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Elevated Expression of the G-Protein-Activated Inwardly Rectifying Potassium Channel 2 (GIRK2) in Cerebellar Unipolar Brush Cells of a Down Syndrome Mouse Model[1]

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SUMMARY

1. Down syndrome (DS) arises from the presence of three copies of chromosome (Chr.) 21. Fine motor learning deficits found in DS from childhood to adulthood result from expression of extra genes on Chr. 21, however, it remains unclear which if any of these genes are the specific causes of the cognitive and motor dysfunction. DS cerebellum displays morphological abnormalities that likely contribute to the DS motor phenotype.

2. The G-protein-activated inwardly rectifying potassium channel subunit 2 (GIRK2) is expressed in cerebellum and can shunt dendritic conductance and attenuate postsynaptic potentials. We have used an interbreeding approach to cross a genetic mouse model of DS (Ts65Dn) with Girk2 knockout mice and examined its relative expression level by quantitative real-time RT-PCR, Western blotting and immunohistochemistry.

3. We report here for the first time that GIRK2 is expressed in unipolar brush cells, which are excitatory interneurons of the vestibulocerebellum and dorsal cochlear nucleus. Analysis of disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and heterozygous-Diploid/Girk2$^{(+/-)}$ mice shows that GIRK2 expression in Ts65Dn lobule X follows gene dosage. The lobule X of Ts65Dn mice contain greater numbers of unipolar brush cells co-expressing GIRK2 and calretinin than the control mouse groups.

[1] While Dr. Julius Axelrod's impact on the development of Neuroscience was significant, one of his major contributions was made indirectly through the people close to him that he influenced. Being a Section Chief and colleague to Julie in the Laboratory of Clinical Science at the National Institute of Mental Health was one of the great honors of my life. It was always a joy observing humility, friendliness and concern of all problems big or small. At laboratory seminars it was a pleasure to watch Julie's ideas and intuitions that often generated a tremendous amount of good science. He taught all of us how to be curious, incisive and imaginative, and above all to "keep it simple." His delight in science was contagious. DMJ

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4. These results demonstrate that gene triplication can impact specific cell types in the cerebellum. We hypothesize that GIRK2 overexpression will adversely affect cerebellar circuitry in Ts65Dn vestibulocerebellum and dorsal cochlear nucleus due to GIRK2 shunting properties and its effects on resting membrane potential.

KEY WORDS: unipolar brush cells; trisomy; cerebellum; Ts65Dn; dorsal cochlear nucleus; vestibulocerebellum; Girk2 knockout mouse; GIRK; Down syndrome; potassium channel; G-protein activated inwardly rectifying potassium channel.

INTRODUCTION

Individuals with Down Syndrome (DS), which is caused by the presence of three copies of chromosome (Chr.) 21, exhibit hypotonia and motor dysfunction related to postural control (Frith, 1974; Shumway-Cook and Woollacott, 1985; Latash and Corcos, 1991). DS children also exhibit a delay in fine motor skills that show little further development with age in spite of the increasingly normal gross motor function (Spano *et al*., 1999). The motor abnormalities in DS are primarily relegated to tasks controlled by the cerebellum, including the coordination of voluntary movement, gait, posture and speech.

A straightforward analysis of single-gene contributions to DS is difficult since many complex compensatory processes are involved to maintain homeostasis. However, genetic mouse models such as the trisomy Ts65Dn mouse provide invaluable tools to study the relationships between the various overexpressed genes in DS. Ts65Dn has triplication of a segment of mouse Chr. 16 that contains more than a hundred genes orthologous to those on human Chr. 21. The Ts65Dn mouse also exhibits fine motor dysfunctions including abnormalities in walking pattern and moderate to severe deficits in balance and motor coordination (Costa *et al*., 1999). Anatomically, the Ts65Dn mouse cerebellum has an abnormal reduction of both the internal granule and molecular layers in addition to a significant decrease in granule cell density (Baxter *et al*., 2000). Thus, the Ts65Dn mouse has anatomical and functional motor deficits reminiscent of the motor dysfunction seen in DS individuals.

GIRK2 is one of four subunits (GIRK1, GIRK2, GIRK3 and GIRK4) found in mammalian tissue (Lesage *et al*., 1994, 1995; Isomoto and Kurachi, 1996; Dascal, 1997; Isomoto *et al*., 1997). GIRK2 is highly expressed in the central nervous system of rodents, including the cerebellum, and plays an important role in cerebellar development (Karschin *et al*., 1996; Signorini *et al*., 1997). Weaver mice with a single amino acid mutation in the GIRK2 pore exhibit various motor disabilities including abnormal gait pattern and ataxia. In these mice, both the granular cells and the dopaminergic neurons in the cerebellum are severely affected (Harkins and Fox, 2002). We have recently reported overexpression of GIRK2 subunit in the hippocampus, frontal cortex and substantia nigra of the Ts65Dn mouse (Harashima *et al*., 2006) and have suggested that cognitive and memory impairments in Ts65Dn may be causally related to GIRK2 expression or function. We therefore sought to extend our studies to characterize GIRK2 expression in the cerebellum as it might produce cues related to deficits in Ts65Dn motor function.

Unipolar brush cells (UBCs) are unique interneurons with a brush-like dendrite structure that are found in the granule cell layer of the vestibular cerebellum and in the dorsal cochlear nucleus (DCN) (Floris *et al*., 1994; Abbott and Jacobowitz, 1995; Mugnaini *et al*., 1997). Calretinin (CR), an intracellular calcium-binding protein that exhibits brain region specificity (Winsky *et al*., 1989; Rogers, 1989; Arai *et al*., 1991), has become the established marker for UBCs. UBCs are highly concentrated in the flocculo-nodular lobe (lobule X), the ventral uvula, the ventral paraflocculus (PF), and lingula, whereas they are moderately concentrated in the vermis (Floris *et al*., 1994). UBCs usually have a single, relatively thick dendrite of varying length, which forms a brush tip; the axon is thin and can achieve lengths of 250 μm or more (Floris *et al*., 1994; Marini *et al*., 1997). UBCs act as feedforward excitatory interneurons, which receive excitatory input from extrinsic and intrinsic mossy fibers (MF) that originate from a subpopulation of first-order UBCs (Dino *et al*., 2000). The UBC-MF "giant synapse" is glutamatergic and contains all three major classes of the ionotropic glutamate receptors: AMPA (ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazole-propioninc acid), NMDA (*N*-methyl-*D*-aspartate) and kainate (KA) in the postsynaptic density (Mugnaini and Floris, 1994; Rossi *et al*., 1995; Marini *et al*., 1997; Kinney *et al*., 1997; Billups *et al*., 2002). The metabotropic receptors mGluR1 and mGluR2/3 are also localized to somatic and extrasynaptic sites of UBCs (Nunzi *et al*., 2002).

In this study, we investigated GIRK2 and CR co-expression using quantitative real-time PCR, Western blot and immunohistochemistry in the Ts65Dn mouse vestibular cerebellum and DCN. Moreover, to further investigate the impact of other genes from the Ts65Dn chromosomal fragment on the pattern of GIRK2 expression, we generated a new mouse model, the disomic-Ts65Dn/Girk2$^{(+/+/-)}$ mouse, that has a diploid number of Girk2 genes whilst possessing three copies of all other genes encoded on the Ts65Dn segment of Chr.16. The co-expression of GIRK2 with CR was compared among all investigated mouse groups.

METHODS

Mice

Ts65Dn and control Diploid littermates were bred to have the mixed genetic background C57BL/6JEi × C3H/HeSnJ as used in our previous studies (Siarey *et al*., 1997, 1999; Harashima *et al*., 2006). The Girk2 null mouse was generated as previously reported (Signorini *et al*., 1997). The disomic-Ts65Dn/Girk2$^{(+/+/-)}$ mouse that is diploid for the Girk2 gene and trisomy for the rest of the Ts65Dn segment of Chr.16 was generated as follows. Males heterozygous for the Girk2 gene (heterozygous-Diploid/Girk2$^{(+/-)}$) were bred with Ts65Dn females to yield the following mice: homozygous-Diploid/Girk2$^{(+/+)}$ = Diploid (like wild-type); heterozygous-Diploid/Girk2$^{(+/-)}$; disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Ts65Dn. The mice used in this research were genotyped by fluorescence *in situ* hybridization and PCR screening using tail DNA as described before (Wickman *et al*., 1998; Strovel *et al*., 1999).

Immunohistochemistry

Mice 85 ± 7 days (Ts65Dn = 5, Diploid = 5; disomic-Ts65Dn/Girk2$^{(+/+/-)}$ = 3; heterozygous-Diploid/Girk2$^{(+/-)}$ = 3) were anesthetized with pentobarbital (50 mg/kg) and perfused intracardially with 10% formalin (Fisher, Pittsburgh,PA) in PBS (pH 7.4). Cerebella were removed and placed in formalin for 30 min and followed by dehydration in 20% sucrose in PBS at 4°C for 2 days, then frozen. Cerebella stored at -80°C were cut into 20 μm sections on a cryostat, thaw-mounted onto poly-L-lysine coated slides (Sigma, St. Louis, MO) and stored at -80°C until immunohistochemical analysis. GIRK2 polyclonal antibody (epitope corresponds to residues 374–414 of mouse GIRK2) (Chemicon, Temecula, CA) and monoclonal antibodies against CR (Chemicon, Temecula, CA) were used at 1:1000 dilution. FITC-conjugated AffiniPure Goat Anti-rabbit IgG (H + L) or Texas Red® dye-conjugated AffiniPure Goat Anti-mouse IgG (H + L) (Jackson Laboratory, PA) were used at 1:100 dilution, as secondary antibodies. The sections were incubated for 48 h with primary antibodies in a blocking solution of PBS (pH 7.4) containing 0.3% TritonX-100 and 1% goat serum. After washing in PBS (pH 7.4) containing 0.2% TritonX-100 (20 min), the sections were incubated for 30 min at room temperature with secondary antibodies in 0.3% TritonX-100. After washing in PBS (pH 7.4) containing 0.2% TritonX-100 and then PBS (pH 7.4) for 5 min, sections were mounted with mounting medium containing p-phenylenediamine, PBS and glycerol (pH 8.0 by carbonate-bicarbonate buffer). Specificity of antibody was confirmed by absorption test and by Western blot with cortical tissue derived from Diploid and Girk2$^{(-/-)}$ mice. No significant staining was detected when the primary antibodies were incubated with the antigenic peptide or on Girk2$^{(-/-)}$ tissue.

Images were collected using a Leica (Bannockburn, IL) DM RXA microscope and a scion corporation (Frederick, MD) FW-1310M camera. Confocal images were acquired using a Zeiss LSM 510 confocal microscope system. Care was taken to ensure that all excitation and detection parameters were equivalent for each subject.

For co-expression analysis of GIRK2 and CR in lobule X of the cerebellum, the numbers of GIRK2 positive, CR positive, or GIRK2 and CR positive cells were counted within a whole lobule X (three sections per each mouse). Data are presented as percentage of total counts (mean ± SEM).

Differences between groups were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons (Tukey's test). A *P*-value less than 0.05 was considered to be significant.

Quantitative Real-Time RT-PCR

Mice 81 ± 5 days of age (Ts65Dn = 6 and Diploid = 6) were sacrificed by decapitation under anesthesia. Cerebella were quickly removed and dissected free. Tissues were quickly frozen on dry ice, and kept at -80°C until RNA isolation. The cerebellar sections including vestibulocerebellum were selected and punched using a 500 μm canula in the lobule X under a microscope. Total RNA was

isolated from a 15 mg block of cerebellum or material obtained from Lobule X micro-punches using Rneasy Mini Kit (Qiagen Inc., Valencia, CA, USA) with DNase I (Qiagen, Inc., Valencia, CA). For reverse transcription, 0.5 μg of total RNA was used to synthesize cDNA by TagMan RT-Reagents (Applied Biosystem, Foster City, CA). The cDNA was stored at -20°C until subsequent analysis. GeneAmp 5700 sequencing detection system (Perkin-Elmer) and SYBR-Green quantitative PCR kit from PE Biosystems (Foster City, CA) were used for Quantitative Real-time RT-PCR. For the PCR step, reaction volumes of 25 μL contained 10 ng of cDNA, 1 X SYBR Green I buffer, 200 μM of each dATP, dCTP, dGTP and 400 μM dUT, and 0.05 U/μl AmpliTag Gold, 0.01 U/μl AmpEraseUNG (uracil-N-glycosylase), 5.5 mM $MgCl_2$ and 200 mM of each primer. The primers were designed based on the published sequences of GIRK2 and ribosomal RNA using the Primer Express 1.0 Software program (Perkin-Elmer, Wellesley, MA) and synthesized by the Biomedical Instrumentation Center at Uniformed Services University for the Health Sciences. The primers sequences were; GIRK2: F^{664}5′GATGGGAAGTGCAACGTTCA3′,R^{728}5′AAGATGTCCGTCAGGTAT CGGT3′(NM_010606) and ribosomal RNA: Ribosomal RNA:F5′CGGCTA CCACATCCAAGGAA3′,R5′GCTGGAATTACCGCGGCT3′. The PCR reaction was followed by the protocol from the company. As a negative control, PCR reactions without template cDNA were added to reaction wells. All samples were run in triplicate. The absolute copy numbers of GIRK2 and Ribosomal RNA, used as an internal control, were generated by the standard curve of cDNA synthesized from AtT-20 cells (mouse pituitary cell line) (ATCC, Manassas, VA). Relative quantitation was performed using each absolute copy numbers as described by (Harashima *et al*., 2006).

Western Blotting

Mice 2–3 months old (Ts65Dn = 6 and Diploid = 6) were sacrificed by decapitation under anesthesia with CO_2. Cerebella were quickly removed and frozen on dry ice, and kept at -80°C until protein isolation. Alternatively, cerebella were quickly removed, frozen and then cut into 300 μm sections on a cryostat and mounted onto slides which were stored at -80°C until processing. The cerebellum sections including vestibulocerebellum were selected and punched using a 500 μm canula in the lobule X under a microscope. Those punches or homogenates from whole cerebellum were quickly placed in RIPA buffer containing: 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris-Cl (pH 8.0) 1:4 (v/v), homogenized, sonicated and allowed to lyse for 20 min and then frozen. The homogenate was centrifuged at 18,000 g for 5 min and protein concentration in the supernatant was determined. Subsequently samples were prepared according to the Novex NuPage Bis Tris/MOPS protocol. Protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL).

The sample extracts were mixed with NuPage™ LDS (Invitrogen, Carlsbad, CA) sample running buffer at 1:4 (v/v) of sample volume and reducing agent at 1:10 (v/v) of sample volume and heated to 70°C for 10 min. Protein aliquots (5, 10 and

15 μg) were loaded onto precast NuPage Tris-Bis 10% gel and electroblotted onto Immobilon-P blotting membrane. After blocking the membranes for 1 h in 5% non-fat dry milk in PBS with 0.1% Tween 20, the blots were incubated with anti-GIRK2 antibody (1:200) (Alomone Inc., Jerusalem, Israel) at room temperature for 2 h or at 4°C overnight. Membranes were washed in PBS with 0.01% Tween 20 for 5 min and buffer changed 3 times. The membranes were then incubated in goat anti-rabbit IgG HRP for at least 45 min in PBS/Milk, washed and signal detected using Super-Signal West Pico Chemilunminescent kit (Pierce Biotechnology, Rockford, IL) and quantified. In order to verify equal loading, the blot was stripped and incubated with β-actin antibody (Sigma, St. Louis, MO). The relative protein levels were calculated as a ratio of optical density of the band corresponding to β-actin (whole cerebellum) or tubulin (lobule X).

RESULTS

Two copies of the Girk2 gene are localized to mouse Chr. 16 and one extra copy is localized to the Ts65Dn Chr., therefore GIRK2 is likely overexpressed in DS and Ts65Dn brain. In fact, we reported that GIRK2 is overexpressed in Ts65Dn cortex and hippocampus in comparison to Diploid littermates (Harashima *et al.*, 2006). In this study we investigated the GIRK2 level of expression in the cerebellum. The abnormal expression of other triplicated genes could affect the expression pattern of GIRK2 in Ts65Dn brain through complex genetic interaction. To test this possibility and to investigate a specific role of GIRK2 subunit in Ts65Dn brain we created a mouse with the Girk2 gene "subtracted" from the trisomic background (disomic-Ts65Dn/Girk2$^{(+/+/-)}$).

We verified the expected gene dosage overexpression of GIRK2 mRNA initially in whole cerebellum and then subsequently in lobule X of Ts65Dn and our newly created mouse. Lobule X of vestibulocerebellum was selected since immunohistochemical analysis show strong GIRK2 immunopositive signal in UBCs located in this area.

Using real-time RT-PCR, the level of GIRK2 mRNA in the whole cerebellum was analyzed and a significant increase was found ($\sim$30%; Fig. 1(A)). To see the impact of the gene dosage effect on GIRK2 expression in lobule X of Ts65Dn, disomic-Ts65Dn/Girk2$^{(+/+/-)}$, heterozygous-Diploid/Girk2$^{(+/-)}$ and Diploid mice, real-time RT-PCR study was carried out on tissues obtained from micro-punches. Lobule X of the vestibulocerebellum in the Ts65Dn mouse showed significant overexpression of GIRK2 mRNA ($\sim$45% more) in comparison to other littermates. In four different mouse types, GIRK2 mRNA levels correlated with the number of Girk2 alleles (Fig. 1(B)) and all detected changes were significant. GIRK2 protein levels were also measured by Western blot with an $\sim$60% and 50% increase found in whole cerebellum and Lobule X, respectively (Fig. 2). Quantitative evaluation of the level of expression of GIRK2 in cerebellum or even in the lobule X with various cell types by real-time RT-PCR and Western blots have been complemented with fluorescent immunohistochemistry.

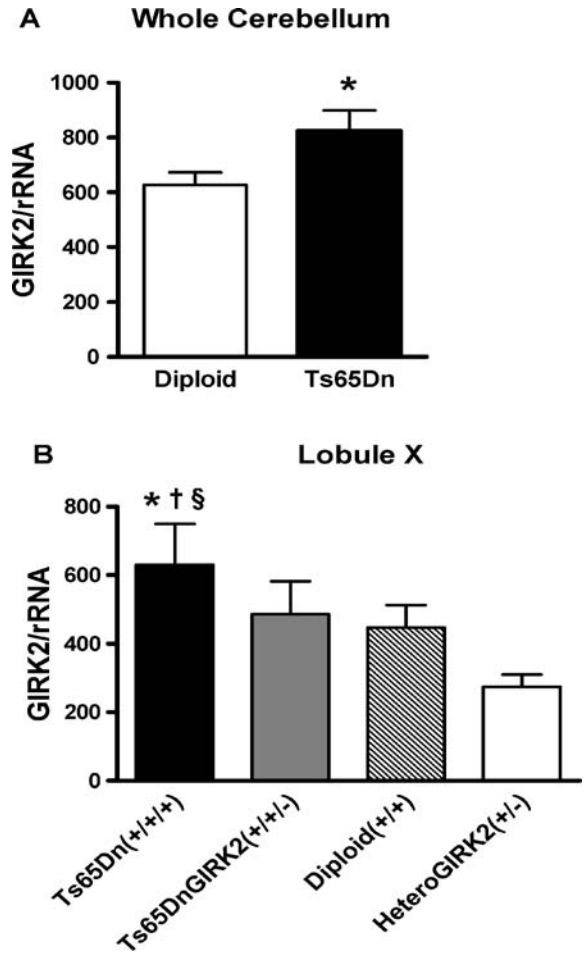

Fig. 1. A. Real-time RT-PCR analysis of GIRK2 mRNA from whole cerebellum of Ts65Dn mice show significant increase in GIRK2 mRNA level in comparison to diploid level ($p<0.05$; Ts65Dn = 5, Diploid = 5). B. Real-time RT-PCR analysis of GIRK2 mRNA from punch-out sections of lobule X of Ts65Dn mice ($n=3$) show significant increase in GIRK2 mRNA level when compared to disomic-Ts65Dn/Girk2$^{(+/+/-)}$ ($n=3$; $^{*}P < 0.05$), Diploid ($n=3$; $^{\dagger}P < 0.05$), and heterozygous-Diploid/Girk2$^{(+/-)}$ ($n=3$; $^{\S}P < 0.05$) level.

Fluorescence immunohistochemistry demonstrated an interesting GIRK2 expression pattern in the lobule X of vestibulocerebellum with a strong GIRK2 immunopositive signal detected in the UBCs. We found that UBCs in granule cell layers of the Ts65Dn lobule X showed stronger GIRK2 immunofluorescence signal than detected in Diploid controls (Fig. 3(A)). In addition, the GIRK2 immunofluorescence signal of UBCs was stronger than that found in granule

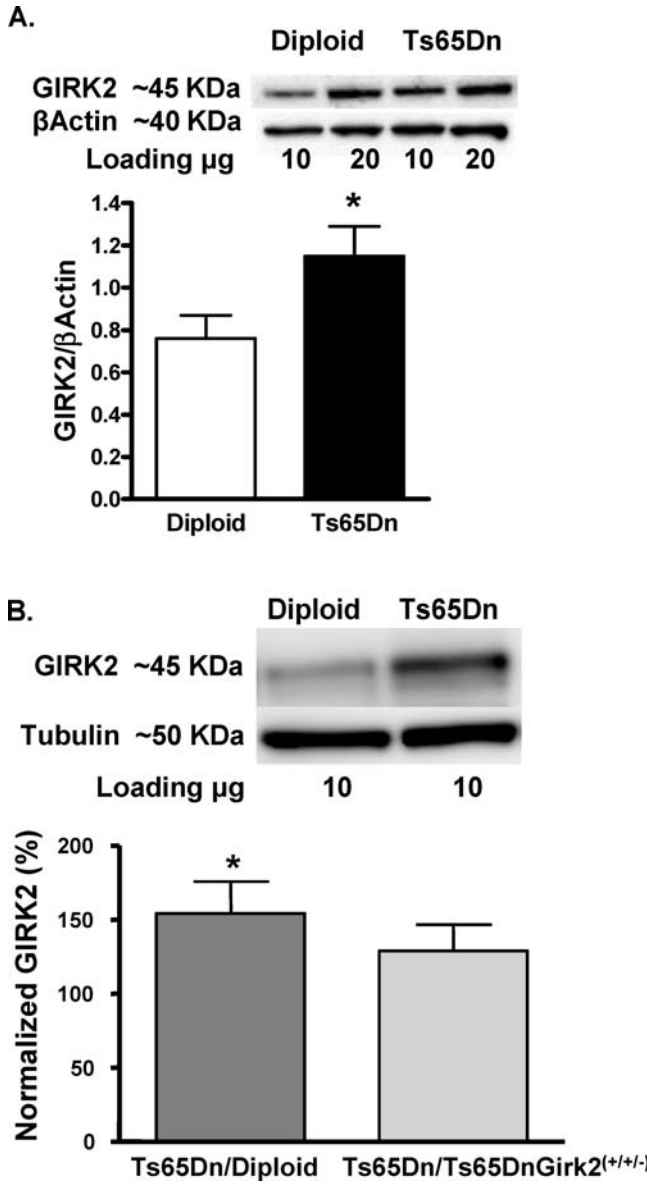

Fig. 2. (A) Western blot analysis indicates that the GIRK2 protein is significantly overexpressed in Ts65Dn whole cerebellum when compared to diploid littermates ($n = 5$; $^*p < 0.05$). (B) GIRK2 normalized (Ts65Dn/Diploid) ratios from punch-outs from Ts65Dn lobule X indicate marginally significant overexpression compared to Diploid littermates ($n = 4$ pair). The GIRK2 protein levels in lobule X of disomic-Ts65Dn/Girk2$^{(+/+/-)}$ mice were decreased ($n = 2$ pairs, significance has not been tested).

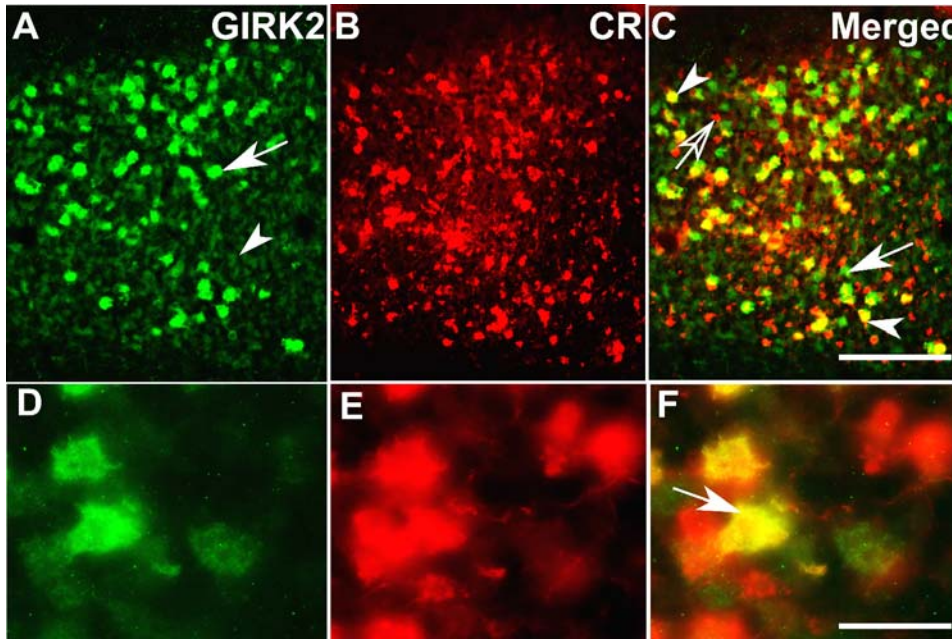

Fig. 3. Co-expression of GIRK2 (A) and CR (B) in UBCs of Ts65Dn lobule X. The GIRK2 immunofluorescence signal of UBCs (arrows) was stronger than that of granule cells (arrow heads) (A). Double immunofluorescence staining for GIRK2 and CR (C) showed only GIRK2 positive UBCs (arrow), only CR positive UBCs (open arrow) and UBCs co-expressed GIRK2 and CR (arrowheads). High magnification image (D)–(F) show GIRK2 expression in soma (arrow in F) and co-expression with CR. Scale bars = 75 μm (C), 20 μm (F).

cells. UBCs were identified morphologically using the CR antibody as previously reported (Floris *et al.*, 1994; Abbott and Jacobowitz, 1995) (Fig. 3(B)). Double immunofluorescence staining for GIRK2 and CR revealed that UBCs are immunopositive for both GIRK2 and CR (Fig. 3(C)). Higher magnification images of GIRK2 and CR staining patterns of UBCs in lobule X of Ts65Dn are depicted in Fig. 3(D)–(F).

To investigate if GIRK2 high expression levels and co-expression patterns in Ts65Dn UBCs are caused by a gene dosage effect, we evaluated its expression pattern in sibling littermates of Diploid; heterozygous-Diploid/Girk2$^{(+/-)}$; disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Ts65Dn mice. Lobule X from Ts65Dn mice showed an increased number of GIRK2 and CR positive UBCs ($\sim$2-fold increase) and stronger immunofluorescence of GIRK2 than other littermates (Fig. 4(A), (D), (G) and (J), Table I). Disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Diploid littermates had a similar GIRK2 subunit expression pattern (Fig. 4(D) and (G)). Furthermore, heterozygous-Diploid/Girk2$^{(+/-)}$ animals showed a very weak signal of GIRK2 due to the presence of only one allele of the Girk2 subunit (Fig. 4(J)). A greater number of cells that co-express GIRK2 and CR were detected in Ts65Dn compared to controls (Fig. 4(C), (F), (I) and (L) Table I). Not all UBCs show GIRK2 signal. About 1/3 of Ts65Dn UBCs show co-expression of GIRK2 and CR. However 1/5 or less of

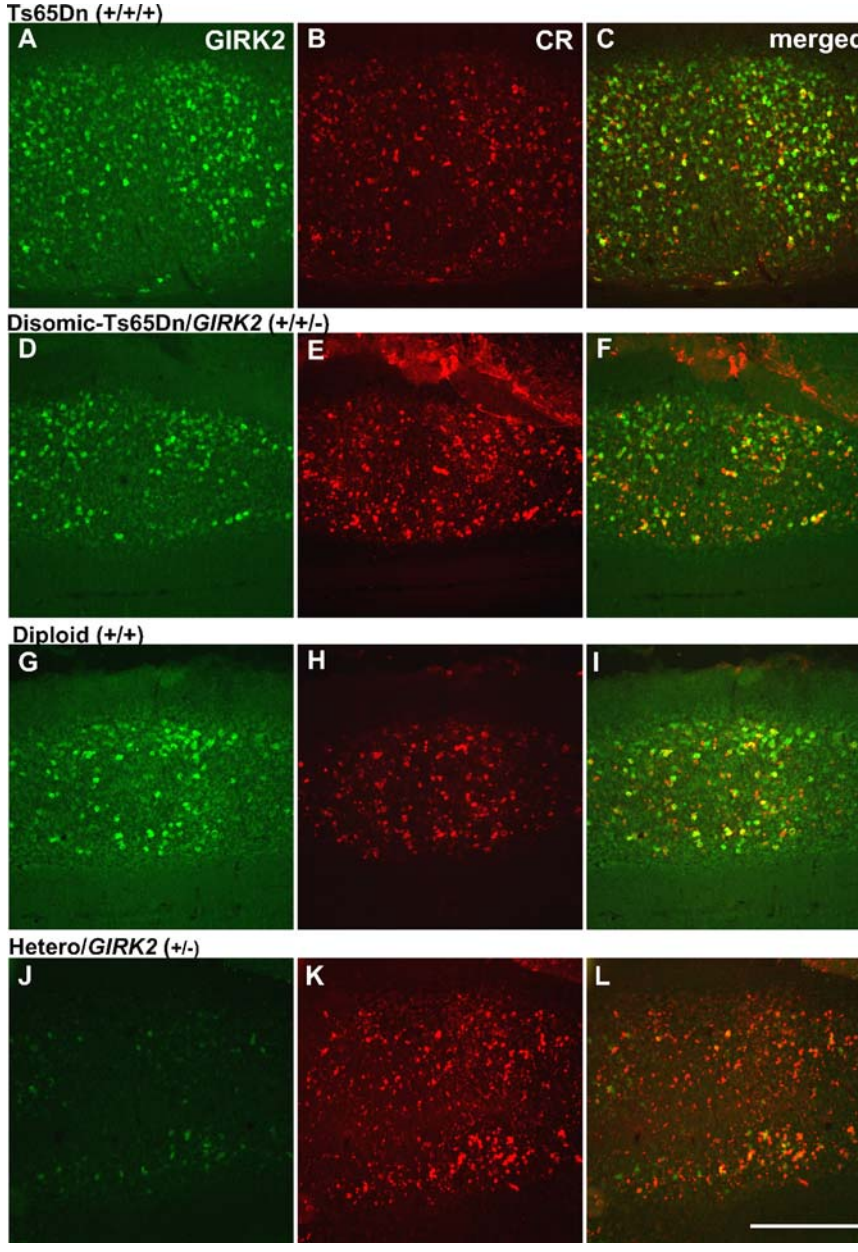

Fig. 4. GIRK2 and CR co-expression in Ts65Dn (A)–(C), disomic-Ts65Dn/Girk2$^{(+/+/-)}$ (D)–(F), Diploid (G)–(I) and heterozygous-Diploid/Girk2$^{(+/-)}$ (J)–(L). Ts65Dn lobule X showed an increased number of GIRK2 positive UBCs compared to other littermates (A), (D), (G) and (J). Disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Diploid littermates had a similar GIRK2 subunit pattern (D) and (G). Double immunofluorescence staining study for GIRK2 and CR confirmed that a greater number of UBCs were co-expressing GIRK2 and CR as shown in Ts65Dn lobule X compared to the other mice groups (C), (F), (I) and (L). Scale bar = 150 μm.

Table I. Number of Unipolar Brush Cells Showing GIRK2 and/or Calretinin in LobuleX Areas of Ts65Dn, Disomic-Ts65Dn/Girk2 $^{(+/+/-)}$, Homozygous-Diploid/Girk2 $^{(+/+)}$ and Heterozygous-Diploid/Girk2 $^{(+/-)}$ Mice

	Number of cells	Only GIRK2 positive cells (%)	Only CR positive cells (%)	GIRK2 & CR positive cells (%)
Ts65Dn	184 ± 8	38 ± 1	28 ± 3#	$34 \pm 2^{\#}$
Disomic-Ts65Dn/GIRK2$^{((+/+/-)}$	102 ± 2	39 ± 1	46 ± 2	15 ± 2
Diploid/GIRK2$^{(+/+)}$	125 ± 10	42 ± 2	41 ± 3	17 ± 3
Heterozygous-Diploid/GIRK2$(^{(+/-)})$	165 ± 19	5 ± 1	$94 \pm 12^{\#\#}$	1 ± 0

Note. Cells immunopositive to: only GIRK2 are displayed in green (FITC), only CR are displayed in red (Texas Red) and both GIRK2 and CR yellow/orange (FITC + Texas Red). Values are percentage of cells from the total number counted

$^{\#}P < 0.001$, statistically different from GIRK2 diploid mice groups

$^{\#\#}P < 0.001$, statistically different from other three mice groups as to CR positive cells.

UBCs in disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Diploid mice were immunopositive for both GIRK2 and CR. In heterozygous-Diploid/Girk2$^{(+/-)}$ mice almost all cells are immunopositive only for CR.

Higher magnification images obtained by confocal microscopy revealed more detailed localization of GIRK2 and CR. GIRK2 is mostly expressed on the UBC somatic membrane and on the distal dendritic brush of UBCs, whereas CR expression pattern is consistent with homogenous expression of CR in the somatic region and in the dendrite (Fig. 5).

In the DCN and flocculus (FL) of Ts65Dn, cell bodies of UBCs had GIRK2 and/or CR immunofluorescence signals (Fig. 6(A)–(C)). The granule cell layers in the PF also contained UBCs co-expressing GIRK2 and CR at a similar level as in lobule X (Fig. 6(D)–(F)). Overall intensity of GIRK2 immunofluorescence signals in these areas were similar to intensity of signal detected in lobule X but with fewer cells showing co-expression.

While the physiological and behavioral impact of GIRK2 overexpression in Ts65Dn UBCs remains to be determined in future physiological experiments, we

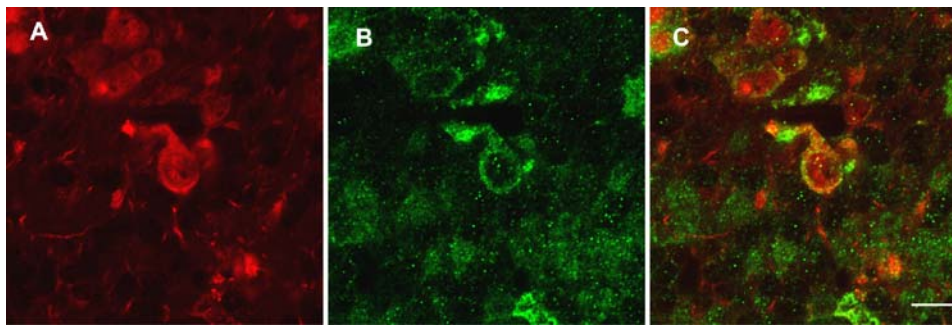

Fig. 5. Double immunofluorescence staining shows coexpression of CR (A) and GIRK2 (B), overlay in (C), in Ts65Dn UBCs. CR is expressed in the dendrite and in the soma whereas GIRK2 expression is localized to the brush, the distal part of the dendrite, and the cell soma. Scale bar = 10μm.

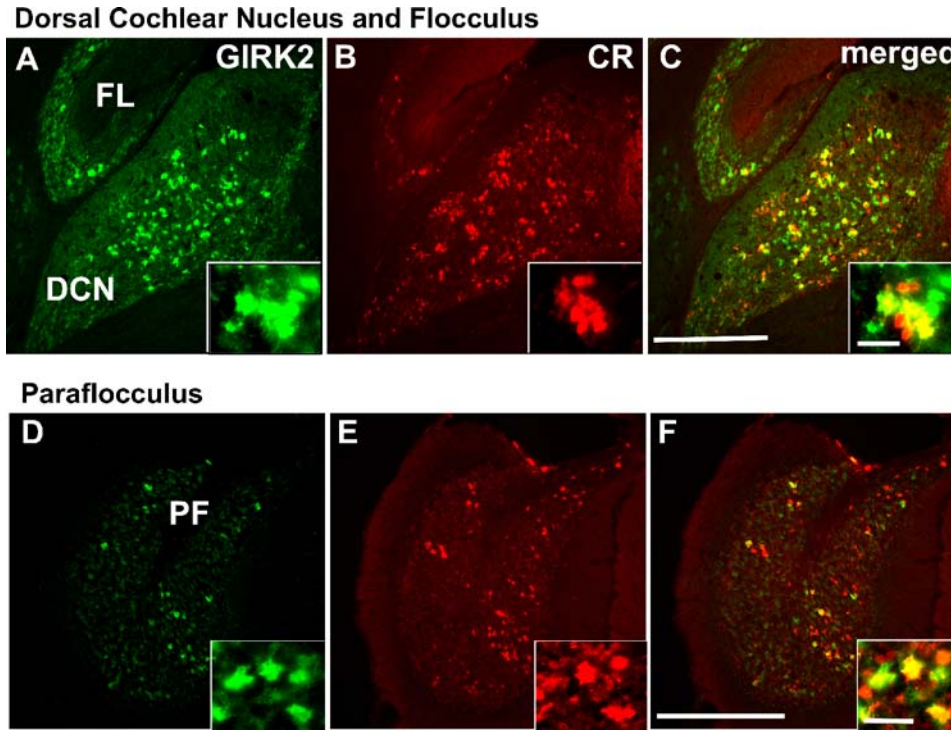

Fig. 6. GIRK2 expression in Ts65Dn DCN and FL (A)–(C) and Ts65Dn PF (D)–(F). The cell bodies of UBCs had GIRK2 and/or CR immunofluorescence levels similar to that seen in lobule X. Scale bars = 100 μm (C), 150 μm (D) and 20 μm (higher magnification inserts (A)–(F).

can speculate that its role would be related to control of synaptic and extrasynaptic shunting, and of control of membrane potential in UBCs. This constitutive GIRK2 activity could be tonically modulated by released neurotransmitters acting via G-protein coupled receptors.

DISCUSSION

Various neuroimaging techniques revealed morphological abnormalities in DS brains including a reduction in the size of the posterior fossa and a growth delay of the cerebellar field prenatally (Ieshima *et al.*, 1984; Lomholt *et al.*, 2003). Furthermore, DS children and adults were shown to have a smaller brain volume, a disproportionally smaller cerebellum and a reduced volume of the temporal lobe (Aylward *et al.*, 1997, 1999; Frangou *et al.*, 1997; Pinter *et al.*, 2001). Similar approach applied to mice have detected abnormalities in the Ts65Dn mouse cerebellum that are comparable to those found in DS individuals (Baxter *et al.*, 2000). Behavioral data obtained from the Ts65Dn mouse using the rotorod test, which assesses sensorimotor coordination, also revealed cerebellar dysfunction

phenotypes in these mice (Costa *et al*., 1999), although no significant motor deficit have been detected using accelerating rotorod and footprint analysis (Baxter *et al*., 2000). The cerebellum is thought to play an important role in the cognition and motor coordination (Daum and Ackermann, 1995; Dolan, 1998; Schmahmann and Sherman, 1998). Taken together, the dysfunction in motor coordination in the Ts65Dn mouse and the important role of the cerebellum suggest that increase in cerebellar expression of proteins encoded by genes from Ts65Dn Chr. could contribute to the DS cerebellar phenotype. However, the extra Ts65Dn mouse Chr. includes $\sim$120 genes of the $\sim$240 known and predicted genes on human Chr. 21 (Gitton *et al*., 2002). The Saran *et al.* (2003) examination of the cerebellar transcriptome in Ts65Dn mice suggests that the expression of many genes that are not present on mouse Chr. 16 is also perturbed. Thus, triplication of specific genes may have far-reaching and unexpected consequences on the global gene expression. Our development of the disomic-Ts65Dn/Girk2$^{(+/+/-)}$ mouse, which has an entire extra Ts65Dn Chr. and the diploid number of Girk2 genes, provides an invaluable tool to study how the elevated expression of GIRK2 affects neurological functions in Ts65Dn mice.

In this study for the first time we find that the GIRK2 is expressed in mouse UBCs localized in lobule IX and X, DCN, FL, and PF. Moreover, Ts65Dn UBCs showed a gene dosage increase in the GIRK2-specific immunofluorescent signal. A higher number of UBCs co-expressed CR and GIRK2 in Ts65Dn compared to Diploid littermates. UBCs are uniquely shaped excitatory interneurons expressing various nervous system specific receptors and proteins including GluR2, heavy neurofilament protein (NF-H), secretogranin II, chromatogranin A and mGluRs which distinguish them from cerebellar granule and Golgi cells (Braak and Braak, 1993; Jaarsma *et al*., 1998; Abbott and Jacobowitz, 1995; Takacs *et al*., 2000; Nunzi and Mugnaini, 2000; Nunzi *et al*., 2002). UBCs form glomeruli with terminals of MF and granule cells in the cerebellar cortex and therefore regulate the input of granule cells to Purkinje cells (Kalinichenko and Okhotin, 2005). We hypothesize that an overexpression of the GIRK2 subunit in Ts65Dn will likely lead to hyperpolarization of the membrane; moreover, since GIRK2 can function as an effector of mGluRs activity, higher levels of GIRK2 could play a significant electrophysiological and biochemical role in the excitatory circuitry as a positive feedback element in the cerebellar cortex.

The DCN, which has a laminar cerebellum-like organization, relays auditory information into inferior colliculus and has been associated with tinnitus occurrence (Levine, 1999; Oertel and Young, 2004). The pathways that go through the DCN are thought to detect sound wave spectral cues and combine them with somatosensory, vestibular and higher-level auditory information through parallel fiber inputs into an auditory-circuit (Kanold and Young, 2001; Oertel and Young, 2004). The DCN contains granule cells in the fusiform cell layer which receive excitatory input from UBCs also conveying multiple sensory information from dorsal column and pontine nuclei, as well as vestibular and auditory afferents (Oertel and Young, 2004). Our results revealed strong GIRK2 and CR-positive immunofluorescent signal in UBCs in Ts65Dn DCN suggesting a physiological role of GIRK2 in these neurons. The

consequences of this would lead to abnormal shunting properties of UBCs and further influence processing of vestibular afferents. Taken together, abnormal overexpression of GIRK2 in DCN could play an important role in the auditory circuitry which could then contribute to hearing impairments of conductive and/or sensorineural origin frequently occurring in DS subjects (Balkany *et al*., 1979; Mazzoni *et al*., 1994; Hassmann *et al*., 1998).

More comprehensive studies will be required to identify a specific role of GIRK2 expressed in UBCs in cerebellar circuitry and in cerebellum of DS in particular but we hope that this finding will stimulate further work on the role GIRK2 overexpression in DS cerebellar phenotype.

In this initial report no behavorial studies have been accomplished, which limits the final conclusion, however this is the first time that neuronal specific immunohistochemical DS-relevant phenotype (increase number of GIRK2 and CR co-expressing UBCs) has been reversed through a breeding scheme. Interestingly, a recently published report shows that systemic treatment of newborn Ts65Dn mice with an agonist of the Hedgehog pathway increases mitosis and restores cerebellar granule cell precursor populations (Roper *et al.*, 2006). There is no known coupling between the hedgehog pathway and GIRK channel function at present.

In conclusion, we found that GIRK2 is expressed in UBCs and its levels are elevated in the UBCs of Ts65Dn mouse in comparison to disomic-Ts65Dn/Girk2$^{(+/+/-)}$ and Diploid littermates. The increased expression levels of the GIRK2 subunit may contribute to the motor and sensorimotor cognitive sequelae seen in the Ts65Dn mouse and DS individuals by impacting physiological properties of cerebellar and auditory neuronal circuits.

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