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Evidence that increased *Kcnj6* gene dose is necessary for deficits in behavior and dentate gyrus synaptic plasticity in the Ts65Dn mouse model of Down syndrome

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Abstract

Down syndrome (DS), trisomy 21, is caused by increased dose of genes present on human chromosome 21 (HSA21). The gene-dose hypothesis argues that a change in the dose of individual genes or regulatory sequences on HSA21 is necessary for creating DS-related phenotypes, including cognitive impairment. We focused on a possible role for *Kcnj6*, the gene encoding Kir3.2 (Girk2) subunits of a G-protein-coupled inwardly-rectifying potassium channel. This gene resides on a segment of mouse Chromosome 16 that is present in one extra copy in the genome of the Ts65Dn mouse, a well-studied genetic model of DS. Kir3.2 subunit-containing potassium channels serve as effectors for a number of postsynaptic metabotropic receptors including GABAB receptors. Several studies raise the possibility that increased *Kcnj6* dose contributes to synaptic and cognitive abnormalities in DS. To assess directly a role for *Kcnj6* gene dose in cognitive deficits in DS, we produced Ts65Dn mice that harbor only 2 copies of *Kcnj6* (Ts65Dn:*Kcnj6*++− mice). The reduction in *Kcnj6* gene dose restored to normal the hippocampal level of Kir3.2. Long-term memory, examined in the novel object recognition test with the retention period of 24h, was improved to the level observed in the normosomic littermate control mice (2N:*Kcnj6*++). Significantly, both short-term and long-term potentiation (STP and LTP) was improved to control levels in the dentate gyrus (DG) of the Ts65Dn:*Kcnj6*++− mouse. In view of the ability of fluoxetine to suppress Kir3.2 channels, we asked if fluoxetine-treated DG slices of Ts65Dn:*Kcnj6*+++ mice would rescue synaptic plasticity. Fluoxetine increased STP and LTP to control levels. These results are evidence that increased *Kcnj6* gene dose is necessary for synaptic

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and cognitive dysfunction in the Ts65Dn mouse model of DS. Strategies aimed at pharmacologically reducing channel function should be explored for enhancing cognition in DS.

Keywords

Down syndrome critical region; *Kcnj6*; Kir3.2; Genotype-phenotype relationship; Mouse models; Ts65Dn; Cognition; Learning; Novel object recognition; Y-maze; Locomotor activity; Synaptic plasticity; Fluoxetine; Long-term potentiation

Introduction

Down syndrome (DS), due to triplication of human chromosome 21 (HSA21) (Lejeune et al., 1959), results in a number of significant neurobiological and somatic phenotypes. The most salient are cognitive and behavioral impairments in children and the emergence of Alzheimer's disease in the elderly (Belichenko et al., 2016; Dierssen, 2012; Dykens, 2007; Kleschevnikov et al., 2012c; Lott, 2012; Nadel, 2003; Roizen and Patterson, 2003; Roper and Reeves, 2006; Sabbagh and Edgin, 2016). The gene-dosage hypothesis proposes that all DS-related phenotypes are due to the presence in excess of one or more genes or regulatory sequences on HSA21 (Epstein et al., 1981). Tests of this hypothesis has in recent years resulted in insights into a number of the genes and mechanisms responsible for DS phenotypes (Belichenko et al., 2009; Dierssen et al., 2011; Fotaki et al., 2002; Kleschevnikov et al., 2004; Olson et al., 2004b; Salehi et al., 2006; Vesa et al., 2005).

Mouse genetic models of DS provide an opportunity to investigate the neurobiology of DS (Belichenko et al., 2015; Das and Reeves, 2011; Edgin et al., 2012; Gardiner et al., 2003; Mojabi et al., 2016; Reeves et al., 1995; Rueda et al., 2012; Yu et al., 2010) and to explore the role of individual genes in DS phenotypes (Altafaj et al., 2013; Salehi et al., 2006). Similar to DS, mouse genetic models of DS exhibit deficient hippocampus-dependent long-term memory, working memory, and other physiological and behavioral changes consistent with deficits in cognition (Fernandez et al., 2007). Mouse models that contain different as well as overlapping sets of triplicated genes have been genetically engineered and examined to explore phenotype-genotype relationship (Davisson et al., 1990; Jiang et al., 2015; O'Doherty et al., 2005; Olson et al., 2004a; Pereira et al., 2009; Sago et al., 1998; Yu et al., 2010). The most intensively investigated mouse is the Ts65Dn model, whose creation 26 years ago was instrumental in advancing this field of study (Davisson et al., 1990). Ts65Dn mice exhibit many phenotypes characteristic of DS including deficient cognition (Braudeau et al., 2011; Busciglio et al., 2013; Costa, 2011; Demas et al., 1998; Faizi et al., 2011; Fernandez et al., 2007; Gutierrez-Castellanos et al., 2013; Martinez-Cue et al., 2014; Smith et al., 2014).

Studies in humans partially trisomic for HSA21 identified a region whose presence in three copies was sufficient to create many DS phenotypes, including intellectual disability; it was thereby labeled the Down Syndrome Critical Region (DSCR) (Korenberg, 1990; Peterson et al., 1994; Rahmani et al., 1989; Yamamoto et al., 2011). Recent studies in mice carrying all or part of the homologous region of mouse Chromosome 16 have shown that many DS-like phenotypes are linked to genes in this region (Belichenko et al., 2009; Dierssen and de

Lagran, 2006; Jiang et al., 2015; Reeves et al., 1995). Within the DSCR, *Kcnj6* is a candidate for contributing through increased dose to cognitive deficits. *Kcnj6* is present in 3 copies in both people with DS and Ts65Dn mice. This gene encodes the Kir3.2 (Girk2) subunit of inwardly rectifying potassium channels which serve as effectors for a number of postsynaptic metabotropic receptors (Luscher et al., 1997; Mark and Herlitze, 2000; Yamada et al., 1998). As predicted by increased dose, the Kir3.2 product of *Kcnj6* is increased in Ts65Dn mice (Harashima et al., 2006; Kleschevnikov et al., 2012b; Kleschevnikov et al., 2005). Suggesting a physiologically meaningful contribution for increased Kir3.2 in these mice, there was increased signaling through postsynaptic GABAB receptors in both primary cultures of hippocampal neurons (Best et al., 2007) and acute hippocampal slices (Best et al., 2012; Kleschevnikov et al., 2012b). In addition, suppressing enhanced GABAB/Kir3.2 signaling by treating with selective GABAB receptor antagonists restored synaptic plasticity and long-term memory in Ts65Dn mice (Kleschevnikov et al., 2012a). Recently, cognitive assessment in a series of mouse genetic models bearing distinct sets of genes within DSCR pointed to a contribution of *Kcnj6* (Jiang et al., 2015).

A direct test of the impact of increased dose of specific genes is essential for defining contribution(s) to phenotypes. To address the impact of *Kcnj6* triplication on cognitive phenotypes in DS, we genetically deleted the third copy by producing Ts65Dn mice with 2 copies of *Kcnj6* (i.e., Ts65Dn:*Kcnj6*++−). Littermate Ts65Dn mice with three copies of *Kcnj6* (Ts65Dn:*Kcnj6*+++) and normosomic mice (2N:*Kcnj6++)* served as controls. Reduction of the *Kcnj6* gene dose restored to normal the level of Kir3.2, long term memory, and short- and long-term potentiation in the DG. Remarkably, pharmacologically inhibiting Kir3.2-containing channels also restored synaptic plasticity. The findings are evidence that increased expression of *Kcnj6* is necessary for the significant cognitive impairment in this model of DS and suggests that strategies aimed at pharmacologically reducing channel function should be explored for enhancing cognition in DS.

Materials and Methods

Animals

Segmental trisomy 16 (Ts65Dn) mice were purchased from the Jackson Laboratory, Bar Harbor, ME, stock #001924. Heterozygous *Kcnj6*+/− mice (129/sv–C57BL/6 hybrid crosses) were a gift from Dr. L. Jan (UCSF). These mice were characterized in a number of previous studies (Blednov et al., 2001; Mitrovic et al., 2003; Signorini et al., 1997). Ts65Dn:*Kcnj6*++− mice were the result of crossing female Ts65Dn with male *Kcnj6*+/− mice. Diploid (2N:*Kcnj6*++) and trisomic (Ts65Dn:*Kcnj6*+++) mice served as controls. To minimize the possible effect of the mixed genetic background, only littermate male mice were used in all experiments. The body weight was examined in 1–12 month old mice. All other experiments were performed using 4–6 month old mice. Ts65Dn and 2N mice were used to investigate fluoxetine effects on synaptic plasticity. The animals were housed 2 to 4 per cage with a 12 h light-dark cycle and *ad lib* access to food and water. Genotype of all animals was confirmed after completing experiments. For genotyping, tail samples were used to extract genomic DNA. A quantitative polymerase chain reaction protocol developed by the Jackson Laboratory, Bar Harbor, ME (http://www.jax.org/cyto/quanpcr.html) was

used to measure expression of the *Mx1* gene, which is present in three copies in Ts65Dn. To determine number of gene copies of *Kcnj6*, PCR reaction for this gene was performed using primers RK2-1: 5′- TAT GGC TAC CGG GTC ATC AC −3′; RK2-3: 5′- GAT CAA CTT GGC TCT GAT GG −3′, and G2KO-A: 5′- GAG TAG AAG TGG CGC GAA GG −3′, as described (Signorini et al., 1997). In addition, all mice were prescreened for *Pde6brd1* homozygosity, a recessive retinal degeneration mutation that results in blindness (Bowes et al., 1993), and only animals free of retinal degeneration were used.

The experiments were conducted in accordance with the National Institutes of Health guidelines and with an approved protocol from the University of California San Diego (UCSD) Institutional Animal Care and Use Committee.

Behavioral testing

Behavioral studies were performed during the light cycle between 7:00 a.m. and 7:00 p.m. Before experiments, the animals were handled for 5 min every day for 2 weeks. On the day of testing, to habituate subjects, mice were left in their home cages in the room used for the experiment at least 1 hour prior to the onset of the study. To minimize olfactory cues, each apparatus was thoroughly cleaned with 10% ethanol after each animal. Three cohorts of mice were tested and the results averaged. Total number of animals per genotype: 2N:*Kcnj6*++, $n = 19$; Ts65Dn:*Kcnj6*+++, $n = 12$; and Ts65Dn:*Kcnj6*++−, $n = 14$. All behavioral tests and procedures were performed by personnel blinded to genotype.

Spontaneous locomotor activity

Spontaneous locomotor activity was evaluated in square Plexiglas activity chambers (43.2 × 43.2 × 20 cm) equipped with three planes of infrared detectors (Med Associates Inc, St. Albans, VT). Four mice were tested concurrently in individual chambers. The chamber was divided into the center (20 cm × 20 cm, zone 1) and periphery (the rest of the chamber, zone 2). Chambers were located within sound-attenuating boxes (66 × 55.9 × 55.9 cm) with a built-in internal fan for background noise (65 dB) and lighting for ambient illumination (40 lux). For testing, each animal was placed in the center of the testing arena and allowed to move freely for 10 minutes. The movements were monitored and recorded by an automated tracking system (Med Associates Activity Monitor, version 5.93.773).

Y-maze

Y-maze testing was performed using an apparatus with three equal arms (30 cm length, 10 cm width, and 20 cm height), made of opaque acrylic (Plexiglas). A mouse was placed at the maze center under ambient illumination (20 lux) and allowed to explore the environment for 5 min. An arm entry was scored when the mouse entered the arm with all four paws. The total number of entries (*N*) and number of ‘correct’ triplets (*M*, consecutive choices of each of the three arms without re-entries) was evaluated. The alternation rate was computed according to the formula: $R\ (\%) = M*100/(N-2)$.

Novel object recognition test

Novel object recognition (NOR) was tested using the Bevins and Besheer protocol (Bevins and Besheer, 2006). Mice were habituated in a black Plexiglas rectangular chamber (31 × 24

cm, height 27 cm) for 10 min on 2 consecutive days under dim ambient lighting conditions (30 lux). The activity of mice was recorded with a video camera. Each test had two phases – acquisition and testing. For the acquisition phase, two identical 'familiar' objects were placed in corners of the chamber, 8–9 cm from the walls. A mouse was placed at the mid-point of the wall opposite the sample objects. After allowing 10 min to explore the objects, the mouse was returned to the colony. The testing phase was performed 24 h after the acquisition. For testing, one of the objects was replaced with a novel object of the same height and volume, but different shape and appearance. The mouse was again placed in the chamber to explore the objects for 3 min. The animal was considered to be exploring the object if its head was directed at the object and the distance between the object and the mouse nose was 1 cm or less. The amount of time spent exploring each object was recorded and evaluated by operators blinded to genotype. The positions (left or right) of the 'new' and the 'familiar' objects during the testing phase were counterbalanced between mice and sessions. The discrimination index was computed as $R = T_{new}*100/(T_{new} + T_{old})$, were T_{new} is the time spent exploring the new object, and T_{old} is the time spent exploring the familiar object.

Electrophysiology

Transverse hippocampal slices were prepared as previously described (Kleschevnikov et al., 2012a; Salehi et al., 2009). In short, a mouse was anesthetized with isoflurane before decapitation. The brain was quickly removed and immersed for 2 min in ice-cold artificial cerebrospinal fluid (ACSF) containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, osmolarity 305 mOsm, continuously bubbled with 95% O_2-5% CO_2, pH 7.4. The hippocampus was dissected and cut into 350-μm-thick slices in ice-cold ACSF with a vibratome (Leica VT1000S). Slices were allowed to recover in oxygenated ACSF at room temperature for at least 2 h prior to experimental recordings.

A slice was transferred into the submerged recording chamber and superfused with ACSF at a constant rate of 2.5 mL/min at 32°C. Recording electrodes made of borosilicate glass capillaries (1B150F, World Precision Instruments, Sarasota, FL) were filled with ACSF (resistance ~0.5 MΩ). Monopolar stimulating electrodes were made of Pt/Ir wires of diameter 25.4 μm (PTT0110, World Precision Instruments, Sarasota, FL) and had 100-μm-long exposed tips. Both the stimulating and recording electrodes were inserted under visual control perpendicular to the slice surface into the middle molecular layer of dentate gyrus (DG) at a distance 250–300 μm from each other. The initial slope of the field excitatory postsynaptic potentials (fEPSP) was measured at latencies 0.1 – 0.9 ms. Testing stimuli (duration 100 μs, current 80 μA) evoked field responses with amplitudes of 70–80% of maximum. Long-term potentiation (LTP) was induced by tetanization with three trains of stimuli (1 sec at 100 Hz; 5 min between the trains). Short-term potentiation (STP) and LTP were evaluated at 1–15 min and 30–60 min after the tetanus respectively. Fluoxetine hydrochloride (Sigma-Aldrich, cat. # F-132) was dissolved in distilled water to a concentration of 2 mM, aliquoted, and stored at −30 °C. Fluoxetine was bath applied, at a final concentration of 10 μM, for 15 min before and 15 min after the tetanus.

Western blot

Three to four month old mice were deeply anesthetized with Isoflurane and sacrificed by decapitation. The hippocampi were collected on an ice-cold preparation table in 0.9% saline solution and stored at −80°C. All samples were processed in parallel. The samples were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF) with 1 mg/mL protease inhibitor cocktail (aprotinin, leupeptin, pepstatin) (Roche, Cat # 11836153001). The homogenates were centrifuged at 14,000rpm for 30 min at 4°C and supernatant was collected. The protein concentration in the supernatant was determined using BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of protein were treated with 2X Laemmli Sample Buffer (Bio-Rad, CAT#161-0737, CA) and 1X PBS without Calcium or Magnesium, pH 7.4 (Gibco-Life Technologies, CAT#10010023), incubated at 95°C for 5 min, and 16 μL (1.25 mg/mL) of total protein per lane was loaded onto 12.5% Tris-Glycine gel. Gel electrophoresis was performed at 100 V for 2 h with 10% Tris-Glycine running buffer (100 μL 10X Tris-Glycine, 10 μL 10% SDS, and 890 μL filtered deionized H_2O). Proteins were transferred to a low-fluorescent, hydrophobic polyvinylidene difluoride (PVDF) membrane at 95 V for 1 h in the cold room with 0.45 μm pore size (Immobilon-P, EMD Millipore, MA) in 10% methanol transfer buffer (100 μL MeOH, 100 μL 10X Tris-Glycine, and 800 μL filtered deionized H_2O) and the membranes were blocked with 5% nonfat milk in TBS-T solution (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with following primary antibodies: rabbit polyclonal anti-Kir3.1 (1:1′000, Alomone Labs, Cat#APC-005), rabbit polyclonal anti-Kir3.2 (1:1′000, Alomone Labs, Cat#APC-006), or rabbit polyclonal anti-Dyrk1A (1:1′000, Cell signaling, Cat#2771S). Comparison of Kir3.1 and Kir3.2 expression was performed on the same blot; Kir3.1 blots were re-probed with Kir3.2. The blots were washed in 1X TBS-T (3 times × 10 min) followed by incubation with secondary antibody goat anti-rabbit IgG-HRP conjugate (1:10′000, Jackson Immunoresearch Laboratories Inc, Cat# 111-035-144, RRID: AB_2307391) for 1 h at room temperature. Mouse monoclonal anti-β-actin primary antibody (1:5000, Sigma-Aldrich, Cat# A5441, RRID: AB_476744) and goat anti-mouse secondary antibody (1:5000, Jackson Immunoresearch Laboratories Inc, Cat# 115-035-146, RRID: AB_2307392) were used to measure levels of β-actin. The blots were washed in 1X TBS-T (3 times × 10 min) and then developed with Clarity Western ECL Substrate (Bio-Rad, Cat#170-5061) and scanned with imaging system (Molecular Imager ChemiDoc XRS+, Bio-Rad, USA). Because there was no overlap of immunoreactivity for the Kir3.1, Kir3.2, and β-actin antibodies, levels of these proteins were measured using the same membranes without stripping and reblotting. The images were analyzed with ImageJ (NIH, USA).

Levels of Dyrk1A in *Kcnj6*+/− mice

Hypothetically, genetic targeting of the *Kcnj6* gene could affect the expression of adjacent genes. One such gene, the dual specificity tyrosine phosphorylation regulated kinase 1A (*Dyrk1A*), affects many important phenotypes in DS including cognition and synaptic plasticity (Altafaj et al., 2013; Souchet et al., 2014). To assess if the genetic alterations of *Kcnj6* dose affected the expression of *Dyrk1A*, we compared levels of Dyrk1A protein in hippocampal samples of *Kcnj6*+/− and *Kcnj6*+/+ mice (Fig S1). As can be seen from both

the representative blots (Fig S1A) and their quantification (Fig S1B), levels of Kir3.2 were reduced proportional to gene dose ($p = 0.0003$), while levels of Dyrk1A were not altered ($p = 0.76$) in *Kcnj6*+− *vs.* *Kcnj6*++ mice. Thus, genetic alterations in *Kcnj6* dose had no impact on the expression of *Dyrk1A*.

Chemicals

All chemicals used for electrophysiological experiments were purchased from Sigma–Aldrich (St. Louis, MO).

Statistical analyses

The data for body weight, behavioral testing, and neurophysiology were exported to Excel (Microsoft, Redmond, WA) for evaluation and statistical comparison. First, all parameters were examined for normality of distributions; they passed this test. Second, a *F*-test was performed to assess an equality of variances. Because some measurements showed unequal variances for different groups, as was expected due to the difference in number of animals per group, '*t*-test with the assumption of unequal variances' was performed. For electrophysiological data, two samples and two-tailed Student's *t*-test was used. All results are expressed as *mean ± SEM*, and reported according to recommendations of the American Psychological Association (APA), with p values < 0.05 considered to be significant.

Results

Kcnj6 dose influences body weight during aging

Body weight is a measure of systemic health. Children with DS show reduced body weight (Cronk, 1978), while adult individuals with DS often have increased body weight due to obesity (de Asua et al., 2014). To assess for an effect of *Kcnj6* gene dose, the body weights of mice of each genotype were examined from 1–12 months of age (Fig. 1). Body weight was significantly reduced in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice at all ages (Fig. 1, red *vs.* blue bar respectively; Table 1). Interestingly, while there was no difference between Ts65Dn:*Kcnj6*++− *vs.* Ts65Dn:*Kcnj6*+++ mice at early ages (Fig. 1, green *vs.* red bar, age intervals 1–2 and 3–4 months; Table 1), body weight was significantly increased in older Ts65Dn:*Kcnj6*++− *vs.* Ts65Dn:*Kcnj6*+++ mice (Fig. 1, green *vs.* red, age intervals 5–6 and 9–12 months; Table 1). These data are evidence that increased *Kcnj6* gene dose may impact overall health status during aging.

Kir3.2 protein levels are increased by increased *Kcnj6* dose

The goal of reducing *Kcnj6* gene dose in Ts65Dn mice was to reduce Kir3.2 protein levels to those observed in 2N animals. To determine if this goal was reached, protein levels in the hippocampi of individual mice were assessed using western blot. For side by side comparison, three experimental series were performed with the samples from the experimental groups loaded serially (Fig. 2). As expected, in Ts65Dn:*Kcnj6*+++ mice the levels of Kir3.2 were increased ($F_{1,6} = 3.04$, $p = 0.01$), while the levels of Kir3.1 unchanged ($F_{1,9} = 0.59$, $p = 0.29$) relative to 2N:*Kcnj6*++ mice (Fig. 2A). The increase in Kir3.2 levels approximated the increase in *Kcnj6* dosage in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice. Removal of the extra copy of *Kcnj6* reduced Kir3.2 levels and had no effect on Kir3.1. Side

by side comparison of Ts65Dn:*Kcnj6*++− *vs.* 2N:*Kcnj6*++ samples showed no difference in the protein levels in these experimental groups (Kir3.1: $F_{1,6} = 0.09$, $p = 0.46$; Kir3.2: $F_{1,9} = 0.01$, $p = 0.49$) (Fig. 2B). Comparison of Ts65Dn:*Kcnj6*++− *vs.* Ts65Dn:*Kcnj6*+++ samples again showed a reduction of Kir3.2 to the levels observed in 2N:*Kcnj6*++ samples ($F_{1,9} = 3.3$, $p = 0.005$) and no change in the levels of Kir3.1 ($F_{1,7} = 0.51$, $p = 0.31$) (Fig. 2C). Thus, in Ts65Dn mice, *Kcnj6* gene dose regulated the levels of Kir3.2 but not Kir3.1.

Spontaneous locomotor activity is unaffected by *Kcnj6* dose

Perceptive-motor slowness and hyperactivity disorders are characteristic of people with DS (Ekstein et al., 2011; Mason et al., 2015). In animal models, this phenotype is reflected in altered parameters of locomotor activity. To assess the effects of *Kcnj6* dose on spontaneous locomotion, behavior in the 'activity box' was examined. Locomotor activity was significantly increased in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice. This was registered as a significant increase in ambulatory distance ($F_{1,16} = 4.78$; $p = 0.0001$) (Fig. 3, A, red *vs.* blue bar) and ambulatory time ($F_{1,17} = 4.83$; $p = 0.0001$) (Fig. 3, B). In addition, resting time was reduced ($F_{1,23} = 3.59$; $p = 0.0001$), but velocity did not differ ($F_{1,15} = 1.13$; $p = 0.14$) in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice. Decrease in *Kcnj6* dose had no effect on locomotor activity in Ts65Dn mice. Indeed, neither the ambulatory distance ($F_{1,24} = 0.14$; $p = 0.44$) nor the ambulatory time ($F_{1,24} = 0.015$; $p = 0.49$) were significantly different comparing Ts65Dn:*Kcnj6*+++ and Ts65Dn:*Kcnj6*++− mice (Fig. 3, A, B, red *vs.* green bar). Likewise, there were no differences in resting time ($F_{1,24} = 0.29$; $p = 0.39$) or velocity ($F_{1,24} = 0.45$; $p = 0.33$).

Thigmotaxis, the tendency to move at the periphery of an open field, is characteristic of Ts65Dn mice; it may be evidence of anxiety or stereotypy (Simon et al., 1994). To estimate the effects of *Kcnj6* dose on thigmotactic behavior, distance and time on arena periphery were assessed (Fig. 3, C, D). Both parameters were greater in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice (ambulatory distance: $F_{1,21} = 2.12$, $p = 0.02$; ambulatory time: $F_{1,24} = 1.91$, $p = 0.03$). Removal of an extra copy of *Kcnj6* had no impact on these parameters, which were equal in Ts65Dn:*Kcnj6*+++ and Ts65Dn:*Kcnj6*++− mice (ambulatory distance: $F_{1,20} = 0.70$, $p = 0.25$; ambulatory time: $F_{1,21} = 0.60$; $p = 0.28$). Thus, *Kcnj6* gene dose had no apparent effect on locomotion in the Ts65Dn mouse.

Normalization of *Kcnj6* dose fails to impact working memory in the Y-maze but normalized exploratory activity

Deficient working memory contributes to cognitive impairment in DS. To assess working memory in Ts65Dn:*Kcnj6*++− mice, the rate of spontaneous alternations in Y-maze was measured. The alternation rate was significantly lower in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ group ($F_{1,36} = 3.06$; $p = 0.002$) reflecting impaired working memory (Fig. 4A, red *vs.* blue bar). Reducing *Kcnj6* gene dose had no effect on the alternation rate ($F_{1,28} = 0.23$; $p = 0.41$ for Ts65Dn:*Kcnj6*+++ *vs.* Ts65Dn:*Kcnj6*++−) thus demonstrating no effect in this test of working memory.

Y-maze testing also allows for an assessment of exploratory activity. The number of arm entries during the test was markedly increased in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice

($F_{1,14}$ = 2.49; p = 0.013) (Fig 4B, red *vs.* blue). However, no increase was evident comparing Ts65Dn:*Kcnj6*++− *vs.* 2N:*Kcnj6*++ mice ($F_{1,46}$ = 0.47; p = 0.32) (Fig 4B, green *vs.* blue). Thus, reducing *Kcnj6* gene dose normalized this facet of exploratory behavior in Ts65Dn mice.

Reducing *Kcnj6* dose enhances long-term memory in Ts65Dn mice

Impairment of hippocampus-dependent long-term memory is a hallmark of DS that is recapitulated in genetic models of DS. To assess the impact of *Kcnj6* dose on long-term memory, we used the novel object recognition test with a retention period of 24 h. In prior studies, this test consistently demonstrated impairment of long-term memory in Ts65Dn mice (Fernandez et al., 2007; Kleschevnikov et al., 2012a; Lysenko et al., 2014). On average, mice of all genotypes spent equal time investigating objects during both the acquisition (p = 0.16–0.39) and testing (p = 0.3–0.75; Fig. 5A). However, time for exploration of new objects tended to be lower, while time for exploration of old objects higher in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice during testing resulting in reduced discrimination index in Ts65Dn:*Kcnj6*+++ mice ($F_{1,13}$ = 2.24; p = 0.02) (Fig. 5B). Similar reduction of discrimination index was observed in Ts65Dn:*Kcnj6*+++ *vs.* Ts65Dn:*Kcnj6*++− mice ($F_{1,13}$ = 2.15; p = 0.025), while there was no difference between Ts65Dn:*Kcnj6*++− and 2N:*Kcnj6*++ groups ($F_{1,35}$ = 0.11; p = 0.46) (Fig. 5B). One factor affecting long-term memory is anxiety. To assess levels of anxiety during the test, we counted numbers of pellets dropped by mice. There was no difference between the groups (p = 0.60–0.93) indicating that the levels of anxiety were equal among all groups. Thus, restoration of *Kcnj6* gene dose led to restoration of long-term memory in Ts65Dn mice.

Reducing *Kcnj6* dose restores synaptic plasticity in DG of Ts65Dn mice

Enhancement of long-term memory suggests that reducing *Kcnj6* gene dose may impact synaptic plasticity. To assess changes in hippocampal synaptic plasticity, we examined STP and LTP in the dentate gyrus (DG) (Fig. 6). Both STP and LTP were significantly reduced (p = 0.016 for STP and 0.018 for LTP) in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ slices (Fig. 6A, B, red *vs.* blue). Restoration of *Kcnj6* to that in 2N mice resulted in synaptic plasticity in Ts65Dn equivalent to that observed in slices from 2N mice (Fig. 6A, green *vs.* blue dots). In fact, neither STP nor LTP were different in Ts65Dn:*Kcnj6*++− *vs.* 2N:*Kcnj6*++ mice (p = 0.29 for STP and 0.44 for LTP) (Fig. 6B, green *vs.* blue bars). Accordingly, both STP and LTP were greater in Ts65Dn:*Kcnj6*++− *vs.* Ts65Dn:*Kcnj6*+++ mice (p = 0.021 for STP and 0.047 for LTP) (Fig. 6, green *vs.* red). Increased *Kcnj6* dose was therefore necessary for deficits in both STP and LTP in the DG of Ts65Dn mice.

Pharmacologically suppressing Kir3.2 channels restores synaptic plasticity in DG of Ts65Dn mice

Fluoxetine impacts a number of aspects of neuronal synaptic function. Best known for its effect on serotonin reuptake (Fuller and Wong, 1977), it has also been shown to inhibit potassium channels containing Kir3.2 subunits (Kobayashi et al., 2003). To assess whether pharmacological suppression of signaling through the Kir3.2 channels affects synaptic plasticity in the Ts65Dn mice, STP and LTP were examined in the Ts65Dn DG during bath application of fluoxetine (Fig. 7). Fluoxetine (10 μM) or vehicle were applied for 30 min (15

min before and 15 min after the tetanization). There was no effect on baseline responses (Fig. 7A). Synaptic plasticity was significantly impaired in vehicle-treated Ts65Dn *vs.* 2N slices (Fig. 7, red *vs.* blue). Application of fluoxetine improved both STP and LTP in Ts65Dn slices (Fig. 7, light green) to levels that were not statistically different than in 2N slices ($p = 0.28$ and 0.20 for STP and LTP respectively). Interestingly, fluoxetine affected neither STP (Veh: 111.1 ± 2.9%, $n = 13$; Flx: 113.7 ± 5.2%, $n = 7$; $p = 0.64$) nor LTP (Veh: 110.5 ± 2.4%, $n = 13$; Flx: 112.8 ± 4.5%, $n = 7$; $p = 0.61$) in 2N mice (Fig. 7, dark green). Together with the findings for *Kcnj6* dose reduction reported above, these data are evidence that Kir3.2 subunit-containing potassium channels contribute to defects in synaptic plasticity in Ts65Dn mice.

Discussion

Herein we examined the effects of the *Kcnj6* gene dose on behavioral and cognitive phenotypes in the Ts65Dn mouse model of DS. Genetically normalizing *Kcnj6* gene dose reduced to 2N levels the Kir3.2 protein subunit of inwardly-rectifying potassium channels, restored synaptic plasticity in the DG, and improved long-term memory. Our findings are evidence that increased gene dose for *Kcnj6* is necessary for deficits in hippocampal synaptic plasticity and hippocampally-mediated tests of long-term memory in a mouse model of DS.

Deciphering the phenotype-genotype relationships in DS provides a powerful approach to examine pathogenesis. Efforts to enhance cognition, *via* studies to decipher the genes and mechanisms responsible for cognitive deficits, are a high priority. Toward this goal, we and others pursue an additive/subtractive approach in mouse genetic models of DS. Studies have documented dose effects for a number of specific genes. A role for increased dosage of *APP* in the pathogenesis of the Alzheimer's disease in DS is now supported by studies in both mouse models and humans (Head et al., 2016; Lott et al., 2006; Prasher et al., 1998; Salehi et al., 2006). We observed that an extra copy of *App* contributed to compromised retrograde axonal transport of NGF and degeneration of cholinergic neurons in Ts65Dn mice (Salehi et al., 2006). Triplication of synaptojanin 1 (*Synj1*), the protein product of which has been shown to regulate clathrin-mediated endocytosis, leads to the enlargement of early endosomes (Cossec et al., 2012). Transgenic mice harboring 4–5 copies of the human *SOD1* gene showed an increase in GABAergic inhibition and diminished LTP in the CA1 region (Gahtan et al., 1998; Levkovitz et al., 1999). Increased *Dyrk1A* dose influenced the expression of proteins involved in GABAergic and glutamatergic neurotransmission (Souchet et al., 2014) as well as other defects characteristic of DS (Toiber et al., 2010). Genetic normalization of *Dyrk1A* by stereotaxic injection of adeno-associated virus containing a short hairpin RNA against *Dyrk1A* improved LTP in the CA1 region (Altafaj et al., 2013). Recently, our studies to genetically dissect the mouse DSCR examined behavioral and synaptic phenotypes in the Dp(16)1/+ model of DS, as well as in mice with smaller segmental trisomies based on this model (Jiang et al., 2015). A clear role for increased *Dyrk1A* was demonstrated. In the current study, we used a direct genetic approach to assess the role of *Kcnj6*, a gene adjacent to *Dyrk1A*.

Kcnj6 expression is increased in mouse models of DS (Laffaire et al., 2009). Encoded by *Kcnj6*, the Kir3.2 subunit of inwardly rectifying potassium channels is widely expressed in brain regions important for cognition, including neocortex and hippocampus (Murer et al., 1997). Kir3.2 forms functional potassium channels as heterotetramers in combination with Kir3.1 and other subunits (Liao et al., 1996; Wischmeyer et al., 1997). The Kir3.2 subunit-containing potassium channels serve as effectors for a number of postsynaptic receptors, including GABAergic GABAB, serotoninergic 5HT-1A, cholinergic m2, and adrenergic A1 (Mark and Herlitze, 2000; Yamada et al., 1998). GABAB receptors mediate 'slow' type IPSCs *via* activation of Kir3.2 channels (Nicoll, 2004). Steady-state activation of Kir3.2-containing potassium channels also contributes to neuronal hyperpolarization (Luscher et al., 1997). Since potassium channels critically control neuronal hyperpolarization, abnormalities in Kir3.2 subunits are readily envisioned as significantly impacting brain functions. Consistent with this view, a missense mutation of *Kcnj6* altering the pore-forming domain of the potassium channel is responsible for the '*weaver*' phenotype, characterized by abnormal development of the cerebellum (Patil et al., 1995). In addition, a heterozygous deletion of three nucleotides or a missense mutation introducing a single amino acid change from glycine to serine in Kir3.2 subunit causes the Keppen-Lubinsky syndrome, a rare genetic disorder characterized by severe developmental delay, intellectual disability, and microcephaly (Basel-Vanagaite et al., 2009; Masotti et al., 2015).

In rodents, Kir3.2 is present in the neocortex, cerebellum, hippocampus, and thalamus as early as p5 (Fernandez-Alacid et al., 2011; Liesi et al., 2000). In the DS brain, Kir3.2 was detected at both 17 and 33 weeks of gestation (Thiery et al., 2003). Thus, increased *Kcnj6* gene dose and its Kir3.2 protein product could contribute to changes in brain function in both mouse models and people with DS as early as the developmental period. In adult Ts65Dn mice, Kir3.2 is overexpressed in many brain regions, including the neocortex and hippocampus (Harashima et al., 2006; Kleschevnikov et al., 2012b; Kleschevnikov et al., 2005). This change results in increased GABAB/Kir3.2 signaling, which plays a defining role in the increased inhibition observed in Ts65Dn mice (Best et al., 2012; Best et al., 2007; Deidda et al., 2015; Kleschevnikov et al., 2012b; Kleschevnikov et al., 2004; Mojabi et al., 2016).

Hippocampus-dependent long-term memory is impaired both in DS (Carlesimo et al., 1997; Vicari et al., 2000) and in mouse genetic models of DS (Escorihuela et al., 1998). In agreement with earlier findings (Fernandez et al., 2007; Kleschevnikov et al., 2012a), novel object recognition memory with the retention period of 24 h was significantly impaired in Ts65Dn mice containing 3 copies of *Kcnj6*. Remarkably, removal of an extra copy of *Kcnj6* restored both synaptic plasticity and long-term memory to the levels observed in 2N controls. This data points to increased expression of Kir3.2 as suppressing hippocampal synaptic plasticity and resulting in cognitive impairment. Complementing our findings documented herein for a necessary role played by *Kcnj6* dose in synaptic plasticity and cognition are studies in which the presence of 4–5 extra copies of *Kcnj6* in the genome of normal mice led to reduced synaptic plasticity and cognitive impairment reminiscent of DS (Cooper et al., 2012). Thus, the evidence points to increased *Kcnj6* dose as necessary and possibly sufficient for deficits in synaptic plasticity in some tests of cognition in models of DS.

In contrast to the evidence that increased *Kcnj6* dose contributes to deficits in synaptic plasticity and long-term memory, we found no evidence that it plays a role in working memory. Impaired working memory is characteristic of DS (Baddeley and Jarrold, 2007; Gathercole and Alloway, 2006; Jarrold and Baddeley, 2001; Lanfranchi et al., 2009) and is also abnormal in Ts65Dn and other genetic models of DS (Bimonte-Nelson et al., 2003; Dowdy-Sanders and Wenger, 2006; Kleschevnikov et al., 2012a; Whitney and Wenger, 2012). Though we failed to detect an effect of increased *Kcnj6* dose on working memory as examined in the Y-maze, we did observe normalization of exploratory behavior during Y-maze testing, suggesting that *Kcnj6* dose may play a role in exploration in the context of this test. We note that deficient working memory has not responded to most of the treatments restoring long-term memory in genetic models of DS (Kleschevnikov et al., 2012a; Lysenko et al., 2014). A likely explanation is that the deficiency in working memory is genetically and mechanistically distinct from the deficits in long-term memory and synaptic plasticity, as studied herein.

Pharmacological strategies to combat the effect of *Kcnj6* gene dose could be considered in an attempt to address changes in cognition in DS. Treatments to reduce the expression of *Kcnj6* or modify the trafficking of Kir3.2 subunits might be considered, but at this time, to our knowledge, these approaches are not being explored. In contrast, inhibiting the activation of GABAB receptors appears feasible (Cramer et al., 2010; Kleschevnikov et al., 2012a), as are attempts to develop antagonists of Kir3.2 containing channels. In this study, we asked if acute suppression of Kir3.2 channel signaling could rescue synaptic plasticity. Like certain other serotonin re-uptake inhibitors, fluoxetine is an effective blocker of the Kir3.2 subunit-containing potassium channels (Kobayashi et al., 2003, 2004). At the concentration used in our experiments (10 μM), fluoxetine is estimated to have blocked about 50% of the current through the Kir3.2 potassium channels. Fluoxetine improved both STP and LTP in the DG of Ts65Dn mice. Since fluoxetine is an inhibitor of serotonin reuptake, it raises the question of whether the finding may be due to increased serotonin levels. However, serotonin in the DG is reported to suppress LTP (Sakai and Tanaka, 1993). Thus, our findings are consistent with the effect of the drug on Kir3.2 channels. Interestingly, early treatment of neonatal (p3–p15) Ts65Dn mice with fluoxetine rescued neurogenesis and cognitive behavior (Bianchi et al., 2010). Although multiple effects may account for this change, suppression of Kir3.2 channels may contribute. Studies using selective inhibitors of Kir3.2 channels will be needed to confirm that pharmacological approaches can be used to explore and treat cognitive deficits in DS.

We conclude that increased expression of *Kcnj6* necessarily contributes to abnormal synaptic plasticity in the Ts65Ds mouse, raising the possibility that excessive signaling through Kir3.2 channels is implicated. Studies of the biology of Kir3.2 subunit-containing potassium channels and development of novel approaches to reduce the effect of increased Kir3.2 expression may offer a means to moderate cognitive deficits in DS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A role for Kcnj6 gene dose in Down syndrome was examined using Ts65Dn mice
- Reduction in Kcnj6 gene dose to normal restored hippocampal level of Kir3.2
- Reduction in Kcnj6 gene dose restored memory in the novel object recognition test
- Reduction in Kcnj6 gene dose restored STP and LTP in dentate gyrus
- Suppression of Kir3.2 channels with fluoxetine restored STP and LTP in DG

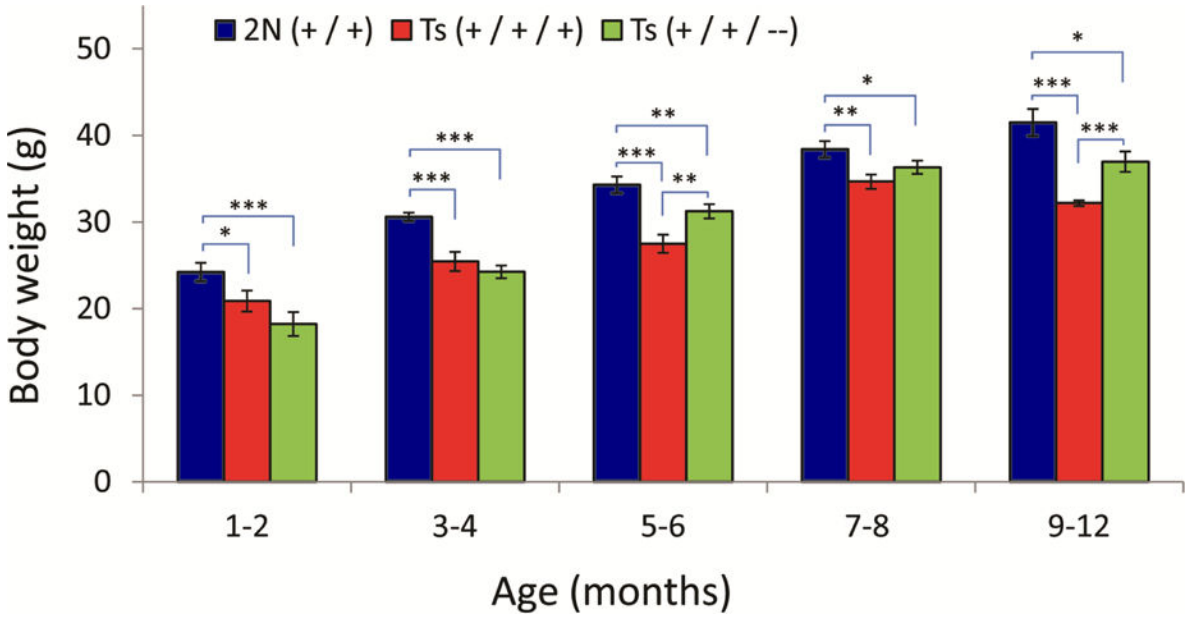

Fig. 1.

Changes in body weight. The body weight was reduced in both Ts65Dn:*Kcnj6*+++ and Ts65Dn:*Kcnj6*++− *vs.* 2N:*Kcnj6*++ mice at all ages. There was no difference between the body weight of Ts65Dn:*Kcnj6*+++ *vs.* Ts65Dn:*Kcnj6*++− in young animals (1–4 months). However, the body weight was increased in older (5–6 mo and 9–12 mo) Ts65Dn:*Kcnj6*++− *vs.* Ts65Dn:*Kcnj6*+++ mice. The animal numbers and other statistical parameters are given in Table 1.

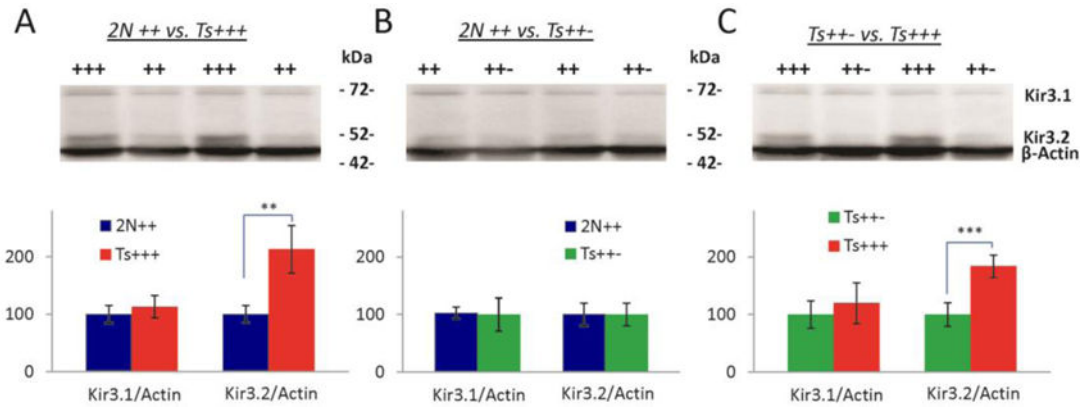

Fig. 2.

Hippocampal levels of proteins. A. Level of Kir3.1 was not altered, while level of Kir3.2 was increased in Ts65Dn:*Kcnj6*+++ ($n = 6$) *vs.* 2N:*Kcnj6*++ ($n = 5$) samples. B. There was no difference in the levels of either Kir3.1 or Kir3.2 in Ts65Dn:*Kcnj6*++− ($n = 5$) *vs.* 2N:*Kcnj6*++ ($n = 6$) samples. C. Kir3.1 level was not different, but Kir3.2 was increased in Ts65Dn:*Kcnj6*+++ ($n = 5$) *vs.* Ts65Dn:*Kcnj6*++− ($n = 6$) mice.

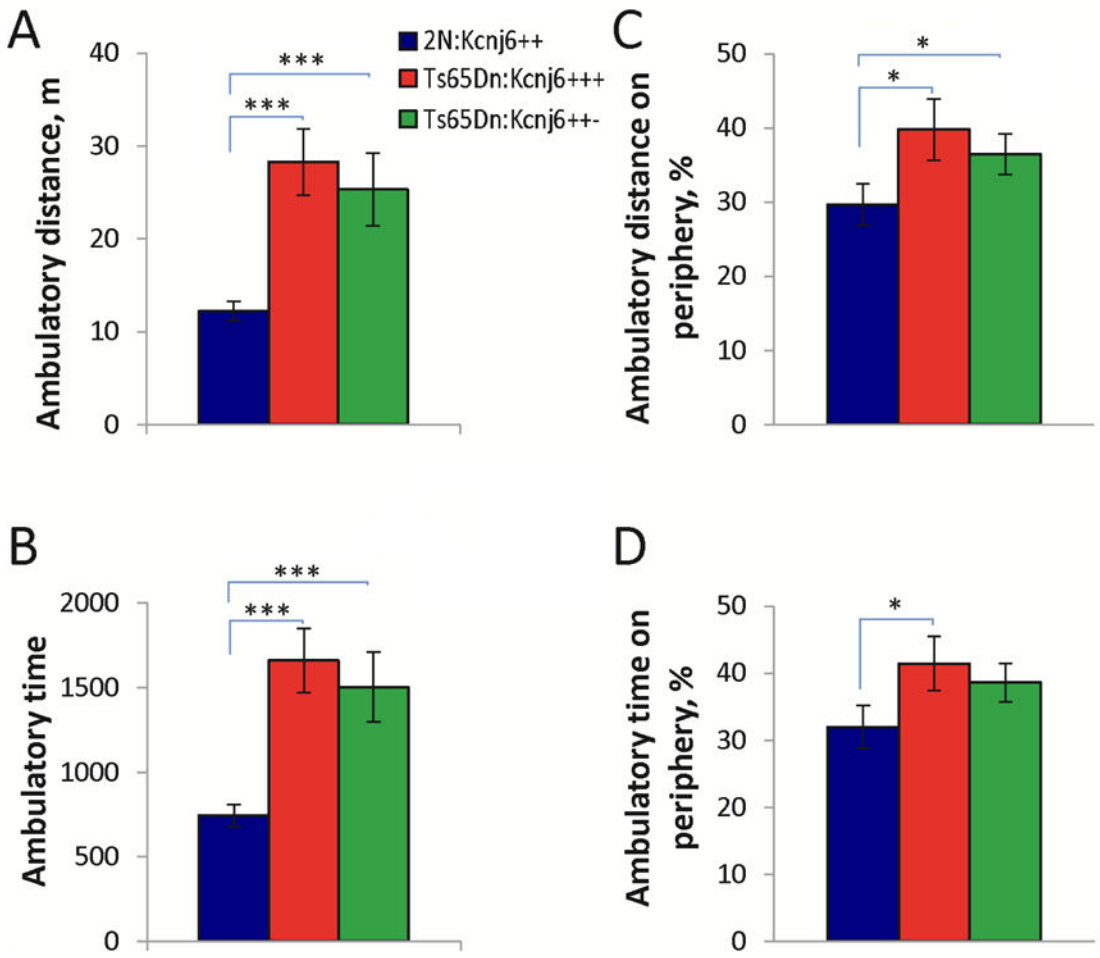

Fig. 3.

Spontaneous locomotion. A. Ambulatory distance was significantly increased in both Ts65Dn:*Kcnj6*+++ ($n = 12$) and Ts65Dn:*Kcnj6*++− ($n = 14$) *vs.* 2N:*Kcnj6*++ ($n = 19$) mice. No difference between Ts65Dn:*Kcnj6*+++ *vs.* Ts65Dn:*Kcnj6*++− mice was observed. B. Ambulatory time was also increased in both Ts65Dn:*Kcnj6*+++ and Ts65Dn:*Kcnj6*++− mice. C, D. Ambulatory distance and ambulatory time spent on periphery were greater in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice, but not different in Ts65Dn:*Kcnj6*++− *vs.* 2N:*Kcnj6*++ animals.

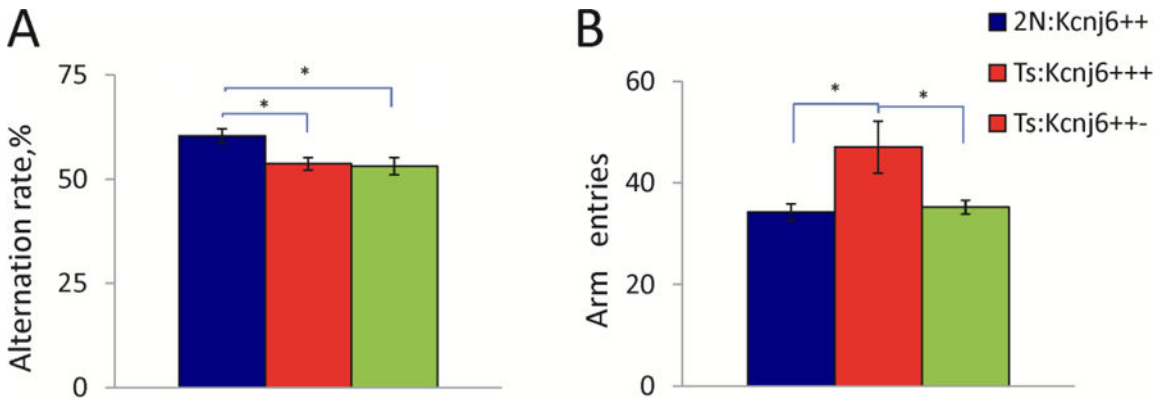

Fig. 4.

Working memory and exploratory activity in the Y-maze. A. The rate of spontaneous alternations was reduced in both Ts65Dn:*Kcnj6*+++ ($n = 12$) and Ts65Dn:*Kcnj6*++− ($n = 14$) *vs.* 2N:*Kcnj6*++ ($n = 19$) mice. Reduction of *Kcnj6* gene dose had no effect on performance of Ts65Dn mice ($p = 0.84$ for Ts65Dn:*Kcnj6*+++ *vs.* Ts65Dn:*Kcnj6*++−). B. Number of 'arm entries' during the Y-maze test was greater in Ts65Dn:*Kcnj6*+++ *vs.* 2N:*Kcnj6*++ mice. The number of arm entries was reduced in Ts65Dn:*Kcnj6*++− mice to the level of 2N:*Kcnj6*++ controls.

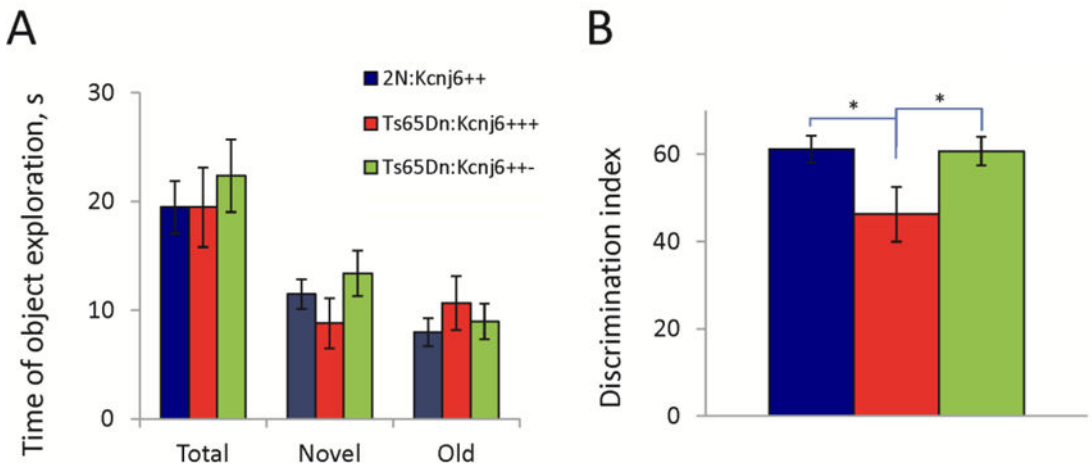

Fig. 5.

Long-term memory. Performance in the novel object recognition test with a retention period of 24 h. A: Time for object exploration was not different between genotypes. B: The discrimination index was reduced in Ts65Dn:*Kcnj6*+++ ($n = 12$) *vs.* 2N:*Kcnj6*++ ($n = 19$) mice, but it was increased in Ts65Dn:*Kcnj6*++− ($n = 14$) mice to the level observed in 2N:*Kcnj6*++ control mice.

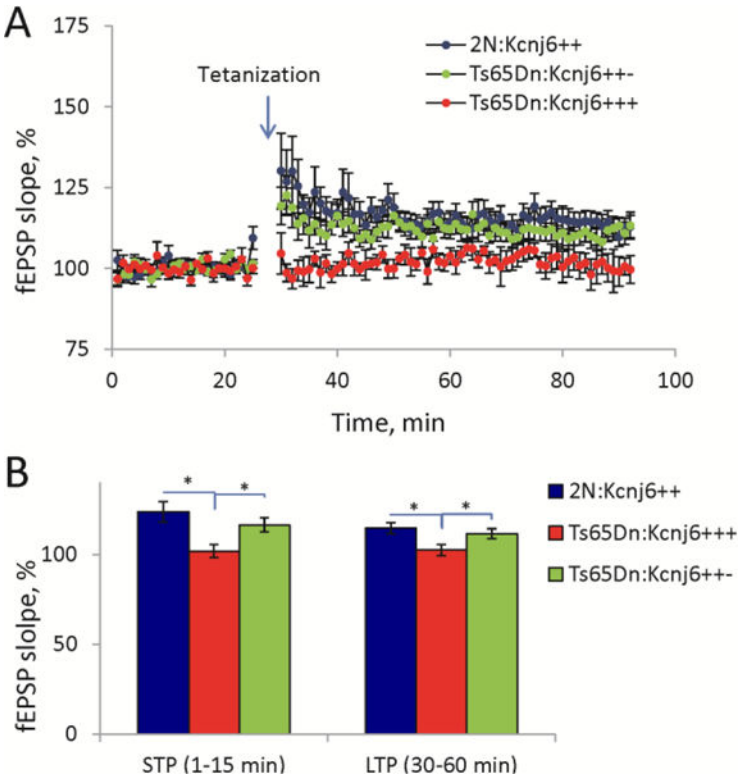

Fig. 6.

Synaptic plasticity: effect of the *Kcnj6* gene dose on STP and LTP in DG. A. Averaged changes in the initial slope of field EPSP during the experiment. B. Averaged values for STP (1–15 min) and LTP (30–60 min). STP and LTP were reduced in Ts65Dn:*Kcnj6*+++ (n = 8) *vs*. 2N:*Kcnj6*++ (n = 12) slices. Both parameters were normalized in slices from Ts65Dn:*Kcnj6*++− (n = 11) mice.

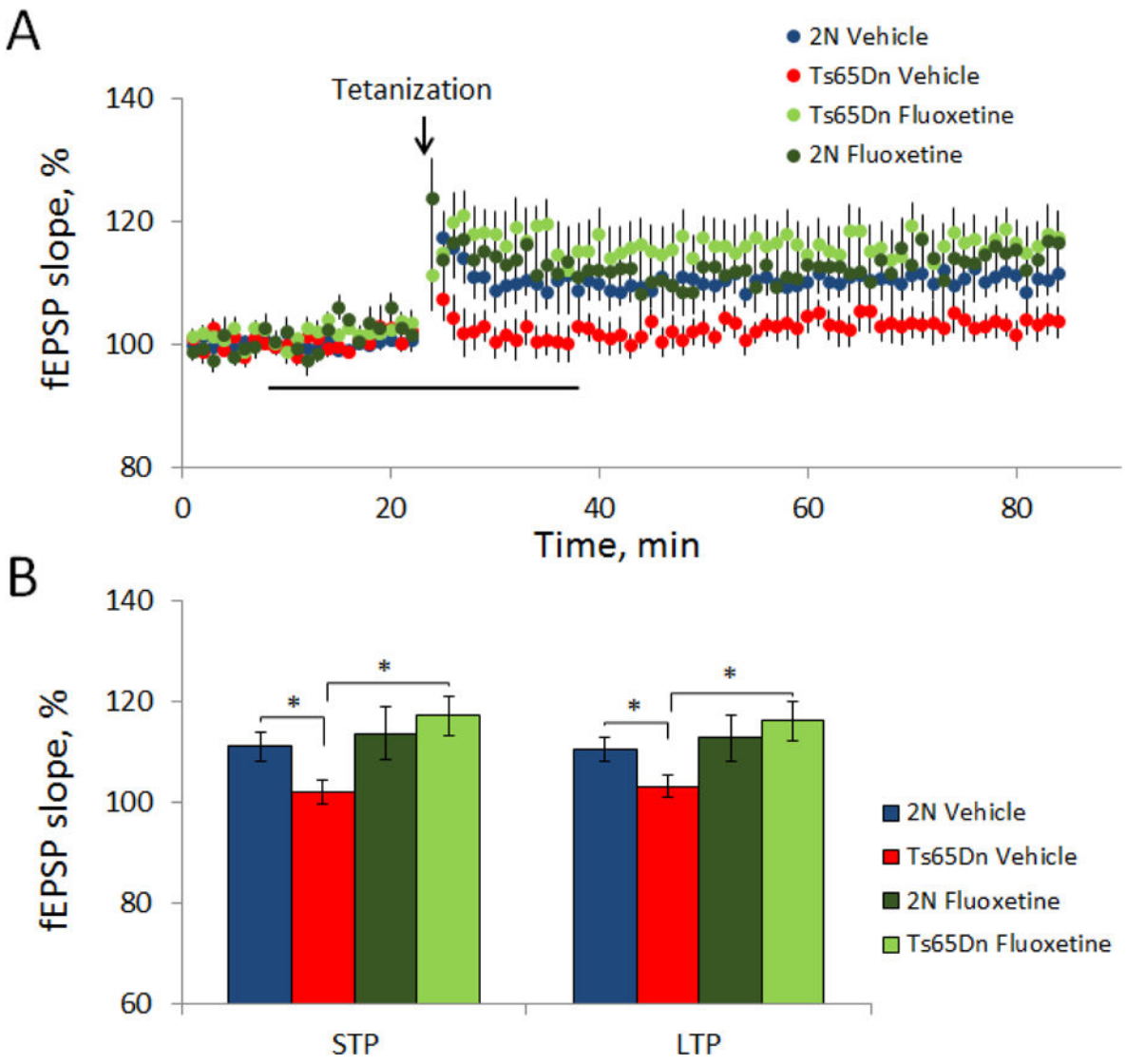

Fig. 7.

Synaptic plasticity: Effect of fluoxetine on STP and LTP in DG. A. Averaged changes in the initial slope of field EPSP during the experiment. Application of fluoxetine or vehicle marked by the black line under the graph. B. Averaged values for STP (1–15 min) and LTP (30–60 min). Both STP and LTP were reduced in Ts65Dn ($n = 14$) *vs.* 2N ($n = 13$) slices. Fluoxetine increased both parameters in Ts65Dn slices ($n = 10$) to control levels, but did not affect these parameters in 2N slices (n = 7).

Table 1

Statistical parameters for the body weight measurements

	2N:*Kcnj6*++ *vs*. Ts65Dn:*Kcnj6*+++			2N:*Kcnj6*++ *vs.* Ts65Dn:*Kcnj6*++−			Ts65Dn:*Kcnj6*+++ vs. Ts65Dn:*Kcnj6*++−		
Age (months)	**F**	**p**	**N ++/+++**	**F**	**p**	**N ++/++−**	**F**	**p**	**N +++/++−**
1–2	2.2	0.021*	13/9	3.6	0.001***	13/10	1.5	0.073	9/10
3–4	4.4	0.000***	30/28	7.4	0.000***	30/30	0.9	0.180	28/30
5–6	4.9	0.000***	18/13	2.5	0.009**	18/23	2.9	0.004**	13/23
7–8	3.0	0.002**	36/13	1.7	0.045*	36/14	1.5	0.068	13/14
9–12	5.9	0.000***	29/11	2.3	0.012*	29/21	4.0	0.000***	11/21