<sub>2</sub>O<sub>2</sub>/oxidative stress treatments were conducted in the slice culture system. After that, slices were homogenized for further analyses.

### Cell cultures

HT22 cells were cultured in DMEM (high glucose, 4.5 g/L) (Invitrogen, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA) and 50  $\mu$ g/ml gentamicin, 0.05 U/ml penicillin, 50  $\mu$ g/ml streptomycin, pH 7.4 at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>.

### Western blot and immunoprecipitation

Western blotting and immunoprecipitation were performed as described previously [4]. For immunoprecipitation assay, cells or tissue homogenates were lysed. After 8,000 x g centrifugation for 15 min the supernatants are used for immunoprecipitation. Anti-HA antibody was purchased from Covance (Berkeley, CA), anti-PKC $\gamma$  was from BD Biosciences (San Jose, CA), anti-Cx57 was purchased from Diatheva (Italy), and antibodies against caspase-3, caspase-12, and  $\alpha$ -tubulin were purchased from Cell Signaling (Danvers, MD).

### PKC $\gamma$ enzyme activity assays

PKC $\gamma$  enzyme activity was measured as previously described [4]. Briefly, equal protein amounts of cerebellar slice extracts from the wild type or PKC  $\gamma$  H101Y transgenic mice were incubated with PKC  $\gamma$  antisera at 4 °C for 4 h to immunoprecipitate endogenous wild type PKC $\gamma$  and/or H101Y mutants. Immunoprecipitated PKC $\gamma$  and/or PKC $\gamma$  H101Y-agarose bead complexes were recovered and incubated with PKC reaction mixture according to the manufacturer's instructions. The fluorescent phospho-PepTag peptides (phosphorylated by PKC $\gamma$  and/or PKC $\gamma$ H101Y) were resolved by 0.8% agarose gel electrophoresis and visualized under UV light. The phosphorylated peptide bands were excised, and their fluorescence intensities were quantified by spectrophotometry at 570 nm.

### Immunohistochemistry and confocal microscopy

To determine the Purkinje cell localization of PKC $\gamma$ , PKC  $\gamma$  H101Y, and Cx57 in the wild type and transgenic mice, cerebellar tissues were fixed in 2 % paraformaldehyde and sagittally sectioned at 20  $\mu$ m in thickness using a cryostat. The cerebellar sections were labeled with primary antisera including anti-Cx57, anti-HA, and/or anti-PKC $\gamma$  for overnight at 4 °C. After washing in PBS, the slice sections were further incubated with secondary antisera with Alexa Fluor 568 or 488 conjugation for 2 hours at room temperature. Then Purkinje cell localization of desired proteins was determined using a Nikon C1 confocal microscope.

## Light microscopy

For the Purkinje cell pathogenesis study, whole cerebellum was fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M cacodylate and then post-fixed in osmium tetroxide. Dehydration was in 70 % ethanol for 12 h, overnight with 100 % ethanol, and 12 h with 100 % acetone and finally embedding in epon resin at room temperature. Sagittal cerebellar sections were made with an ultramicrotome and thick sections (1  $\mu$ m) were cut with a diamond knife and stained with 1% toluidine blue. Purkinje cell layers including soma and dendrites were viewed and images were captured by a Nikon light microscope.

## Tail suspension photography

Neurological dysfunction of transgenic mice was exhibited as hindlimb and/or forelimb clasping determined by tail suspension test [31, 32]. A tape was applied to the surface of the tail to set a metal clip which is anchored to the fixed lever. Mice first struggled to escape but sooner or later attained a posture of immobility. The response of each mouse to 1 min of vertical suspension from the tail was tested and photographed. Total 12 transgenic and 12 wild type mice at 12 months of age were tested. Of this, over 90 % of the transgenic mice exhibited a clasping response as illustrated in Figure 1D.

## Measurement of cell surface Cx57 gap junction plaques

HT22 cells were treated with 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA), 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, or phosphate-buffered saline (PBS) for 30 min. Endogenous Cx57 gap junctions was determined as described previously [4]. Briefly, the cells were fixed with 2.5 % paraformaldehyde for 5 min and labeled with anti-Cx57 overnight at 4 °C. After washing, the fixed cells were incubated with the secondary antibody with Alexa Fluor 568 conjugation. The cells were then examined using a Nikon confocal microscope. We photographed ten points per slide, three slides for each treatment. For quantitation, the cell surface Cx57 gap junctions from single cells in single sections in each image were counted. The number of gap junction plaques was expressed as mean  $\pm$  SEM.

## Statistical analysis

The statistical analysis employed in this paper is the unpaired Student's t-Test. All analyses represent at least triplicate experiments. The level of significance (\*) was considered at p 0.05. All data are mean  $\pm$  S.E.M.

## Results and Discussion

### Neurological phenotype of the PKC $\gamma$ H101Y transgenic mouse

According to our in vitro cell culture study, PKC $\gamma$  H101Y mutant cells have milder cell apoptosis phenotype while S119P and G128D mutants are more severe [4, 6]. Therefore, we expected that PKC $\gamma$  H101Y mutation would not be lethal to the transgenic mutant mouse. In the current study, we have successfully created five PKC $\gamma$  H101Y transgenic founders as determined by PCR (Fig. 1A, arrowed). We also determined the expression of PKC $\gamma$  H101Y mutant proteins in the transgenic cerebellum by immunohistology/confocal microscopy (Fig. 1B). Confocal microscopy results demonstrated that endogenous PKC $\gamma$  is mainly localized

in Purkinje cells of the wild type C57BL/6J at 4 weeks of age (Fig. 1B, left panel). Compared to expression pattern of the wild type of PKC $\gamma$ , a similar Purkinje cell localization of HA-tagged PKC $\gamma$  H101Y was observed in transgenic mice (Fig. 1B, right panel). Of interest, altered Purkinje cell soma were found in the transgenic mice at 4 weeks of age (Fig. 1B, arrowed).

Light microscopy of sections from sagittal cerebella of the control and PKC $\gamma$  H101Y transgenic mice is shown in Fig. 1C. As predicted from the cell culture studies, transgenic mice overexpressing the PKC $\gamma$  H101Y SCA14 mutants were observed with altered morphology of and loss of Purkinje cells at four weeks of age (Fig. 1C, right panel). Further, we determined motor function using tail suspension photography. In current experiments 11 out of 12, 12-month old wild type C57BL/6J mice showed splaying out of hind limbs (Fig. 1D, left panel). However, over 90 % of the PKC $\gamma$  H101Y transgenic mice (11 out of 12 mice) manifested the stereotypical clasping response (Fig. 1D, right panel) in the hind limbs, indicating that the presence of a H101Y SCA14 mutant may confer a neurological phenotype which is more severe than a knockout for PKC $\gamma$  which has a normal Purkinje cell layer (data not shown).

Although the PKC $\gamma$  H101Y SCA14 transgenic mouse expressed a HA-tagged SCA14 mutant PKC $\gamma$  H101Y under CMV promoter control, a universal promoter, PKC $\gamma$  H101Y was expressed specifically in the Purkinje cell layers particularly in the soma and dendrites. We chose this promoter after discussion with others who had failed to find an effect using a Purkinje cell promoter. We have the only colony showing Purkinje neuron degeneration and others are not currently commercially available.

### **Dominant negative effect of PKC $\gamma$ H101Y mutation on wild type endogenous PKC $\gamma$ and Cx57 phosphorylation in the transgenic mice**

We have previously demonstrated that PKC $\gamma$  H101Y mutation causes dominant negative effects on endogenous wild type PKC $\gamma$  enzyme activity which leads to uncontrolled, open gap junctions in the HT22 cells overexpressing PKC $\gamma$  SCA14 mutants [4, 6]. In this study we used HT22 cells and cerebellar slice culture in vitro to determine control of Cx57 gap junctions by PKC $\gamma$ . Almost 90 % confluence of HT22 cells were treated with 200 nM TPA or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min, cell surface Cx57 gap junction plaques were visualized by immunocytochemistry/confocal microscopy as demonstrated previously [4]. Plaque number was counted and graphed (Fig. 2A). Results indicated that application of PKC $\gamma$  activators, such as TPA and H<sub>2</sub>O<sub>2</sub>, caused decreases in the Cx57 gap junction plaques (Fig. 2A, graph). TPA or H<sub>2</sub>O<sub>2</sub>-activation of PKC $\gamma$  caused Cx57 phosphorylation on serines in HT22 cells as determined by immunoprecipitation/Western blotting. Inhibition of Cx57 gap junction plaques and phosphorylation of Cx57 on serines results in inhibition of gap junction activity.

Cx57 was newly characterized in the Purkinje cell layers of wild type C57BL/6B mouse (Fig. 2B). To determine whether control of Cx57 gap junctions was altered in the cerebella of PKC $\gamma$  transgenic mice, we treated four-week old mouse cerebellar slices in culture with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min and then determined Cx57 phosphorylation. As shown in Fig. 2C, PKC $\gamma$ H101Y was only expressed in the transgenic mice though endogenous PKC $\gamma$  were present in both types of animals. Lack of Cx57 phosphorylation on serines was observed in

the transgenic mice. Enzyme activity assay further confirmed that overexpression of PKC $\gamma$  H101Y mutant in vivo caused lack of PKC $\gamma$  enzyme activity in the transgenic cerebella (Fig. 2D). Taken together, overexpression of PKC $\gamma$  H101Y mutants caused malfunction of endogenous wild type PKC $\gamma$  which may further lead to loss of control of gap junctions such as Cx57 in vivo, with or without stress stimuli.

### Activation of caspase-12 in the transgenic mouse

Based on the observation from cell cultures [6, 7, 18, 33], we next determined endoplasmic reticulum (ER) stress by activation of caspase-12 in the PKC $\gamma$  H101Y transgenic mouse. Results from Fig. 3 showed that caspase-12 was greatly activated in the PKC $\gamma$  H101Y mutant cerebellar tissues. In vitro application of 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min significantly enhanced activation of caspase-12, suggesting that ER stress may be a key event for pathogenesis of SCA14.

These data suggest that overexpression of PKC $\gamma$  SCA14 mutants, e.g., H101Y, triggered cellular ER stress and Purkinje cell apoptosis in vivo. Loss of control of gap junctions may further propagate ER stress-linked cell death signals to adjacent Purkinje cells. This is a novel transgenic mouse model which provides an important model system to study ER stress-initiated, dysfunction of gap junctions-mediated neurodegenerative disorders. This may be a suitable model for developing potential treatments that will prevent or delay neurodegeneration through restoration of control of gap junction and/or elimination of ER stress in the brain.

### Acknowledgments

The authors would like to thank Dr. Denis Medeiros for critical reading of the manuscript. The transgenic production was done at the University of Missouri-Columbia Transgenic Animal Facility. The work was supported by NIH R01 EY013421 to D.J.T., NIH COBRE Grant P20-RR-017686, NIH K-INBRE Major Starter grant P20-RR-016475, and the National Organization for Rare Disorders grant to D.L. The publication contribution number from K-State Research and Extension is.

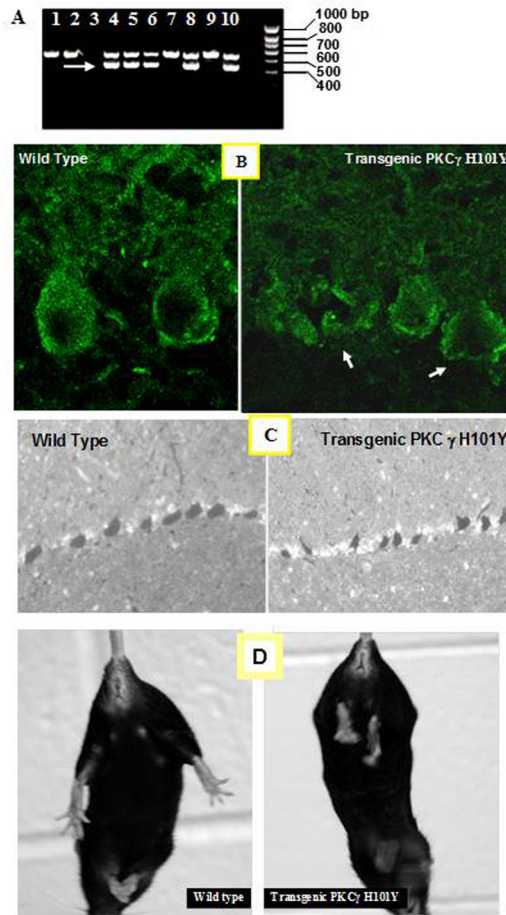
### References

1. Duenas AM, Goold R, Giunti P. Molecular pathogenesis of spinocerebellar ataxias. *Brain*. 2008; 129:1357–1370. [PubMed: 16613893]
2. Chen DH, Brkanac Z, Verlinde CL, Tan XJ, Bylenok L, Nochlin D, Matsushita M, Lipe H, Wolff J, Fernandez M, Cimino PJ, Bird TD, Raskind WH. Missense mutations in the regulatory domain of PKC gamma: a new mechanism for dominant nonepisodic cerebellar ataxia. *Am J Hum Genet*. 2003; 72:839–849. [PubMed: 12644968]
3. Vlak MH, Sinke RJ, Rabelink GM, Kremer BP, van de Warrenburg BP. Novel PRKCG/SCA14 mutation in a Dutch spinocerebellar ataxia family: Expanding the phenotype. *Mov Disord*. 2006; 21:1025–1028. [PubMed: 16547918]
4. Lin D, Takemoto DJ. Oxidative activation of protein kinase Cgamma through the C1 domain: Effects on gap junctions. *J Biol Chem*. 2005; 280:13682–13693. [PubMed: 15642736]
5. Lin D, Takemoto DJ. Loss of control of gap junctions by protein kinase C  $\gamma$  spinocerebellar ataxia type 14 mutations leads to neural cell apoptosis. *Annals Neurol*. 2006; 60 (S10):S33.
6. Lin D, Takemoto DJ. Protection from ataxia-linked apoptosis by gap junction inhibitors. *Biochem Biophys Res Commun*. 2007; 362:982–987.
7. Seki T, Adachi N, Ono Y, Mochizuki H, Hiramoto K, Amano T, Matsubayashi H, Matsumoto M, Kawakami H, Saito N, Sakai N. Mutant protein kinase Cgamma found in spinocerebellar ataxia

- type 14 is susceptible to aggregation and causes cell death. *J Biol Chem.* 2005; 280:29096–29106. [PubMed: 15964845]
8. Stevanin G, Hahn V, Lohmann E, Bouslam N, Gouttard M, Soumphonphakdy C, Welter ML, Ollagnon-Roman E, Lemainque A, Ruberg M, Brice A, Durr A. Mutation in the catalytic domain of protein kinase C gamma and extension of the phenotype associated with spinocerebellar ataxia type 14. *Arch Neurol.* 2004; 61:1242–1248. [PubMed: 15313841]
  9. van de Warrenburg BP, Verbeek DS, Piersma SJ, Hennekam FA, Pearson PL, Knoers NV, Kremer HP, Sinke RJ. Identification of a novel SCA14 mutation in a Dutch autosomal dominant cerebellar ataxia family. *Neurol.* 2003; 61:1760–1765.
  10. Verbeek DS, Knight MA, Harmison GG, Fischbeck KH, Howell BW. Protein kinase C gamma mutations in spinocerebellar ataxia 14 increase kinase activity and alter membrane targeting. *Brain.* 2005; 128:436–442. [PubMed: 15618281]
  11. Chen DH, Cimino PJ, Ranum LP, Zoghbi HY, Yabe I, Schut L, Margolis RL, Lipe HP, Feleke A, Matsushita M, Wolff J, Morgan C, Lau D, Fernandez M, Sasaki H, Raskind WH, Bird TD. The clinical and genetic spectrum of spinocerebellar ataxia 14. *Neurol.* 2005; 64:1258–1260.
  12. Yabe I, Sasaki H, Chen DH, Raskind WH, Bird TD, Yamashita I, Tsuji S, Kikuchi S, Tashiro K. Spinocerebellar ataxia type 14 caused by a mutation in protein kinase C gamma. *Arch Neurol.* 2003; 60:1749–1751. [PubMed: 14676051]
  13. Alonso I, Costa C, Gomes A, Ferro A, Seixas AI, Silva S, Cruz VT, Coutinho P, Sequeiros J, Silveira I. A novel H101Q mutation causes PKCgamma loss in spinocerebellar ataxia type 14. *J Human Genet.* 2005; 50:523–529. [PubMed: 16189624]
  14. Klebe S, Durr A, Rentschler A, Hahn-Barma V, Abele M, Bouslam N, Schols L, Jedynak P, Forlani S, Denis E, Dussert C, Agid Y, Bauer P, Globas C, Wullner U, Brice A, Riess O, Stevanin G. New mutations in protein kinase Cgamma associated with spinocerebellar ataxia type 14. *Ann Neurol.* 2005; 58:720–729. [PubMed: 16193476]
  15. Fahey MC, Knight MA, Shaw JH, Gardner RJ, du Sart D, Lockhart PJ, Delatycki MB, Gates PC, Storey E. Spinocerebellar ataxia type 14: study of a family with an exon 5 mutation in the PRKCG gene. *J Neurol Neurosurg Psychiatry.* 2005; 76:1720–1722. [PubMed: 16291902]
  16. Adachi N, Kobayashi T, Takahashi H, Kawasaki T, Shirai Y, Ueyama T, Matsuda T, Seki T, Sakai N, Saito N. Enzymological analysis of mutant protein kinase Cgamma causing spinocerebellar ataxia type 14 and dysfunction in Ca<sup>2+</sup> homeostasis. *J Biol Chem.* 2008; 283:19854–19863. [PubMed: 18499672]
  17. Doran G, Davies KE, Talbot K. Activation of mutant protein kinase Cgamma leads to aberrant sequestration and impairment of its cellular function. *Biochem Biophys Res Commun.* 2008; 372:447–453. [PubMed: 18503760]
  18. Seki T, Takahashi H, Adachi N, Abe N, Shimahara T, Saito N, Sakai N. Aggregate formation of mutant protein kinase C gamma found in spinocerebellar ataxia type 14 impairs ubiquitin-proteasome system and induces endoplasmic reticulum stress. *Eur J Neurosci.* 2007; 26:3126–3140. [PubMed: 18005063]
  19. Miura S, Nakagawara H, Kaida H, Sugita M, Noda K, Motomura K, Ohyagi Y, Ayabe M, Aizawa H, Ishibashi M, Taniwaki T. Expansion of the phenotypic spectrum of SCA14 caused by the Gly128Asp mutation in PRKCG. *Clin Neurol Neurosurg.* 2008 Nov 3. Epub ahead of print.
  20. Verbeek DS, Goedhart J, Bruinsma L, Sinke RJ, Reits EA. PKC gamma mutations in spinocerebellar ataxia type 14 affect C1 domain accessibility and kinase activity leading to aberrant MAPK signaling. *J Cell Sci.* 2008; 121:2339–2349. [PubMed: 18577575]
  21. Doran G, Davies KE, Talbot K. Activation of mutant protein kinase Cgamma leads to aberrant sequestration and impairment of its cellular function. *Biochem Biophys Res Commun.* 2008; 372:447–453. [PubMed: 18503760]
  22. Adachi N, Kobayashi T, Takahashi H, Kawasaki T, Shirai Y, Ueyama T, Matsuda T, Seki T, Sakai N, Saito N. Enzymological analysis of mutant protein kinase Cgamma causing spinocerebellar ataxia type 14 and dysfunction in Ca<sup>2+</sup> homeostasis. *J Biol Chem.* 2008; 283:19854–19863. [PubMed: 18499672]

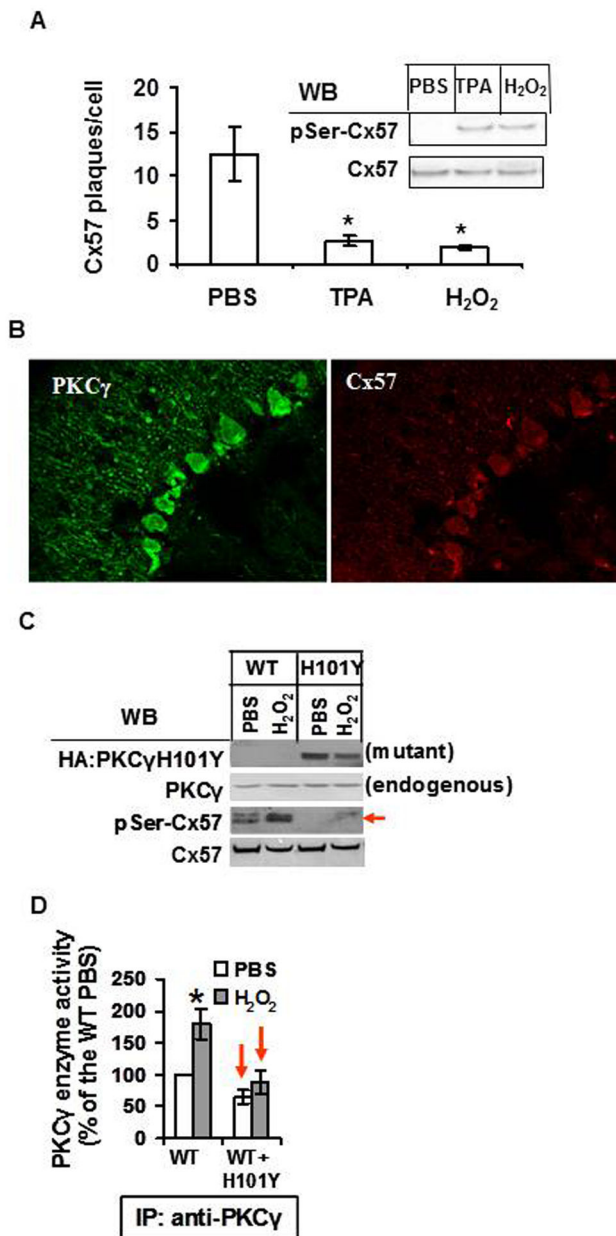
23. Klebe S, Faivre L, Forlani S, Dussert C, Tourbah A, Brice A, Stevanin G, Durr A. Another mutation in cysteine 131 in protein kinase C gamma as a cause of spinocerebellar ataxia type 14. *Arch Neurol.* 2007; 64:913–914. [PubMed: 17562946]
24. Lin D, Shanks D, Prakash O, Takemoto DJ. Protein kinase C gamma mutations in the C1B domain cause caspase-3-linked apoptosis in lens epithelial cells through gap junctions. *Exp Eye Res.* 2007; 85:113–122. [PubMed: 17493614]
25. Visser JE, Bloem BR, van de Warrenburg BP. PRKCG mutation (SCA-14) causing a Ramsay Hunt phenotype. *Mov Disord.* 2007; 22:1024–1026. [PubMed: 17343273]
26. Morita H, Yoshida K, Suzuki K, Ikeda S. A Japanese case of SCA14 with the Gly128Asp mutation. *J Hum Genet.* 2006; 51:1118–1121. [PubMed: 17024314]
27. Hiramoto K, Kawakami H, Inoue K, Seki T, Maruyama H, Morino H, Matsumoto M, Kurisu K, Sakai N. Identification of a new family of spinocerebellar ataxia type 14 in the Japanese spinocerebellar ataxia population by the screening of PRKCG exon 4. *Mov Disord.* 2006; 21:1355–1360. [PubMed: 16763984]
28. Meller K, Krahe K, Theiss C. Dye coupling in Purkinje cells of organotypic slice cultures. *Brain Res Dev Brain Res.* 2005; 160:101–105.
29. Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D. Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem.* 2004; 279:21749–21758. [PubMed: 15031298]
30. Xiang Z, Hrabetova S, Moskowitz HS, Casaccia-Bonnett P, Young SR, Nimmrich VC, Tiedge H, Einheber S, Karnup S, Bianchi R, Bergold PJ. Long-term maintenance of mature hippocampal slices in vitro. *J Neurosci Methods.* 2000; 98:145–154. [PubMed: 10880828]
31. Ju YI, Sone T, Okamoto T, Fukunaga M. Jump exercise during remobilization restores integrity of the trabecular architecture after tail suspension in young rats. *J Appl Physiol.* 2008; 104:1594–1600. [PubMed: 18420719]
32. Jiao Y, Yan J, Zhao Y, Donahue LR, Beamer WG, Li X, Roe BA, Ledoux MS, Gu W. Carbonic anhydrase-related protein VIII deficiency is associated with a distinctive lifelong gait disorder in waddles mice. *Genetics.* 2005; 171:1239–1246. [PubMed: 16118194]
33. Zhao L, Ackerman SI. Endoplasmic reticulum stress in health and disease. *Current Opinion Cell Biol.* 2006; 18:444–452. [PubMed: 16781856]





**Fig. 1. Characterization of PKC $\gamma$  H101Y SCA14 transgenic mice**

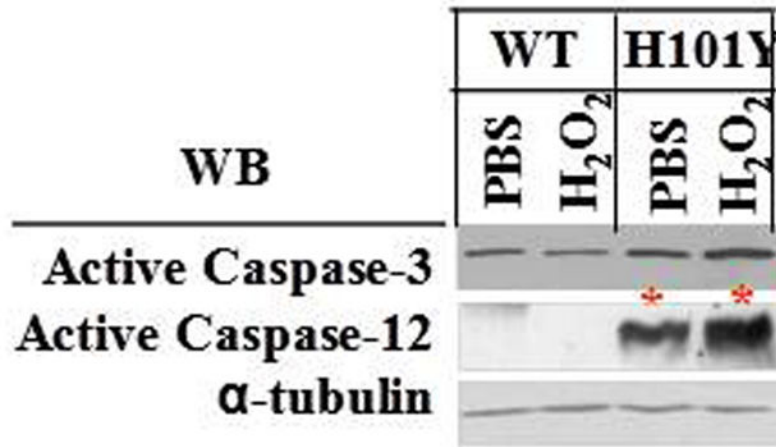
**A.** Five founders were identified by PCR using PKC $\gamma$  H101Y specific primers. Transgenic PCR products are arrowed. **B.** Endogenous PKC $\gamma$  is mainly localized in Purkinje cells of the wild type mouse (left panel), and a similar Purkinje cell localization of HA-tagged PKC $\gamma$  H101Y proteins in the PKC $\gamma$  H101Y transgenic mouse at the age of 4 weeks (right panel). Altered Purkinje cell body is observed in transgenic mice (as arrowed). **C.** Light microscopy of Purkinje cell layers in the wild type (left panel) and PKC $\gamma$  H101Y transgenic mice (right panel) at four weeks of age, showing an altered morphology of Purkinje cell layer in the transgenic mice. **D.** Tail suspension photography. Almost all 12-month old nontransgenic mice demonstrated splaying out of hind limbs (Left panel). However, PKC $\gamma$  H101Y transgenic mice manifested stereotypical clamping responses (Right panel)



**Fig. 2. The dominant negative effect of PKC $\gamma$  H101Y on Cx57 phosphorylation and endogenous PKC $\gamma$  activation in PKC $\gamma$  H101Y SCA14 transgenic mice**

**A.** HT22 cells were treated with 200 nM TPA, 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, or PBS for 30 min, cell surface Cx57 gap junction plaques were measured by immunocytochemistry/confocal microscopy. Gap junction plaques per cell were graphed. Phosphorylation of Cx57 on serines was determined by immunoprecipitation/Western blotting (see insert at right). **B.** Sagittal sections of C57BL/6J mouse cerebella at age of 4 weeks were made and endogenous PKC $\gamma$  and Cx57 were labeled and visualized by immunohistology/confocal microscopy. **C.** Four-week old cerebellar slices in culture from the wild type and transgenic mice were treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min. After treatments, whole tissue extracts

were used to determine phosphorylation of Cx57 by Western blotting. PKC $\gamma$  H101Y SCA14 mutant proteins are revealed by anti-HA antisera. Total cellular PKC $\gamma$  is revealed by anti-PKC $\gamma$  antisera.  $\alpha$ -tubulin is used as a loading control. **D.** The whole tissue extracts from **C** were used to immunoprecipitate both endogenous PKC $\gamma$  and HA-tagged PKC $\gamma$  H101Y by anti-PKC $\gamma$  antibodies. The precipitates (wild type and mutant H101Y PKC $\gamma$  together, see **C**) were used for the enzyme sources to determine total cellular PKC $\gamma$  enzyme activity. WT= control mouse cerebellar slice cultures; H101Y= PKC $\gamma$  H101Y transgenic mouse slices in culture; WT+H101Y= transgenic mouse cerebellar slice cultures which have both endogenous PKC $\gamma$  and the H101Y mutated PKC $\gamma$ . Note: We can not make a quantitative estimate of mutant PKC $\gamma$  expression vs endogenous wild type PKC $\gamma$  since PKC $\gamma$  and HA antisera can not be compared to each other. WB= Western blot



**Fig. 3. Activation of caspase-12 in the transgenic mouse**

Four-week old mouse cerebellar slices in culture were treated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min. The samples were collected after treatments. The whole tissue extracts were used to determine active caspase-3 and caspase-12 by Western blotting. WT= control mouse cerebellar slice cultures; H101Y= PKC $\gamma$  H101Y transgenic mouse slices in culture.