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Abnormal Expression of the GIRK2 Potassium Channel in Hippocampus, Frontal Cortex and Substantia Nigra of Ts65Dn Mouse: A Model of Down Syndrome

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Abstract

Ts65Dn, a mouse model of Down syndrome (DS), demonstrates abnormal hippocampal synaptic plasticity and behavioral abnormalities related to spatial learning and memory. The molecular mechanisms leading to these impairments have not been identified. In this study, we focused on the G-protein-activated inwardly rectifying potassium channel 2 (GIRK2) gene that is highly expressed in the hippocampus region. We studied the expression pattern of GIRK subunits in Ts65Dn and found that GIRK2 was over-expressed in all analyzed Ts65Dn brain regions. Interestingly elevated levels of GIRK2 protein in the Ts65Dn hippocampus and frontal cortex correlated with elevated levels of GIRK1 protein. This suggests that heteromeric GIRK1-GIRK2 channels are over-expressed in Ts65Dn hippocampus and frontal cortex, which could impair excitatory input, modulate spike frequency and synaptic kinetics in the affected regions. All GIRK2 splicing isoforms examined were expressed at higher levels in the Ts65Dn in comparison to the diploid hippocampus. The pattern of GIRK2 expression in the Ts65Dn mouse brain revealed by *in situ* hybridization and immunohistochemistry was similar to that previously reported in the rodent brain. However, in the Ts65Dn mouse a strong immunofluorescent staining of GIRK2 was detected in the lacunosum molecular layer of the CA3 area of the hippocampus. In addition, tyrosine hydroxylase containing dopaminergic neurons that co-express GIRK2 were more numerous in the substantia nigra compacta and ventral tegmental area in the Ts65Dn compared to diploid controls. In summary, the regional localization and the increased brain levels coupled with known function of the GIRK channel may suggest an important contribution of GIRK2 containing channels to Ts65Dn and thus to DS neurophysiological phenotypes.

Keywords

Down syndrome; mental retardation; immunohistochemistry; *in situ* hybridization; dopaminergic neurons; GIRK1

Introduction

Down syndrome (DS) is a major cause of mental retardation that arises from the presence of three copies of chromosome (Chr.) 21. A gene encoding the G-protein-activated inwardly rectifying potassium channel subunit 2 (GIRK2) has been found within the so-called DS critical region (DSCR) of Chr. 21 (Korenberg et al., 1994; Dahmane et al., 1998; Toyoda et al., 2002). It is the only known channel-coding gene from genetically defined DSCR that is highly expressed in the central nervous system (CNS). Recently, the concept of the DSCR has been challenged with respect to at least DS craniofacial dysmorphology (Olson et al., 2004). The development of the surviving segmental trisomy mouse models Ts65Dn, Ts1Cje and most recently Ts1Rhr has provided windows into the effects of abnormal Chr. number and over-expression of multiple genes neuronal functions during development and adulthood (Davisson et al., 1990; Reeves et al., 1995; Sago et al., 1998; Olson et al., 2004). Each of these mouse models contains decreasingly shorter trisomic segments of mouse Chr. 16 (Figure 1), which can be associated with various aspects of the DS neurological phenotype. However, it can be argued that the Ts65Dn mouse demonstrates the strongest phenotype match.

The Ts65Dn mouse contains the longest triplicated segment of murine Chr.16. In this mouse, phenotypic abnormalities include postnatal developmental delay, muscular trembling, male sterility and specific skeletal malformations corresponding directly to the craniofacial dysmorphogenesis in DS (Reeves et al., 1995; Holtzman et al., 1996; Richtsmeier et al., 2002). Abnormal cholinergic function and degeneration of cholinergic basal forebrain neurons have been reported in the Ts65Dn mouse (Holtzman et al., 1996; Granholm et al., 2000). These characteristics are also found in DS individuals and are thought to be initial signs of Alzheimer disease pathology (Schneider, 1998). The Ts65Dn mouse is considered a model of Alzheimer disease and/or accelerated aging. In spite of elevated levels of amyloid in Ts65Dn mouse brain, pathological hallmarks like plaques and tangles have not been found (Hunter et al., 2003b; Hunter et al., 2004; Cooper et al., 2001). The Ts65Dn mouse demonstrates learning and memory behavioral deficits in spatial tasks, abnormal nociception and motor dysfunction (Reeves et al., 1995; Escorihuela et al., 1995; Coussons-Read and Crnic, 1996; Holtzman et al., 1996; Demas et al., 1996; Martinez-Cue et al., 1999; Costa et al., 1999; Hyde et al., 2001; Hunter et al., 2003a; Bimonte-Nelson et al., 2003). For more critical reviews of these and other behavioral studies and their relevance to DS neurological phenotypes see (Pennington et al., 2003; Nadel, 2003; Dierssen, 2003; Galdzicki and Siarey, 2003).

Deficits in learning and memory spatial tasks suggest dysfunction of the hippocampus. In fact, decreased cAMP production was reported in the hippocampus of Ts65Dn animals (Dierssen et al., 1996). In the young adult (3 mo. old) Ts65Dn mice cholinergic, catecholaminergic, and serotonergic systems that project to the hippocampus are not found to be dramatically affected in the Ts65Dn brain (Megias et al., 1997) however as we mentioned earlier age-dependent cholinergic impairment have been reported by (Holtzman et al., 1996; Granholm et al., 2000). Alterations in neuronal number has been reported in the dentate gyrus (DG) (decrease) and CA3 area (increase), whereas hippocampal volumes of the Ts65Dn mice are normal (Kurt et al., 2000). In agreement with behavioral hippocampal deficit abnormal hippocampal synaptic plasticity, including reduced long-term potentiation (LTP) and increased long-term depression (LTD), has been demonstrated in the CA1 area and DG of the Ts65Dn mouse hippocampus (Siarey et al., 1997b; Siarey et al., 1999; Kleschevnikov et al., 2004) and more recently in the CA1 area of the Ts1Cje mouse (Siarey et al., 2005). Dendritic abnormalities also have been found in the Ts65Dn mouse (Belichenko et al., 2004) and in DS brains (Marin-Padilla, 1972), further suggesting abnormal synaptic function. The presence of learning deficits and abnormal synaptic

plasticity in both Ts65Dn and Ts1Cje mice, although less dramatic in Ts1Cje, suggest that overlapping gene sets are sufficient to cause impairments of neuronal networks in these mice. The Ts1Cje mouse possesses a smaller extra segment of mouse Chr. 16 than the Ts65Dn mouse (Sago et al., 1998) yet; the GIRK2 gene is located within the extra Chrs. of Ts65Dn, Ts1Cje and Ts1Rhr mice (Figure 1). The reported pattern of expression of GIRK2 (Karschin et al., 1996; Chen et al., 1997) and physiological functions of channels containing GIRK2 suggest that it can significantly contribute to a variety of CNS deficits in DS (for review (Nadel, 2003)). Moreover, over-expression of these channels can alter homeostasis of neuronal networks and impact dendritic excitability (e.g. through hyperpolarization and shunting mechanisms) and contribute to the impaired learning disability and synaptic plasticity seen in Ts65Dn and Ts1Cje mice. It is unlikely that over-expression of a single gene like GIRK2 can explain all of the abnormal functions of DS neuronal networks. However, quantitative assessment of its pattern of expression may suggest its potential role in neurophysiological and behavioral impairments.

GIRK containing channels belong to a superfamily of the inwardly rectifying K⁺ channels (Kir) that are predominantly expressed in CNS neurons, atrial myocytes and neuroendocrine cells (Yamada et al., 1998). GIRK channels are activated by various neurotransmitters, hormones and other neuropeptides through a number of G protein-coupled receptors (GPCRs) (Wickman and Clapham, 1995; Yamada et al., 1998). Four GIRK subunits (GIRK1, GIRK2, GIRK3 and GIRK4) have been identified in mammals (Dascal, 1997; Lesage et al., 1994; Lesage et al., 1995; Isomoto and Kurachi, 1996; Isomoto et al., 1997). GIRK1–3 but not GIRK4 are expressed at high levels in many overlapping CNS regions (Karschin et al., 1996; Karschin et al., 1994; Kobayashi et al., 1995; Ponce et al., 1996; Inanobe et al., 1999; Morishige et al., 1996; Drake et al., 1997; Signorini et al., 1997). GIRK4, although predominantly expressed in heart, is also expressed in several regions of the brain. However, the details of its CNS expression remain controversial (Wickman et al., 2000). GIRK subunits form homo- or hetero-tetrameric channels with homo-tetrameric channels forming non-conductive or slightly-conductive ion pores (Kofuji et al., 1995; Corey and Clapham, 1998; Corey et al., 1998; Schoots et al., 1999). The GIRK2 and GIRK4 subunits include endoplasmic reticulum (ER) export signals for plasma membrane expression of GIRK2/GIRK1 and GIRK4/GIRK1 hetero-tetramers (Kennedy et al., 1999; Ma et al., 2002). The GIRK2 gene generates splicing isoforms that share the same N-terminus but vary in their C-termini (Isomoto et al., 1997; Wei et al., 1998). Each splice isoform imparts a variety of physiologically and pharmacologically identified channels, based on which isoform is more dominant, have been reported (Leaney, 2003; Milovic et al., 2004).

Important roles of GIRK channels in neuronal development and maintenance have been investigated in *weaver* mutant and GIRK1, GIRK2, GIRK3 and GIRK4 knockout mice (Signorini et al., 1997; Wickman et al., 2000; Torrecilla et al., 2002). The *weaver* mouse has a G156S mutation within the GIRK2 pore selectivity filter that results in nonspecific ion conductance and membrane depolarization (Patil et al., 1995). As a result, these mice undergo extensive granular cell death and neuronal migration abnormalities in the cerebellum, age-dependent loss of dopaminergic neurons within the substantia nigra (SN) and sporadic seizures (Rakic and Sidman, 1973; Hatten et al., 1984; Schmidt et al., 1982; Roffler-Tarlov and Graybiel, 1984; Eisenberg and Messer, 1989). Some of these neurological irregularities have also been reported in the GIRK2 knockout mouse, including impaired K⁺-dependent inhibition and spontaneous development of seizures with no gross anatomical abnormality (Signorini et al., 1997). Taken together, these reports suggest that GIRK channels, especially the GIRK2 subunit, play important roles in regulating neuronal excitability and likely influence signal transduction mechanisms related to learning and memory.

A better understanding of the role of GIRKs in the network of dysfunctional DS neurons can be gained if the expression pattern of GIRK subunits is determined in the mouse model of DS. Over-expression of the GIRK2 subunit may affect a number of neuronal properties, including the functional arrangements between GPCRs and effector GIRK2 containing channels, membrane potential, excitability and firing rates (Ehrensgruber et al., 1997). Although expression patterns have been detected in rodent, including GIRK2 knockout and *weaver* mice (Karschin et al., 1996; Karschin et al., 1994; Kobayashi et al., 1995; Ponce et al., 1996; Inanobe et al., 1999; Morishige et al., 1996; Drake et al., 1997; Signorini et al., 1997; Chen et al., 1997), it has not been studied in the Ts65Dn mouse.

The aims of the present study were to investigate whether expression of GIRK subunits is altered in the Ts65Dn mouse and whether predicted over-expression of GIRK2 affects the expression of other GIRK subunits. *In situ* hybridization and real-time RT-PCR were used to investigate brain distribution of GIRK mRNAs. In addition, we used immunoblotting and immunohistochemistry, to determine whether GIRK mRNA expression pattern affects protein expression levels and patterns.

Materials and Methods

Mice

Male Ts65Dn mice and control diploid littermate mice were generated as previously reported (Reeves et al., 1995). In brief, Ts65Dn animals were bred to have the mixed genetic background C57BL/6J × C3H/HeSnJ used in our previous studies (Siarey et al., 1997a; Siarey et al., 1999). Results from Ts65Dn mice were always compared to diploid littermates. All protocols were approved by the USUHS Institutional Animal Care and Use Committee.

Total RNA isolation and reverse transcription

Mice 75 ± 10 day old (N = 5 pairs (Ts65Dn and diploid littermates)) were sacrificed by decapitation while deeply anesthetized with CO₂.

Brains were removed and frontal cortex, hippocampus and cerebral cortex (remaining cortex) were dissected free. (Cerebellum was also obtained for analysis in a separate study). Tissue was quickly frozen on dry ice and kept at -80°C until RNA or protein isolation. For all experiments, total RNA was isolated from 15–30 mg brain areas using Rneasy Mini Kit (Qiagen Inc., Valencia, CA, USA) with DNase I (Qiagen, Inc., Valencia, CA). Purified RNA was quantified using 260 nm absorbance and assessed for purity using the 260/280 nm ratio with a UV spectrophotometer. For reverse transcription, 1.0 µg of total RNA was used to synthesize cDNA with TagMan RT-Reagents (Applied Biosystem, Foster City, CA). The RT reaction mixture contained 2.5 µM oligo-d(T)₁₆ primers, 2.5 µM Random hexamers, 500 µM of each dNTP, 1.25 U/µl of Multiscribe reverse transcriptase, 0.4 U/µl Rnase inhibitor, 5.5 mM MgCl₂ and 1 X TaqMan® RT buffer except for total RNA. The reaction mixture was incubated at 25°C for 10 min to maximize primer-RNA template binding. The RT reaction was performed for 30 min at 48°C, followed by heating for 5 min at 95°C for RT incubation in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Wellesley, MA). The cDNA was stored at -20°C until subsequent analysis.

Conventional reverse transcription (RT)-PCR analysis

RT-PCR analysis was performed as previously described (Stoll and Galdzicki, 1996). Primers were designed using the published sequences of GIRK2A (Gen Bank accession no. U51122), GIRK2B (Gen Bank accession number U51125), GIRK2C-1 (Gen Bank accession number U51123), GIRK2C-2 (Gen Bank accession number U51124) as templates. Primers sequences were: GIRK2A forward 5'-3' CTCTTAATCCAGTCCGTGTTG and reverse 5'-3'

CAGGCCGTCTGCAAGAACAAT, which amplified a cDNA fragment of 969 bp; GIRK2B forward 5'-3' CTCTTAATCCAGTCCGTGTTG and reverse 5'-3' AACCACACCACCAACCAAAT, which amplified a cDNA fragment of 597bp; GIRK2C-1 forward 5'-3' CTCTTAATCCAGTCCGTGTTG and reverse 5'-3' GAGGGGCAGGTAGGTATTTT, which amplified a cDNA fragment of 1012bp; and GIRK2C-2 forward 5'-3' CTCTTAATCCAGTCCGTGTTG and reverse 5'-3' CAGACTTGCTAGATGAAGTGAGAG, which amplified a cDNA fragment of 2043bp. Searching of the sequences using Blast (<http://www.ncbi.nlm.nih.gov/BLAST/>) confirmed their specificity. PCR amplifications of GIRK2A, GIRK2B, GIRK2C-1 and GIRK2C-2 were performed using these primers. Amplified products were analyzed using 1.0–2.0% agarose gel electrophoresis.

Quantitative real-time RT-PCR

Quantitative RT-PCR was performed using a GeneAmp 5700 sequencing detection system (Perkin-Elmer, Wellesley, MA) and SYBR-Green quantitative PCR kit from PE Biosystems (Foster City, CA). For the PCR step, reaction volumes of 25 µl contained 10 ng of cDNA, 1 × 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 MgCl₂ and 200 mM of each primer (Table 1). The primer pairs for GIRK2C (Accession Number NM_010606) were designed to detect both splicing isoforms GIRK2C-1 (Accession Number U511123) and 2C-2 (Accession Number U511124). PCR primers designed using the Primer Express 1.0 Software program (Perkin-Elmer, Wellesley, MA) were set between exons of each cDNA and primers were synthesized by the Biomedical Instrumentation Center at Uniformed Services University for the Health Sciences. Searching of the sequences using Blast confirmed their specificity. The PCR protocol was done by denaturing for 15 sec at 95°C, annealing for 10 sec at 57°C, and elongation for 1 min at 72°C for 40 cycles. Prior to the PCR step, reaction samples were incubated for 2 min at 50°C and then for 10 min at 95°C. To exclude contamination of unspecific PCR products, dissociation curve analyses were performed on all final PCR products after the cycling protocol. To avoid genomic DNA and cDNA, PCR reactions without template cDNA were added to reaction wells. All samples were run in triplicate. The PCR products were run on a 2% agarose gel to confirm product size and then sequenced with an ABI Prism 310 automated sequencer (Perkin-Elmer, Wellesley, MA). This system allows the increase in PCR product to be monitored based on the threshold number of cycles (CT) required to produce a detectable change in fluorescence from SYBR[®] Green I Dye. The absolute copy numbers of GIRKs and neuronal specific enolase (NSE), used as an internal control, were generated by standard curve that cDNA synthesized from AtT-20 cells (mouse pituitary cell line) (ATCC, Manassas, VA). Relative quantitation was performed using each absolute copy number as provided by (Ueno et al., 2002).

In situ hybridization

80 ± 13 day old mice (N= 7 pairs (Ts65Dn and diploid littermates)) were sacrificed by decapitation were sacrificed by decapitation while deeply anesthetized with CO₂. Brains were removed, quickly frozen on dry ice, and kept at –80°C until sectioning. Coronal sections of 16 µm thickness were cut through the whole brain with a cryostat at –16°C and air-dried on a warm plate at 37°C for 30 min. Sections were fixed in 4% formaldehyde in phosphate buffer solution (PBS) for 5 min, then rinsed twice with PBS. The sections were acetylated with acetic anhydride in TEA for 10 min at room temperature, rinsed with 2 × SSC and dehydrated through an ascending alcohol series (70, 80, 90, 95 and 100%). ³⁵S-UTP-labeled antisense- and sense-riboprobes were generated using the Maxiscript kit (Ambion, Austin, TX). Riboprobes were synthesized using either T7 or T3 (antisense and sense) RNA polymerase. GIRK2 probe, designed to detect all splice isoforms, corresponds

to nucleotides 645–1014 of mouse GIRK2 cDNA (accession number NM_010606), GIRK2A to nucleotides 1722–2126 (accession number U51122), GIRK2B to nucleotides 1446–1908 (accession number U51125), GIRK2C-1 to nucleotides 1781–2265 (accession number U51123), GIRK2C-2 to nucleotides 2536–3011 (accession number U51124), and GIRK1 to nucleotides 1075–1483 (accession number NM_008426) (see Table 1 for detailed primers sequence). After incubation at 37°C for 1 h, probes were treated with DNase I, precipitated, resuspended, and visualized on an ethidium bromide-stained gel to confirm size and estimate concentration.

Sense- and antisense-labeled probes were separately hybridized to tissue sections in hybridization buffer (50% deionized formamide, 300 mM NaCl, 20 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% dextran sulfate, 1 × Denhardt's solution) at 55°C overnight.

The sections were rinsed in 4 × SSC at room temperature to remove coverslips and then washed four times for 5 min each in 4 × SSC containing 1 mM DTT. Free probes were removed by rinsing with 20 µg/ml RNase (Sigma, St. Louis, MO) in buffer (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, and 0.25 mM EDTA) for 45 min at 37°C. Sections were then rinsed for 5 min each time twice in 2 × SSC containing 1 mM DTT, once for 5 min in 1 × SSC containing 1 mM DTT and once for 5 min in 0.5 × SSC containing 1 mM DTT. Then they were incubated twice in 0.1 × SSC with 1 mM DTT for 30 min at 65°C. The container with slides was placed on wet ice to cool for 15 min. Sections were dehydrated through an ascending alcohol series for 1 min in each concentration up to 100%.

Sections were exposed to autoradiographic films (Kodak Hyperfilm Biomax MR) and developed after appropriate time of exposure from 24 to 72 h. For cellular resolution, selected slides were then coated with nuclear track emulsion (NTB-3, Kodak, New Haven, CT). After 4–8 weeks of exposure at 4°C, the slides were developed in Dektol (Kodak, New Haven, CT), then fixed and counterstained with 0.5% Giemsa or Nissl with cresyl violet (Sigma, St. Louis, MO). The sections were then air dried and mounted. Data on the mRNA expression of GIRK1 and GIRK2 including splicing isoforms was compiled from examination of X-ray film images and emulsion-coated sections of brain sections cut in the coronal plane for each mouse.

Quantitative analysis of *in situ* hybridization—The quantification of hybridization signals for GIRK2, GIRK2A, GIRK2B, GIRK2C-1, GIRK2C-2 and GIRK1 mRNAs was performed using a the NIH Image program (Wayne Rasband, Research Services Branch, NIMH, Bethesda, MD). The optical densities were measured within same sized circular frame for each specific brain region. A minimum of 3 sections was analyzed for each selected region for each brain. The tissue measurements were within the linear portion of the natural log of silver grain density curve, as determined from the standards (¹⁴C ARC, St. Louis, MO). Background density was measured over tissue areas not expressing specific signal and then subtracted from the value measured. Average values of investigated groups were compared statistically by Welch two-sample *t*-test. The statistical significance level was set at *P* < 0.05.

Western blotting

GIRK1 and GIRK2 protein levels were measured by Western blot analysis. Ts65Dn and diploid littermates 79 ± 11 day old (N= 5 pairs (Ts65Dn and diploid littermates)) were sacrificed by decapitation while deeply anesthetized with CO₂. Brains were quickly removed and dissected into frontal cortex, remaining cerebral cortex and hippocampus. Tissues were quickly frozen on dry ice, and kept at −80°C until homogenization. Frozen tissue was resuspended in RIPA buffer containing: 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, and 50 mM Tris-Cl (pH 8.0) 1:4 (v/v), they were then

homogenized, sonicated and allowed to lyse for 20 min. The homogenate was centrifuged at 18,000 g for 5 min and protein concentration of the supernatant determined. Subsequently samples were prepared according to the Novex NuPage Bis Tris/MOPS protocol. Protein concentrations were determined using the 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% nonfat dry milk in PBS with 0.1% Tween 20, the blots were incubated with either anti-GIRK1 (1:400) or anti-GIRK2 antibody (1:200) (Alomone Inc., Jerusalem, Israel) at room temperature for 2 h or at 4° C degree overnight. In order to verify equal loading, the lower part of the blot was incubated for 1 h with NSE (sigma, St. Louis, MO). Membranes were washed 3 times for 5 min each in PBS with 0.01% Tween 20 for 5 min. The membranes were then incubated in goat anti-rabbit IgG HRP for at least 45 min in PBS/Milk, washed and signal was detected using SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and quantified. The relative protein levels were calculated as a ratio of optical density of the bands corresponding to GIRK1 and GIRK2 and the optical density of the band corresponding to NSE.

Immunohistochemistry

90 ± 10 day old mice (N= 7 pairs (Ts65Dn and diploid littermates)) were anesthetized with pentobarbital (50 mg/kg) and perfused intracardially with 4% formaldehyde (Fisher, Pittsburgh, PA) in PBS (pH 7.4). Brains were removed, post-fixed in the same solution at +4°C overnight and then dehydrated in 20% sucrose in PBS at 4°C. Brains were stored at -80°C for more than 24 h, cut into 20 µm sections on a cryostat, thaw and mounted onto poly-L-lysine coated slides (Sigma, St. Louis, MO) and stored at -80°C until immunohistochemical examination.

GIRK2 polyclonal antibody (Chemicon, Temecula, CA) and monoclonal antibodies against tyrosine hydroxylase (TH) (Chemicon, Temecula, CA) were used at 1:1000 dilutions. The epitope of the GIRK2 antibody corresponds to residues 374–414 of mouse GIRK2 (Accession Number [P48542](#)) located at the C-terminal domain. 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 or 1:300 dilutions, respectively as secondary antibodies. The specificity of GIRK2 antibody was confirmed by absorption test (see below) and by Western blot with normal and GIRK2^(-/-) tissues. The sections were incubated for 48 h with primary antibodies in a blocking solution of 1 × PBS (pH 7.4) containing 0.3% Triton× 100 and 1% goat serum. After washing in 1 × PBS (pH 7.4) containing 0.2% Triton× 100, the sections were incubated for 30 min at room temperature with secondary antibodies in 0.3% Triton× 100. After washing in 1 × PBS (pH 7.4) containing 0.2% Triton 100 and then in 1 × PBS (pH 7.4), sections were mounted with mounting medium including p-phenylenediamine, PBS and glycerol (pH 8.0 by carbonate-bicarbonate buffer). Images were collected using a Leica (Bannockburn, IL) DM RXA microscope and a Scion Corporation (Frederick, MD) FW-1310M camera C using XYZ Grabber software (Jozsef Szege, PhD; BIC, USUHS). Images presented in Fig. 7 were collected with Zeiss Pascal Laser Scanning Confocal Microscope (Thornwood, NY). Figures were assembled with CREATE software (Stone Design Corporation, Albuquerque, NM). The absorption test was carried out using 100µM/ml of antigen and 1:500, 1:1000 or 1:2000 concentration of GIRK2 antibody for 48 hours incubation as described above. No staining was detected when the primary antibodies were pre-incubated with the antigenic peptide.

Statistical analysis

Data are expressed as mean $\pm$ SEM of values relative to those found in corresponding diploid controls. Differences between groups were analyzed for statistical significance using Welch two-sample *t*-test. *P*-value less than 0.05 were considered to be significant.

RESULTS

Increased levels of GIRK2 mRNA in Ts65Dn mouse brain by real-time PCR

The GIRK2 subunit gene is localized to human Chr. 21 and mouse Chr. 16, and is therefore likely to be over-expressed in DS and in the Ts65Dn mouse. Hence, we verified the expected gene dosage overexpression of GIRK2 mRNA in the Ts65Dn mouse. Furthermore, we examined the levels of GIRK1 and GIRK3 mRNAs to corroborate reports that GIRK2 expression level is correlated with the expression of other GIRK channel subunits (Marker et al., 2004; Signorini et al., 1997)

Using real-time RT-PCR, the levels of GIRK1, GIRK2 and GIRK3 mRNAs were analyzed in: frontal cerebral cortex, (non-frontal) cerebral cortex and hippocampus of Ts65Dn and diploid littermates (*n* = 5 pairs). PCR primers are shown in Table 1. NSE mRNA expression level was not significantly different between Ts65Dn and diploid littermates in any brain area studied. Therefore NSE mRNA was used to normalize each sample for loading and to evaluate GIRKs mRNA expression (Chicurel et al., 1993; Takahashi et al., 1992).

In 5 pairs of mice, an average of 1.5-fold higher mRNA level of GIRK2 was found in the Ts65Dn mouse brains compared with diploid male littermate brains. The specific brain regions increases (Fig. 2: B, E and H) were cerebral cortex 1.5-fold (*P*<0.001), frontal cortex 1.3-fold (*P*=0.017) and hippocampus 1.7-fold (*P*=0.012). This demonstrates that the expression of GIRK2 gene message in the Ts65Dn mouse reflects a gene dosage effect with an approximate 1.5-fold increase. In contrast GIRK1 and GIRK3 mRNA expression in these brain regions of the Ts65Dn mouse was at similar levels to diploid littermates (Fig. 2: A, D, G and C, F, I).

Our results (Fig. 2I) showed significant expression of GIRK3 mRNA in both Ts65Dn and control diploid hippocampi, as has been reported in the rat hippocampus (Karschin et al., 1996). GIRK3 mRNA had not previously been found in the mouse hippocampus (Kobayashi et al., 1995), a discrepancy, which may be due to mouse strain differences.

Overexpression of GIRK2 and GIRK1 protein subunits in Ts65Dn frontal cortex and hippocampus

Temporal and spatial expression patterns of GIRK1 and GIRK2 overlap to a great degree, since they form heteromultimeric channel assemblies (Slesinger et al., 1996; Liao et al., 1996). In the GIRK2 knockout mouse, lack of GIRK2 expression led to dramatic reduction in GIRK1 protein, similarly in the GIRK1 knockout mouse the GIRK2 protein levels were also reduced (Marker et al., 2004; Signorini et al., 1997). We hypothesized that the expression level of GIRK1 or GIRK2 subunits affects the level of the other GIRKs subunits and therefore influences ratios of homo- and/or hetero-tetrameric channels composed of GIRK1 and/or GIRK2 subunits, changing the electrophysiological profiles of recorded inwardly rectifying K⁺ currents (Marker et al., 2004; Signorini et al., 1997; Leaney, 2003). A subunit specific membrane trafficking pattern has been proposed as the possible underlying mechanism (Ma et al. 2002).

Western blot analysis of GIRK subunits was performed to investigate whether the gene dosage increase in mRNA expression translated to an increase in protein. The GIRK2 (2.44-

fold, $P < 0.01$), but not GIRK1 subunit was significantly over-expressed in the Ts65Dn cerebral cortex (total cortex-frontal cortex) when compared with diploid littermate cerebral cortex (Fig. 3: B, E, H). The hippocampus and frontal cortex of Ts65Dn showed significant over-expression of both GIRK1 (1.43-fold $P = 0.047$ and 1.50-fold $P = 0.032$, respectively) and GIRK2 (1.37-fold $P = 0.011$ and 1.45-fold $P = 0.048$, respectively) subunits (Fig. 3: A, D, G and C, F, I). These data either demonstrate that in the cortex the mechanism of channel assembly is different to that in the frontal cortex and hippocampus, or that the heterogeneity and size of the remaining cortex makes it more difficult to accurately detect relationships between an increase in GIRK1 and an increase in GIRK2 protein by the Western blot technique. Therefore, our data imply that at least in the frontal cortex and hippocampus of the Ts65Dn mouse, GIRK1 protein level is regulated by channel assembly processes where the amount of GIRK2 subunit could play a rate-limiting role.

Expression pattern of GIRK2 and GIRK1 mRNAs in Ts65Dn and control brains by *in situ* hybridization

Since the trisomy condition could differentially affect expression of channels containing GIRK1 and GIRK2 subunits in a brain-area specific manner, we investigated the localization of GIRK1 and GIRK2 transcripts in the Ts65Dn brain by *in situ* hybridization. In order to compare the expression levels of GIRK transcripts, we kept the size and specific activity of riboprobes used for the *in situ* hybridization similar to each other and all the tissue slides were processed in parallel (see Material and Methods section). GIRK2 riboprobe was designed to detect transcripts of all GIRK2 splice isoforms. The sequences of the primer probes for *in situ* hybridization are listed in Table 1.

In general, we found similar region-specific pattern of expression of GIRK1 and GIRK2 in both Ts65Dn and diploid brains. The quantitative results of GIRK1 and GIRK2 transcript expression and region specific quantitative comparisons of expression levels between Ts65Dn and control brain have been summarized in Table 2. GIRK2 mRNA expression was the most abundant in the DG but intense radiolabeling was also found in pyramidal cell regions (CA1-CA3) of both the Ts65Dn and diploid hippocampi (Fig. 4: C, D, I, J and Table 2). In both Ts65Dn and diploid mice the midbrain substantia nigra compacta (SNc) revealed a very high expression of GIRK2 transcripts whereas the ventral tegmental area (VTA) showed slightly lower levels (Fig. 4: D, J and Table 2). Levels of GIRK2 transcripts, similar to the ones detected in VTA, were also detected in pontine nuclei and in the granule cell layer of the olfactory bulb in each Ts65Dn and control mouse (Fig. 4: A, F, G, L and Table 2). Moderate expression levels of GIRK2 transcript were observed in the olfactory bulb, cortex, septum, habenula, amygdala, mammillary nucleus of the basal ganglia, and a portion of the thalamus, midbrain and the remaining portion of brain stem in both Ts65Dn and diploid littermates (Fig. 4: A–L and Table 2). GIRK1 transcripts also demonstrated differences among brain regions but they were not as dramatic as observed for GIRK2 transcripts in Ts65Dn compared with diploid littermates. The granule cell layer of the olfactory bulb and the hippocampal granular layer of DG showed the highest expression levels of GIRK1 transcripts (Fig. 4: M–Q and Table 2). The remaining parts of the olfactory bulb, cortex, hippocampus (other than the granular layer of DG), mammillary nucleus and many nuclei of the thalamus revealed a moderate density of GIRK1 transcripts (Fig. 4: M–R). The patterns of expression of GIRK1 and total GIRK2 mRNAs in Ts65Dn was qualitatively similar to that previously reported in the normal mouse and rat (Karschin et al., 1996; Ponce et al., 1996) as well as the *weaver* mouse brain (Chen et al., 1997).

The principle finding was that GIRK2 mRNA expression levels in Ts65Dn were much higher in specific brain regions compared to diploid littermates, whereas GIRK1 levels were similar to controls except in the ethmoid thalamic and oculomotor nuclei. In particular, the glomerular layer of the olfactory bulb, subiculum, septum, caudate putamen, thalamus and

midbrain showed the highest regional increases in the message level of GIRK2 in Ts65Dn brain in comparison to diploid brain (Table. 2). The areas that showed >2-fold relative increases in GIRK2 mRNA levels in Ts65Dn in comparison to diploid mice are those that have moderate to low GIRK2 mRNAs levels in diploid mice. In the VTA, the Ts65Dn/diploid ratio of GIRK2 was >2, in spite of the high level in diploid mice, which would be consistent with lack of signal saturation. There were similar regional expression levels of GIRK1 mRNA in Ts65Dn and diploid littermates in all analyzed brain areas (Table 2).

Comparison of expression patterns of GIRK2 splice isoforms and GIRK2 specific immunohistochemistry in Ts65Dn and diploid hippocampus

The electrophysiological properties of heteromultimeric GIRK1 subunit containing channels are similar (Jelacic et al., 1999; Leaney, 2003) but different trafficking pattern and membrane localization within the same neuron have been reported for heteromultimeric channel assemblies containing GIRK2A and GIRK2C (Ma et al., 2002; Leaney, 2003). It has been suggested that GIRK1/GIRK2A channel assemblies are the chief functional heteromeric GIRK channels in hippocampal neurons, whereas GIRK1/GIRK2C containing channel appear to be trafficked to special neuronal compartments within a cell (Leaney, 2003). The GIRK2C isoform has a postsynaptic density (PSD) binding domain and therefore may be localized in the synaptic compartments (Nehring et al., 2000; Hibino et al., 2000; Leaney, 2003). The GIRK3 subunit plays a regulatory role in the expression of GIRK1 containing heteromultimeric channels by limiting trafficking to the membrane (Kofuji et al., 1995).

Four types of previously known splice isoforms; GIRK2A, 2B, 2C-1 and 2C-2 were detected in both the Ts65Dn and diploid littermates by RT-PCR using specific primer pairs in Ts65Dn and diploid hippocampi. This result suggested a normal splice process in Ts65Dn brain (Fig. 5A). GIRK2 splice isoforms were quantified in the hippocampus by real-time RT-PCR using specific primer pairs (Table 1). Real-time RT-PCR data from the whole hippocampi of Ts65Dn (n=5) and diploid littermates (n=5) showed that four GIRK2 isoforms were significantly over-expressed in Ts65Dn in comparison to diploid littermates with the following ratios: GIRK2A; 1.8-fold (p=0.029), GIRK2B; 1.9-fold (p=0.0067), GIRK2C; 1.6-fold (p=0.031) and GIRK2C-2; 1.7-fold (p=0.041) (Fig. 5B–E). The primer pairs for GIRK2C, by design, can detect both splicing isoforms GIRK2C-1 and 2C-2. The absolute quantity of GIRK2B mRNA was the lowest of the four splice isoforms.

Real-time RT-PCR studies were followed with *in situ* hybridization to determine expression patterns of GIRK2 splice isoforms. In Ts65Dn hippocampus total GIRK2 signal that included all splice isoforms was heavily concentrated in the granular layer of DG and the pyramidal layer of CA1, CA2 and CA3 areas, but the expression levels were lower in the pyramidal layer (Fig. 4: C, I). The indusium griseum in the Ts65Dn brain, a rudimentary component of the hippocampus, expressed GIRK2 mRNA at level similar to CA1, CA2 and CA3 areas (Fig. 4: B, H). *In situ* hybridization study with specific RNA probes to each of the four GIRK2 splice isoforms revealed that pyramidal cells in the CA1 and CA2 areas of Ts65Dn hippocampus had a stronger signal for all examined splicing isoforms than did the same areas in the diploid hippocampus (Fig. 6AH, Table 3). GIRK2A, 2C-1 and 2C-2 were >1.5-fold overexpressed in the CA3 area of Ts65Dn hippocampus compared to diploid littermates (Fig. 6: A–H, Table 3). GIRK2C-1 and 2C-2 were >1.5-fold overexpressed in the Ts65Dn granular layer of the DG compared to diploid littermates (Fig. 6: A–H, Table 3).

Fluorescence immunohistochemistry demonstrated an interesting GIRK2 expression pattern in Ts65Dn hippocampus (Fig. 7: A, B). Coronal sections of hippocampus from Ts65Dn and diploid littermates were stained with GIRK2 antibody that detects all splice isoforms with a long C-terminus (i.e. all but GIRK2B). In the diploid brain the strongest fluorescence was

observed in granule cells in the DG. Moderate intensity was seen in areas of the pyramidal cell layer in CA1, stratum oriens (OR) and lacunosum molecular layer (LM) of CA1, CA2 and CA3 areas and molecular layer of DG. Weak fluorescence was observed in stratum radiatum (RD). The Ts65Dn hippocampus showed, as expected from *in situ* hybridization results, stronger immunostaining in the area lateral to the DG (“hot spot”) and LM (terminal field of the perforant pathway). The most ventral portion of the molecular layer (ML) of the DG (“ventral leaf”) was also intensely fluorescent. Interestingly the pyramidal cell layer in CA3 was shown only weak immunofluorescence of GIRK2 although strong GIRK2 mRNA expression was observed in the CA3 area of Ts65Dn and diploid mice. The indusium griseum in Ts65Dn had more abundant immunofluorescence in cell bodies and fibers compared with diploid littermates (Fig. 7C–F).

Our results indicated that the pattern and level of expression of GIRK2 subunits are abnormal in Ts65Dn hippocampus compared to diploid littermates and, therefore, it is likely that GIRK2 can contribute to abnormal hippocampal function reported in behavioral (see Introduction) and neurophysiological studies (Siarey et al., 1997b; Siarey et al., 1999; Kleschevnikov et al., 2004).

Comparison of expression patterns of GIRK2 splice isoforms and GIRK2 specific immunohistochemistry in Ts65Dn and diploid substantia nigra

The SN is a midbrain region severely affected in the homozygous *weaver* mouse (Ponce et al., 1996; Liao et al., 1996). Our results, similar to previous reports (Karschin et al., 1996), showed no detectable mRNA expression of GIRK1 but extremely high levels of GIRK2 mRNA expression in SNC and VTA (see Figs. 4 and 8). In Ts65Dn SNC and VTA, the GIRK2 mRNA expression pattern revealed higher GIRK2 mRNA expression levels than in diploid littermates (Fig. 8: A, B). Therefore we examined in GIRK2 subunit expression patterns in detail by *in situ* hybridization and fluorescence immunohistochemistry.

Ts65Dn mice showed abundant GIRK2 mRNA (total GIRK2, irrespective of splice isoforms) expression differences in the SNC and VTA compared to diploid littermates (Fig. 8: A, B). High magnification images revealed more GIRK2 positive grains in Ts65Dn SNC and VTA than in diploid littermates (Fig. 8: C, D, E, F). In addition these high power images revealed that Ts65Dn SN reticulata (SNR) neurons contain GIRK2 mRNA while expression in diploid littermates SNR was just above background (Fig. 8: G, H).

In the Ts65Dn SNC, SNR and VTA GIRK2 splice isoforms show differential over-expression. In Ts65Dn SNC and VTA, GIRK2B, 2C-1 and 2C-2 are over-expressed to a higher degree than GIRK2A compared to diploid littermates (Fig. 9A–H and Table 3). Yet, in the SNR, GIRK2B is more highly over-expressed than other splicing isoforms (Fig. 9A–H and Table 3). The potential physiological impact on SN/VTA of these specific abnormal spliced mRNA expression patterns is not clear.

Immunohistochemical fluorescence revealed GIRK2 expression in both cell bodies and fibers in SNC and VTA but mainly in fibers in SNR. The Ts65Dn areas showed higher fluorescence intensity than similar areas in diploid littermates (Fig. 10A–C, G–I). TH and GIRK2 co-expression revealed a population of GIRK2 positive dopaminergic neurons in the SNC and VTA. Not all TH positive neurons were found to be GIRK2 positive in Ts65Dn and diploid mice. However, the Ts65Dn mice had more neurons that expressed both TH and GIRK2 proteins in SNC/VTA than their diploid littermates (Fig. 10J–U). Similarly to what has been found for wild mouse (Schein et al., 1998) VTA of diploid controls contains fewer cells that co-express TH and GIRK2 than VTA of Ts65Dn mice. This result suggests that also in diploid mice co-expression of TH and GIRK2 may occur but GIRK2 is expressed at low levels (Fig. 10J–U). Abnormal patterns of GIRK2 expression in midbrain areas of SN

and VTA suggest a potential physiological impact on neurons that aid in control of movement and those in specific mesolimbic reward pathways.

GIRK2 expression patterns in selected brain areas that may contribute to DS phenotype

Since, under normal conditions, amygdala, habenula, thalamus and brain stem areas reveal significant levels of GIRK channel expression and may potentially contribute to the spectrum of neurological deficits of DS, we also examined GIRK2 expression in these regions in Ts65Dn mice.

Amygdala—The amygdaloid nucleus, including cortical amygdala, revealed a moderately higher level of GIRK2 transcripts in Ts65Dn (Fig. 11A, B). The mRNA expression was confirmed by GIRK2 specific immunofluorescence staining (data not shown). Since the amygdala modulates the acquisition and consolidation of memory via reciprocal connections with the prefrontal cortex and with the hippocampus abnormal GIRK2 expression in DS could further impact hippocampal function and contribute to DS neuropathology in the amygdala (Mann et al., 1986; Krasuski et al., 2002) but also (Pinter et al., 2001) and play a role in occurrence of seizures in DS, for review see (Stafstrom, 1993).

Habenula—Habenula a small epithalamic nucleus that receives fibers (stria medullaris) from medial septal nuclei and project via habenulointerpeduncular tract to the midbrain reveal elevated expression of GIRK2 mRNA. The medial habenular nucleus (MHb) in Ts65Dn had a stronger GIRK2 hybridization signal than did diploid littermates (Fig. 11E, F). Immunofluorescence showed abundant signal in the cell bodies of MHb and moderate signal in fibers of the lateral habenular nucleus (LHb) (Fig. 12A–F). The fluorescence signals of cell bodies and fibers in MHb and LHb were both stronger in Ts65Dn than in diploid littermates. A role of GIRK2 in DS habenula is unclear.

Thalamus—GIRK2 mRNA *in situ* hybridization revealed moderate expression in the medial geniculate nucleus (MGN), anterior pretectal nucleus, laterodorsal nucleus and mediodorsal nucleus, but a lower level of expression in other areas (Fig. 11C, D, Table 2). Again the signal from these regions in the Ts65Dn mouse was stronger than from diploid littermates (Table 2). By comparison, GIRK1 mRNA signal was also moderate, but only in the medial geniculate nucleus, anterior nuclei, ventrolateral nucleus, ventroposterior and lateral nuclei, reticular nucleus, laterodorsal nucleus and mediodorsal nuclei (Table 2). GIRK2 immunofluorescence was weak throughout the thalamus (data not shown), consistent with previous reports (Liao et al., 1996).

Brainstem—In pontine nuclei GIRK2 mRNA was expressed abundantly, but GIRK1 mRNA was weakly expressed (Fig. 4F, L). This result is opposite to that described in rat brain (Karschin et al., 1996). The parvicellular reticular nucleus expressed GIRK2 at a low level and GIRK1 at a moderate level (data not shown). In the motor trigeminal nucleus (Mo5) and laterodorsal tegmental nucleus, GIRK2 transcript was moderately expressed, but no detectable expression of GIRK1 message was found (Table 2).

Abnormal patterns of GIRK2 expression in the above mentioned areas may contribute to DS motor and sensory neurological phenotypes governed by neuronal pathways associated with or controlled by these regions.

Discussion

DS is the most prevalent cause of mental retardation at ~1 in 700 births (Epstein et al., 1991; Yoon et al., 1996). The presence of an extra maternal Chr. 21 (trisomy 21) results in DS and

it is presumed that products of the extra gene copies contribute to the DS phenotype (Lejeune, 1959; Korenberg et al., 1990; Antonarakis, 1991). Mental retardation is one of the more debilitating of the numerous neurological deficits afflicting DS individuals and their hippocampal-dependent learning and memory systems (Nadel, 2003). DS individuals also have disabilities in short-term memory, delayed gross and fine motor development and, with age, develop neurodegenerative diseases such as Alzheimer's disease (Burger and Vogel, 1973; Wisniewski et al., 1985). Mouse models of DS have been developed which genetically model the human condition (Epstein et al., 1985; Davisson et al., 1990; Reeves et al., 1995; Sago et al., 1998; Olson et al., 2004). The distal segment of mouse Chr. 16 is homologous to nearly the entire long arm of human Chr. 21 (Fig. 1). Full trisomy 16 (Ts16) mice die *in utero* (for review see (Galdzicki et al., 2001)) whereas segmental trisomy 16 (Ts65Dn, Ts1Cje, Ts1Rhr) mice survive to adulthood and mimic many of the behavioral, learning and developmental deficits characteristic of DS (Reeves et al., 1995; Holtzman et al., 1996; Sago et al., 1998; Olson et al., 2004).

GIRK subunits are expressed primarily in neurons and are localized to specific brain areas, including cortex, hippocampus, SN, and the VTA (Karschin et al., 1996; Liao et al., 1996; Signorini et al., 1997). Their over-expression, evidenced in this report, may impact the physiological role that GIRK channels play in those brain regions and likely contribute to the neurological phenotypes seen in DS individuals. In the GIRK2 knockout, hippocampal neurons are known to be depolarized (Luscher et al., 1997), thus we can expect that over-expression of GIRK2 would lead to neuronal hyperpolarization. This would not only shunt synaptic current but also affect T-type Ca^{2+} channels, A-type K^{+} channels and H-channels and therefore participate in the dendritic processing of subthreshold signals (Hoffman et al., 1997; Magee et al., 1998). In rat hippocampal cultures in which GIRK1 and GIRK2 subunits were over-expressed, baclofen induced GIRK activity impedes firing under current induced spike trains, an effect which mimics that of acetylcholine on atrial myocytes (Ehrengruber et al., 1997). GIRK channel activity also shown suppresses afterhyperpolarization and has significant effect on the processing of repetitive excitatory inputs (Takigawa and Alzheimer, 2003). Therefore, it is plausible that the over-expression of GIRK2 demonstrated here could result in abnormal processing of repetitive excitatory signals and lead to miscoding of synaptic information (Chen and Johnston, 2005).

The present data shows that the Ts65Dn mouse brain over-expresses GIRK2 message and, subsequently, GIRK2 protein in all investigated areas, findings consistent with a gene dosage effect. GIRK1 protein is also over-expressed in Ts65Dn hippocampus and frontal cortex. GIRK1 over-expression, however, cannot be explained by a gene dosage effect but may be due to heteromeric channel trafficking involving both GIRK1 and GIRK2 subunits. In GIRK1 and GIRK2 knockout mice absence of one subunit results in the concomitant downregulation of the other subunit (Marker et al., 2004; Signorini et al., 1997). This leads us to conclude that the protein expressions of GIRK1 and GIRK2 subunits within the hippocampus and frontal cortex are reciprocally related.

Behavioral studies with Ts65Dn mice have demonstrated cognitive and behavioral abnormalities that include higher levels of spontaneous locomotor and exploratory activity and impaired hippocampal function (reviewed in (Galdzicki and Siarey, 2003)). In Ts65Dn mice hyperactivity has been related to defective control of exploratory behavior (Coussons-Read and Crnic, 1996; Demas et al., 1996; Escorihuela et al., 1995). These findings imply that both hippocampal and prefrontal function are impaired in Ts65Dn mice and that abnormal GIRK2 expression in the hippocampus and frontal/prefrontal cortex could contribute to these deficits. However, Ts1Cje mice are hypoactive and show a reduction in exploratory activity (Sago et al., 1998). A simplistic explanation that the region not triplicated in Ts1Cje Chr., i.e. gene(s) between *App* and *Sod1* (that does not include *Girk2*

gene) is (are) responsible for hyperactivity has been rejected since mice that carry this limited App-Sod1 triplication have normal exploratory activity (Sago et al., 2000). These data suggest that we cannot explain the complex DS related traits without investigating complex interactions among genes. Nonetheless, all these findings are consistent with a contribution of abnormal GIRK2 expression to defective hippocampal (Reeves et al., 1995) and frontal/prefrontal cortex function in both DS mouse models (Shetty et al., 2000; Bimonte-Nelson et al., 2003; Dierssen, 2003).

In fact, decreased LTP and increased LTD have been found in both Ts65Dn and Ts1Cje CA1 areas of hippocampus, (Kleschevnikov et al., 2004; Siarey et al., 1997b; Siarey et al., 1999; Siarey et al., 2005). Furthermore, in the CA1 area of Ts1Cje mouse trains of pulses at both 20 Hz and 100 Hz produced a short-term attenuation of excitatory postsynaptic potentials in comparison to diploid responses (Siarey et al., 2005). Thus, the extra gene copies from the Ts1Cje Chr., including GIRK2, and their respective products lead to abnormal CA1 short- and long-term plasticity. These electrophysiological abnormalities in Ts65Dn and Ts1Cje hippocampus suggest that abnormal expression of GIRK2 and its splice isoforms might positively correlate with impaired physiological and behavioral functions in both mice models. The important role of GIRK2 in the mechanism of LTP induction has been supported by our finding that the GIRK2 knockout mouse exhibits an increase in CA1 LTP ((Adeniji-Adele et al., 2004) unpublished results). Lack of selective GIRK subunit specific antagonists makes direct physiological tests of the role of GIRK2 in the synaptic plasticity difficult. As an alternative to pharmacological analysis of GIRK2 subunits, male GIRK2^{+/-} knockout mice could be mated with Ts65Dn females to produce Ts65Dn mice that have a diploid GIRK2 gene number and all other Ts65Dn genes in triplicate (we have created such mice; study in progress). In addition, a role of synergistic expression of GIRK2 and KIR4.2. in brain development and in DS has been discussed (Thiery et al., 2003).

Quantitative real-time RT-PCR analysis revealed that in Ts65Dn hippocampus, GIRK2 splice isoforms that form longer C-termini are over-expressed to a greater extent than splice isoform forming shorter C-termini. Our *in situ* hybridization analysis showed that GIRK2C-1 and GIRK2C-2 were highly over-expressed in the Ts65Dn granular layer of the DG and in the CA3 area compared to diploid littermates. Only GIRK2C-1 and 2 isoforms have the PSD-95 and PDZ binding domains therefore their abnormal expression could particularly contribute to in abnormal synaptic function in hippocampus.

Immunofluorescent detection of GIRK2 “hot spots” in the lateral extension of the LM layer of CA3 area of Ts65Dn mice suggest that the function of pathways located in these areas could be severely affected. These areas contain several types of GABAergic interneurons that receive excitatory inputs from Schaffer collaterals and perforant path fibers. They also receive septal projections from the medial septal nucleus and the nucleus of the diagonal band of Broca (Freund and Buzsaki, 1996). Therefore input to the hippocampus through these projections may be abnormally processed. Furthermore, irregular GABAergic interneuron function within the DG of the Ts65Dn mouse has been previously reported (Kleschevnikov et al., 2004) and it is likely that LM of CA3 area from these mice also show abnormal GABAergic activity.

In our study expression levels of GIRK1 mRNA do not correlate with expression levels of GIRK2 in the SN. In Ts65Dn and diploid SN we were unable to detect GIRK1 mRNA despite high GIRK2 expression levels. Therefore GIRK2 is a prevailing GIRK channel subunit in the SN and VTA, and GIRK2 homomers may predominate as the functional GIRK channel assembly in these regions. However, previous reports demonstrated that GIRK3 mRNA is expressed throughout entire SN and VTA, albeit at low levels (Karschin et al., 1996; Ponce et al., 1996). Nonetheless, GIRK2 subunit function must be critical to

proper neuronal functioning in these regions since *weaver* mice show abnormal electrical activity in the SNC and VTA (Kofuji et al., 1996; Navarro et al., 1996; Slesinger et al., 1996; Slesinger et al., 1997). If, indeed, GIRK2 homomeric and/or GIRK2/GIRK3 heteromeric channels predominate in these areas under normal conditions, over-expression may have substantial implications. The relative differences in splice isoform over-expression within these regions (Fig. 9, Table 4) may further change GIRK channel function.

Dopaminergic neurons of the VTA that express GIRK2C isoforms, and no GIRK1 subunit channels, respond to GABA_B receptor stimulation with high transient current responses and strong desensitization. These dopaminergic neurons also exhibit low coupling efficacy between GIRK channels and GABA_B receptors. In contrast, GABAergic neurons of VTA that express GIRK2C and GIRK1 subunits have high coupling efficacy between GIRK channels and GABA_B receptors and small non-desensitizing current responses (Cruz et al., 2004). In addition, GABA_B activation in dopaminergic neurons shows bidirectional effects on spiking activity, with low-level GABA_B activation increasing spiking activity and high-level GABA_B activation inhibiting spiking activity (Cruz et al., 2004). Over-expression of GIRK2C in Ts65Dn dopaminergic neurons could, therefore, strongly influence spiking activity and contribute to overall synaptic activity. In both GABAergic and dopaminergic neurons, over-expression of GIRK2 may increase GIRK current density due to an increase in the number of GIRK channels.

The pattern of GIRK2 over-expression can also impact mesolimbic pathways suggesting involvement in motivational and addictive behavior (Cruz et al., 2004). Neurons in the Ts65Dn trigeminal motor nucleus revealed a high expression level of GIRK2, yet none of the typical motor functions of these neurons seems to be affected in DS or in the Ts65Dn mouse.

In conclusion, this study has determined levels and patterns of GIRK2 subunit expression in the DS mouse model Ts65Dn. The data indicate elevated GIRK2 mRNA expression due to the extra GIRK2 gene, including all examined splice isoforms. This increase in mRNA levels led not only to increased levels of GIRK2 protein, but also of GIRK1 protein in the regions that co-expressed both subunits, i.e., in hippocampus and frontal cortex. The physiological impacts of these abnormal GIRK2 levels have been correlated with known learning and behavioral deficits in the Ts65Dn mouse and their potential significance for DS neurological phenotypes have been discussed.

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Fig. 1.

Representation of Ts65Dn (left, ~ 104 genes), Ts1Cje (middle, between Sod1-Mx1, ~81 genes) and Ts1Rhr (right, Cbr1-Mx1, ~33 genes) chromosomal segments that originated from mouse chromosome 16. Right bar also represents homologous segment with the so-called Down syndrome critical region. Note that the Girk2 gene (KCNJ6) is present in each of the Ts65Dn, Ts1Cje and Ts1Rhr fragments. Abbreviations: App; amyloid precursor protein, Glur5; glutamate receptor subunit 5, Sod1; superoxide dismutase-1, Synj1; synaptojanin-1, Sim2; single-minded 2, Cbr1-carbonyl reductase 1, Dyrk1a/Mnbh; dual-specificity tyrosine-(Y)-phosphorylation regulated kinase, Kcnj6/GIRK2; G-protein activated inwardly rectifying potassium channel subunit 2, Ets2; avian leukemia oncogene 2,3' domain, Mx1/Tmprss2; myxovirus resistance-1/transmembrane protease, serine 2.

Fig. 2.

Real time RT-PCR analysis of GIRK1 (A, D, G), GIRK2 (B, E, H) and GIRK3 (C, F, I) expressions in Ts65Dn (solid bars; N = 5) and diploid littermate (open bars; n = 5) cortex (A, B, C), frontal cortex (D, E, F) and hippocampus (G, H, I). GIRK2 mRNA was over-expressed in cerebral cortex (1.5-fold, $P < 0.001$), frontal cortex (1.3-fold, $P = 0.017$) and hippocampus (1.7-fold, $P = 0.012$) compared to diploid littermates. The expression levels of GIRK1 and GIRK3 mRNAs were not significantly different between Ts65Dn and diploid littermates in all brain areas. Results are mean $\pm$ SEM. $0.01 \leq *P < 0.05$, $**P < 0.01$, significantly different from diploid littermates.

Fig. 3.

Western blot analysis indicates that GIRK1 and GIRK2 are over-expressed in Ts65Dn hippocampus and frontal cortex. Western immunoblots of Ts65Dn ($N = 5$) hippocampus (A: lane 1, 2 and 3) and diploid littermates (A: lane 4, 5 and 6; $N = 5$), Ts65Dn cerebral cortex (B: lane 1, 2 and 3) and diploid littermates (B: lane 4, 5 and 6) and Ts65Dn frontal cortex (C: lane 1, 2 and 3) and diploid littermates (C: lane 4, 5 and 6). Anti-GIRK1 or anti-GIRK2 antibodies detected bands of 55 kDa (top of A, B, C) and 47 kDa (third of A, B, C) respectively. Anti-NSE antibody detected bands of 39 kDa (second and bottom of A, B, C). Samples containing approximately 5 (lane 1, 4), 10 (lane 2, 5) and 15 μ g (lane 3, 6) of protein were loaded. Bands of NSE appeared depending on loading amounts. Histograms (D–I) represent the normalized to NSE signals detected from hippocampus, cerebral cortex and frontal cortex immunoblots probed with antibodies specific for GIRK1 (D, E, F) and for GIRK2 (G, H, I) for Ts65Dn (solid bars) and diploid littermates (open bars) samples. Ts65Dn cerebral cortex significantly over-expressed GIRK2 (2.44-fold, $P < 0.01$), but not GIRK1 subunits compared with diploid littermates. In Ts65Dn hippocampus and frontal cortex both GIRK2 and GIRK1 subunits are overexpressed with the respective following increases: GIRK2 (1.37-fold $P = 0.011$ and 1.45-fold $P = 0.048$), GIRK1 (1.43-fold $P = 0.047$ and 1.50-fold $P = 0.032$). The results are mean $\pm$ SEM. * $P < 0.05$, significantly different from littermates.

Fig. 4.

Film autoradiographs show elevated GIRK2 mRNA expression in Ts65Dn (N = 7) brain (A–F) in comparison to diploid littermates (N = 7) (G–L) and increase in GIRK1 mRNA expression in thalamus of Ts65Dn brain (M–O) in comparison to diploid littermates (P–R). A similar region-specific pattern of expression of GIRK2 and GIRK1 subunits was revealed in both Ts65Dn and diploid brains. Abbreviations: OB; olfactory bulb, IG; indusium griseum, DG; dentate gyrus, Cx; cortex, Th; thalamus, CP; caudate putamen, MHb; medial habenula, SN; substantia nigra, SNC; substantia nigra, compacta, VTA; ventral tegmental area, S; subiculum, MMn; mammillary nucleus, Pn; pontine nucleus. Scale bars = 1 mm (G, P), 1.5 mm (H, K, L), 2 mm (I, J, Q, R).

Fig. 5.

All investigated GIRK2 splicing isoforms are elevated in Ts65Dn hippocampus (N = 5) in comparison to diploid littermates (N = 5). The PCR products amplified with primer pairs of GIRK2A, 2B, 2C-1 and 2C-2 show each band of expected size (A). Real-time RT-PCR analysis using primer pairs of GIRK 2A, 2B, 2C or 2C-2 that show four GIRK2 isoforms were significantly over-expressed in the Ts65Dn (solid bars) in comparison to diploid littermates (open bars) with the following levels: GIRK2A, 1.8-fold ($p=0.029$); GIRK2B, 1.9-fold ($p=0.0067$), GIRK2C, 1.6-fold ($p=0.031$); GIRK2C-2, 1.7-fold ($p=0.041$) (B, C, D, E). The results are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, significantly different from diploid littermates.

Fig. 6. GIRK2 splicing isoforms mRNAs expression patterns in Ts65Dn (N = 7) and diploid (N = 7) hippocampi. GIRK2 hippocampi levels are elevated in Ts65Dn in comparison to diploid littermates mice. GIRK2A, 2B, 2C-1 and 2C-2, profiles of expression are similar in Ts65Dn hippocampus (A, C, E, G) and diploid littermates (B, D, F, H). Scale bars = 500 μ m (H).

Fig. 7.

Immunohistochemical detection of GIRK2 in Ts65Dn hippocampus (A: N = 6) shows stronger immunostaining (arrowhead) in the “hot spot” lateral to the dentate gyrus and lacunosum moleculare (LM) than the diploid littermate (B: N = 6). Open arrow in (A) points to the ventral leaf of Ts65Dn molecular layer (ML) area where GIRK2 is highly expressed. Abbreviations: OR; striatum oriens, RD; radiatum, LM; lacunosum moleculare, ML; molecular layer. (C–F) Immunofluorescence signals of indusium griseum are stronger in fibers and some cells of Ts65Dn (C, D) compared to diploid littermates (E, F). Scale bars = 500 μ m (B), 250 μ m (E), 125 μ m (F).

Fig. 8. Darkfield photographs show GIRK2 mRNA pattern of expression in substantia nigra (SN) with a radioprobe that recognizes all isoforms in Ts65Dn (N = 7) (A) and in diploid littermates (N = 7) (B). Brightfield photographs of Nissl-stained sections hybridized for GIRK2 mRNA for SN compacta (SNC), ventral tegmental area (VTA) and SN reticulata (SNR) in Ts65Dn (C, E, G) and diploid littermate (D, F, H). Ts65Dn SN over-expressed GIRK2 mRNA compared with diploid littermates. Scale bars = 750 μ m (B), 50 μ m (H).

Fig. 9.

Darkfield photographs show mRNA pattern of expression in SN of GIRK2A, GIRK2B, GIRK2C-1 and GIRK2C-2 splicing isoforms in Ts65Dn (A, C, E, G) and diploid littermate (B, D, F, H). In Ts65Dn SN each GIRK2 isoform mRNA is over-expressed compared to the diploid littermates, with a different pattern of over-expression for each isoform. Scale bar = 500 μ m (H).

Fig. 10.

GIRK2 immunofluorescence signals in SNC, SNR and VTA of Ts65Dn (A, B, C: N = 6) and diploid littermates (G, H, I: N = 6). Ts65Dn showed stronger immunofluorescent staining than diploid littermates. (D–F) Tyrosine hydroxylase (TH, red) immunofluorescence signals of SNC, SNR and VTA of Ts65Dn. (J–U) Co-expression of TH (red) and GIRK2 (G2, green) in dopaminergic neurons in the SNC (J–O) and VTA (P–U) revealed that not all TH positive neurons are GIRK2 positive in Ts65Dn (J, K, L, P, Q, R) and diploid littermates (M, N, O, S, T, U). Scale bars = 60 μ m (I), 50 μ m (U). Arrowheads point to dopaminergic cells that co-expressed GIRK2 and TH (orange). Arrows point to the cells with only TH (red).

Fig. 11.

High expression of GIRK2 mRNA in Ts65Dn in comparison to diploid littermates in amygdala, medial geniculate nucleus (MGN) and habenula. (A–B) The amygdala in Ts65Dn mice has more GIRK2 mRNA grains (A) than diploid littermate (B). (C–D) The MGN in Ts65Dn mice (C: arrowheads) shows more GIRK2 mRNA than diploid littermates (D). The medial habenular nucleus (arrows) in Ts65Dn (E) has more grains than diploid littermates (F). Scale bar = 1 mm (B, D, F).

Fig. 12.

Fluorescence immunohistochemical study shows abundant signals in cell bodies of medial habenula (MHb) (A, B) and in fibers of lateral habenula (LHb) in Ts65Dn (C) compared with diploid littermates (D, E and F). Scale bars = 750 μ m (D), 200 μ m (F).

TABLE 1

Primer Pairs and Product Sizes

Primers for Real Time RT-PCR Analysis				
Primer Names	Number	Forward Primer(5'3')	Reverse Primer(5'3')	size, bp
GIRK1	NM_008426	TGTGGAAACACAGGAATGACTT	AAAACGATGACCCCAAAGCA	76
GIRK2	NM_010606	GATGGGAAGTGCAACGTTCA	AAGATGTCCGTCAGGTATCGGT	65
GIRK2A	U51122	CAAACTGAACCAACATGCAGAATT	GAGCCCAGCATCACCCATT	89
GIRK2B	U51125	TGGTGAAGCCACAGTAAGA	ACACCTGAGGGTCTGAATGCA	72
GIRK2C	NM_010606	ACATGCAGAATTGGAGACAGAAGAG	AGGTTTGCCACGTCACCATT	78
GIRK2C-2	U51124	CCTTGACTGCTGGCCCTCTA	AGGTGCCCTAAAACTGTCAGTCT	72
GIRK3	NM_008429	TCGAGAGGGACGACTTCGAGAT	CCAGGTACGAGCTTCGAGCTT	90
NSE	NM_013509	TGGGACTGAGAATAAATCCAAGTTT	AGCTGCCCCAGCCTTACAC	79
Primers for <i>in situ</i> Hybridization				
Primer Names	Accession Number	Forward Primer(5'3')	Reverse Primer(5'3')	product size, bp
GIRK1	NM_008426	CAGTTCCATGCAACCTTTGA	CTCCAGCCATCTTTTGAAGC	409
GIRK2	NM_010606	CCAGAGGTACGTGAGGAAGG	CACGGACTGGATTAAGAGGAG	370
GIRK2A	U51122	GTGATGCTGGGCTCCTAGTG	TTGTTTCCGTTTATTTTGTGC	405
GIRK2B	U51125	TCCGACCTTAAACGTGCTCT	TTAACTCCCCTTTGGCTT	463
GIRK2C-1	U51123	CATCAGACATGACCCCTCCT	ACCTGCCCTGTGCTAGGTAA	485
GIRK2C-2	U51124	TTTTTGATCCCCTTGACTGC	GGAAGCTGGCAATGTAGTCC	476

TABLE 2

GIRK1 and 2 Expression Levels of Ts65Dn Mouse Brain by *in situ* Hybridization

	GIRK1		GIRK2	
	Ts65Dn	Diploid	Ts65Dn	Diploid
Brain region				
Olfactory bulb				
Main olfactory bulb				
Glomerular layer	628 ± 60	595 ± 60	619 ± 19***	220 ± 8
Mitral cell layer	889 ± 46	833 ± 58	566 ± 49	441 ± 40
Granular layer	1065 ± 90	1176 ± 117	1346 ± 49***	801 ± 39
Anterior olfactory nucleus	649 ± 73	771 ± 56	1209 ± 9***	686 ± 15
Piriform cortex	800 ± 96	823 ± 77	970 ± 54***	668 ± 14
Cortex				
Retrosplenial granular cortex	534 ± 55	678 ± 45	642 ± 50***	294 ± 52
Primary motor cortex	493 ± 67	630 ± 66	737 ± 95**	337 ± 59
Sensorimotor cortex	459 ± 57	557 ± 72	673 ± 51***	360 ± 41
Ectorhinal cortex	577 ± 55	646 ± 50	704 ± 67	520 ± 58
Island of Calleja	n.d.	n.d.	662 ± 53**	442 ± 59
Hippocampus				
Granular layer, dentate gyrus	1027 ± 72	1125 ± 95	1895 ± 100**	1477 ± 84
CA1	656 ± 63	773 ± 72	1196 ± 84**	815 ± 79
CA2	771 ± 77	796 ± 62	1310 ± 76***	852 ± 81
CA3	587 ± 56	647 ± 55	1238 ± 84*	906 ± 111
Subiculum	591 ± 67	633 ± 64	697 ± 106**	269 ± 75
Indusium griseum	583 ± 57	763 ± 69	1332 ± 84***	601 ± 79
Septum				
Lateral septal nucleus	422 ± 56	530 ± 57	495 ± 60***	182 ± 49
Bed nucleus stria terminalis	348 ± 54	380 ± 66	354 ± 75*	95 ± 57
Nucleus VDB	187 ± 45	204 ± 72	389 ± 78***	139 ± 53
Brain region				
Preoptic area	296 ± 56	426 ± 57	377 ± 62	223 ± 50
Mammillary nucleus	583 ± 68	658 ± 77	592 ± 70**	262 ± 84
Substantia nigra				
SN, compacta	n.d.	n.d.	1378 ± 70***	835 ± 71
SN, reticulata	n.d.	n.d.	472 ± 57*	301 ± 34
Ventral tegmental area	n.d.	n.d.	1024 ± 74***	494 ± 77
Thalamus				
Medial geniculate nucleus	574 ± 55	592 ± 50	496 ± 50**	254 ± 66

	GIRK1		GIRK2	
	Ts65Dn	Diploid	Ts65Dn	Diploid
Anterior pretectal nucleus	419 ± 62	574 ± 68	512 ± 62***	183 ± 50
Ethmoid thalamic nucleus	444 ± 54***	126 ± 53	428 ± 54***	126 ± 53
Paraventricular nucleus	363 ± 67	480 ± 59	393 ± 57*	161 ± 57
Anterior nucleus(AD/AV)	605 ± 52	595 ± 47	444 ± 69**	170 ± 61
Ventrolateral nucleus	608 ± 65	641 ± 59	453 ± 66**	166 ± 52
Ventroposterior, lateral nucleus	636 ± 58	590 ± 61	411 ± 53*	156 ± 60
Reticular nucleus	585 ± 57	568 ± 54	462 ± 64**	197 ± 57
Laterodorsal nucleus	595 ± 72	663 ± 67	541 ± 52*	305 ± 61
Mediodorsal nucleus	480 ± 75	594 ± 56	622 ± 69**	321 ± 65
Posterior thalamic nucleus	412 ± 61	567 ± 54	325 ± 61	167 ± 50
Habenula	361 ± 59	352 ± 58	596 ± 49**	342 ± 53
Midbrain				
Superior colliculus	294 ± 59	430 ± 51	471 ± 57***	157 ± 47
Inferior colliculus	260 ± 45	357 ± 48	n.d.	n.d.
Deep mesencephalic nucleus	n.d.	n.d.	247 ± 50	158 ± 49
Oculomotor nucleus	247 ± 75	594 ± 56*	552 ± 69**	275 ± 64
Interpeduncular nucleus	358 ± 66	374 ± 50	348 ± 59*	140 ± 61

In situ Hybridization signals obtained for ³⁵S-labeled RNA probes on Ts65Dn mice and littermate diploid brain sections were rated according to the grain density based on C¹⁴ standard. The unit is nCi/g tissue. n.d., not determined. The asterisks mean the significance compared Ts65Dn signal density with littermate diploid signal density;

* , $P < 0.05$;

** , $P < 0.01$;

*** $P < 0.001$.

TABLE 3

GIRK1 and 2 Expression Levels of Ts65Dn Mouse Brain by *in situ* Hybridization

	GIRK2A			GIRK2B			GIRK2C-1			GIRK2C-2		
	Ts65Dn	Diploid		Ts65Dn	Diploid		Ts65Dn	Diploid		Ts65Dn	Diploid	
Brain region												
Olfactory bulb												
Main olfactory bulb												
Glomerular layer	397 ± 60*	180 ± 55		713 ± 80****	325 ± 53		752 ± 80**	444 ± 69		719 ± 78****	212 ± 77	
Mitral cell layer	420 ± 51	261 ± 57		718 ± 69****	329 ± 69		655 ± 78*	370 ± 66		756 ± 69****	243 ± 78	
Granular layer	615 ± 58**	343 ± 53		1034 ± 90**	607 ± 74		1397 ± 52****	845 ± 96		1340 ± 117****	697 ± 93	
Anterior olfactory nucleus	567 ± 65	427 ± 51		859 ± 74**	553 ± 49		1078 ± 67****	602 ± 66		1191 ± 129**	702 ± 95	
Piriform cortex	642 ± 69****	214 ± 58		1030 ± 52****	496 ± 80		1213 ± 81****	518 ± 74		1198 ± 108**	760 ± 84	
Cortex												
Retrosplenial granular cortex	440 ± 62*	232 ± 60		611 ± 73**	289 ± 69		788 ± 57****	405 ± 59		711 ± 67****	297 ± 49	
Primary motor cortex	424 ± 46**	218 ± 50		900 ± 59****	467 ± 54		935 ± 62**	586 ± 66		844 ± 57****	459 ± 52	
Sensory motor cortex	522 ± 46**	318 ± 50		780 ± 66****	416 ± 55		887 ± 78****	486 ± 58		780 ± 51****	396 ± 47	
Entorhinal cortex	550 ± 56**	268 ± 53		733 ± 80	581 ± 71		930 ± 89*	640 ± 78		832 ± 76*	529 ± 77	
Island of Calleja	628 ± 60****	296 ± 59		914 ± 65****	406 ± 69		912 ± 87****	440 ± 75		1040 ± 81****	425 ± 79	
Hippocampus												
Granular layer, dentate gyrus	1261 ± 70*	1079 ± 68		1513 ± 82*	1191 ± 77		1902 ± 118****	1265 ± 86		1940 ± 122****	1204 ± 55	
CA1	964 ± 57****	379 ± 56		1227 ± 90****	627 ± 70		1375 ± 107**	785 ± 106		1392 ± 232*	820 ± 68	
CA2	1039 ± 61****	484 ± 58		1164 ± 87****	645 ± 58		1646 ± 99****	1020 ± 60		932 ± 87****	499 ± 55	
CA3	786 ± 60****	403 ± 60		1246 ± 86**	843 ± 63		1396 ± 105****	866 ± 73		1588 ± 158**	914 ± 88	
Subiculum	640 ± 53****	264 ± 63		705 ± 56****	330 ± 51		780 ± 79****	239 ± 52		628 ± 67****	328 ± 54	
Industium griseum	824 ± 70****	329 ± 61		890 ± 59****	449 ± 57		1343 ± 66****	606 ± 49		1400 ± 142****	660 ± 67	
Septum												
Lateral septal nucleus	276 ± 50	173 ± 59		635 ± 68****	233 ± 62		557 ± 69**	229 ± 60		663 ± 83*	350 ± 95	
Bed nucleus stria terminalis	250 ± 59	122 ± 67		633 ± 60****	286 ± 50		658 ± 57**	399 ± 57		501 ± 105	227 ± 83	

	GIRK2A		GIRK2B		GIRK2C-1		GIRK2C-2	
	Ts65Dn	Diploid	Ts65Dn	Diploid	Ts65Dn	Diploid	Ts65Dn	Diploid
Substantia nigra								
SN, compact	537 ± 59**	245 ± 51	816 ± 82****	373 ± 63	937 ± 63****	445 ± 66	1251 ± 101****	594 ± 107
SN, reticular	304 ± 54*	141 ± 53	612 ± 67**	327 ± 56	588 ± 69*	370 ± 59	452 ± 79*	229 ± 60
Ventral tegmental area	602 ± 80**	229 ± 59	558 ± 66****	227 ± 46	928 ± 73****	429 ± 69	1029 ± 89****	534 ± 73

In situ Hybridization signals obtained for ³⁵S-labeled RNA probes on Ts65Dn mice and littermate diploid brain sections were rated according to the grain density based on C¹⁴ standard. The unit is nCi/g tissue. n.d., not determined. The asterisks mean the significance compared Ts65Dn signal density with littermate diploid signal density;

* , $P < 0.05$;
 **, $P < 0.01$;
 **** $P < 0.001$.