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Deficits in cognition and synaptic plasticity in a mouse model of Down syndrome ameliorated by GABA_B receptor antagonists

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Abstract

Cognitive impairment in Down syndrome (DS) is characterized by deficient learning and memory. Mouse genetic models of DS exhibit impaired cognition in hippocampally mediated behavioral tasks and reduced synaptic plasticity of hippocampal pathways. Enhanced efficiency of GABAergic neurotransmission was implicated in those changes. We have recently shown that signaling through postsynaptic GABA_B receptors is significantly increased in the dentate gyrus (DG) of Ts65Dn mice, a genetic model of DS. Here we examined a role for GABA_B receptors in cognitive deficits in DS by defining the effect of selective GABA_B receptor antagonists on behavior and synaptic plasticity of adult Ts65Dn mice. Treatment with the GABA_B receptor antagonist CGP55845 restored memory of Ts65Dn mice in the novel place recognition, novel object recognition and contextual fear conditioning tasks, but did not affect locomotion and performance in T-maze. The treatment increased hippocampal levels of brain-derived neurotrophic factor (BDNF), equally in 2N and Ts65Dn mice. In hippocampal slices, treatment with the GABA_B receptor antagonists CGP55845 or CGP52432 enhanced long-term potentiation (LTP) in the Ts65Dn DG. The enhancement of LTP was accompanied by an increase in the NMDA receptor-mediated component of the tetanus-evoked responses. These findings are evidence for a contribution of GABA_B receptors to changes in hippocampal-based cognition in the Ts65Dn mouse. The ability to rescue cognitive performance through treatment with selective GABA_B receptor antagonists motivates studies to further explore the therapeutic potential of these compounds in people with DS.

Keywords

Down syndrome; GABA_B receptors; Cognition; Contextual fear conditioning; Novel object recognition; Long-term potentiation

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Introduction

Intellectual deficiency in Down syndrome (DS) features abnormalities in learning (Gardiner et al., 2010; Lott and Dierssen, 2010) and, most notably, in hippocampus-dependent explicit memory (Carlesimo et al., 1997; Vicari, 2001). Morphological, physiological and behavioral data consistently point to impaired function of the hippocampus and dentate gyrus (DG) (Sylvester, 1983; Nadel, 2003; Haydar and Reeves, 2012). There is widespread agreement that deficits in memory contribute significantly to the overall burden of intellectual difficulties in people with DS (Contestabile et al., 2010).

Mouse genetic models have provided important insights into the genes and mechanisms responsible for DS-specific changes (Reeves, 2006; Hanson et al., 2007; Moore and Roper, 2007; Salehi et al., 2007; Gotti et al., 2011; Liu et al., 2011; Popov et al., 2011; Kleschevnikov et al., 2012b; Zhang et al., 2012). The Ts65Dn mouse bears an extra copy of approximately half the genes homologous to those on human chromosome 21 (Davisson et al., 1993; Olson et al., 2007; Sturgeon and Gardiner, 2011). These mice recapitulate a number of behavioral and physiological features of DS, including deficits in hippocampal function and impaired learning and memory (Holtzman et al., 1996; Dierssen et al., 2001; Hyde et al., 2001; Galdzicki and Siarey, 2003; Incerti et al., 2011; Shichiri et al., 2011). Long-term potentiation (LTP), a cellular model for learning and memory, is impaired in the CA1 and DG of Ts65Dn and other DS models (Siarey et al., 1997; Kleschevnikov et al., 2004; Costa and Grybko, 2005; Belichenko et al., 2007; Fernandez et al., 2007; Yu et al., 2010). These changes were linked to alterations in the properties of inhibitory neurotransmission (Kleschevnikov et al., 2004). Indeed, it was subsequently shown that hippocampal synaptic plasticity and cognition can be regulated by modulating inhibitory efficiency in mouse models of DS (Kleschevnikov et al., 2004; Costa and Grybko, 2005; Fernandez and Garner, 2007b; Rueda et al., 2008; Belichenko et al., 2009b; Begenisic et al., 2011; Braudeau et al., 2011b).

Recently we demonstrated that signaling through postsynaptic GABA_B receptors is significantly enhanced in the Ts65Dn DG (Kleschevnikov et al., 2012a). Other researchers observed that in the CA1 region the balance between GABA_A and GABA_B receptor-mediated inhibition is altered in favor of the latter in a pathway specific manner (Best et al., 2012). These changes were linked to DS-specific genetic alterations owing to triplication of *Kcnj6* and subsequent overexpression of Kir3.2 subunits of inwardly-rectifying potassium channels, postsynaptic effectors of the GABA_B receptors (Kleschevnikov et al., 2005; Harashima et al., 2006). The role of GABA_B receptors in cognitive impairment in DS has not been yet investigated behaviorally.

Herein we examine the effect of selective GABA_B receptor antagonists on cognition and synaptic plasticity of Ts65Dn mice. Suppression of GABA_B receptors significantly improved performance of Ts65Dn mice in behavioral tests for short-term and long-term memory, but did not affect locomotor activity and working memory. Significantly, the improvement in hippocampus-mediated behaviors seen *in vivo* was complemented by improved LTP and increased NMDA receptor-mediated responses after suppressing GABA_B receptors in hippocampal slices. Thus, GABA_B receptors represent a potential therapeutic target for reversing cognitive deficits in people with DS.

Materials and Methods

Animals

Segmental trisomy 16 (Ts65Dn) mice were obtained by mating female carriers of the 17¹⁶ chromosome (B6EiC3H-a/A-Ts65Dn) with (C57BL/6Jei X C3H/HeJ) F1 (JAX #JR1875)

males (Davisson et al., 1993). Ts65Dn mice are thus maintained on the B6/C3H background. Diploid (2N) littermate mice served as control. 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/quantpcr.html>) was used to measure expression of the *Mx1* gene, which is present in three copies in Ts65Dn. All mice were screened also for retinal degeneration due to *Pde6brd1* homozygosity (Bowes et al., 1993), and only animals free of retinal degeneration were used. Mice were males and housed 2 to 5 per cage with a 12 h light-dark cycle and *ad lib* access to food and water. The experiments were conducted in accordance with the National Institutes of Health guidelines and with an approved protocol from the Stanford University Institutional Animal Care and Use Committee.

Behavioral testing

All mice were exposed to behavioral tests starting at 2-3 months of age. Each mouse was handled for 5 min, twice a day, during the 7 days that preceded testing and for 3 days in between tests. All tests were performed during the light cycle between 7:00 a.m. and 7:00 p.m. To minimize olfactory cues from previous trials, each apparatus was thoroughly cleaned with 10% ethanol after each animal. On the day of testing, mice were left in their home cages in the room used for the experiment for 2 hours prior to the onset of the study for habituation. CGP55845 (0.5 mg/kg suspended in saline, 10 mL/kg), or equivalent volume of saline only, was injected intraperitoneally 2-3 hours before the testing. For chronic tests, the drug was injected once a day for 3 weeks. Body weight, which was measured weekly, was not affected by the treatment. All behavioral tests and procedures were performed by personnel blind to both genotype and treatment group.

Spontaneous locomotor activity was evaluated in square Plexiglas chambers ($43.2 \times 43.2 \times 20$ cm) equipped with three planes of infrared detectors (Med Associates Inc, St. Albans, VT). The area of the chamber was divided virtually on center (20cm x 20cm, zone 2) and periphery (the rest of the chamber, zone 1). The activity chamber was located within sound-attenuating boxes ($66 \times 55.9 \times 55.9$ cm). For testing, an animal was placed in the center of the testing arena under bright ambient light and allowed to move freely for 10 minutes. The movements were monitored by an automated tracking system (Med Associates Activity Monitor, version 5.93.773).

T-maze testing was performed as previously described (Belichenko et al., 2009a; Faizi et al., 2011). The T-maze had three equal arms (30 cm length, 10 cm width, and 20 cm height). It was made of opaque acrylic (Plexiglas) and had three gates: in the start arm and in the two goal arms. A mouse was placed at the beginning of the start arm, with its back to the closed door. The trial started with opening of the start door, after which the mouse ran down the start arm to choose the right or left goal arm. After the mouse had entered one goal arm, defined as the entry of all four limbs, the door to the other goal arm was closed. This allowed the mouse to explore the arm entered and to return to the start arm. When the mouse eventually returned to the start arm, the start door was closed and the doors of the goal arms were then opened. The procedure was repeated 11 times (i.e. 10 alternations) for 3 consecutive days, for a total of 30 alternations. The spontaneous alternation score was defined as the number of left-right and right-left alternations, expressed as a percentage of the total number of possible alternations.

Novel object recognition and *novel place recognition* were 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 light conditions. The activity of mice was recorded with a video camera. Each test had two phases – acquisition and testing. For acquisition, two identical objects were

placed in diagonally opposite corners of the chamber, 8-9 cm from the walls. A mouse was placed at the mid-point between the objects. After allowing for 10 min exploring the objects, the mouse was returned to the colony. The testing was performed 10 min or 24 hours after the acquisition. In the tests for place recognition, one of the objects was moved in a new location in another corner of the chamber, while the other object remained at its former spatial location. In the tests for novel object recognition, one of the objects was replaced with a novel object of the same height and volume, but different shape and appearance. For testing, the mouse was again placed in the chamber to explore the objects for 3 min. The amount of time spent exploring each object (nose sniffing and head orientation within <1.0 cm) was recorded and evaluated by operators blinded to genotype and treatment. The discrimination index was computed as $R(\%) = T_{new} * 100\% / (T_{new} + T_{old})$, where T_{new} is the time spent exploring the new or moved object, and T_{old} is the time spent exploring the familiar or unmoved object.

Fear conditioning

The test was performed using chambers from Coulbourn Instruments (Whitehall, PA). Day 1 training consisted of five pairs of tone and electrical foot shocks given in the conditioning context. Once placed in the conditioning chamber, mice were allowed to freely explore for 3 min. Then a 20 s tone (2 kHz; 70 dB) was played that co-terminated with a foot shock (0.5 mA, 50 Hz, 2 s). This procedure was repeated five times with a 1-min interval. Mice remained in the chamber for 30 s following the last shock. On day 2 (24 h after the training), a recent tone fear memory was measured in a distinct chamber that had new olfactory, tactile and visual cues. The mice were placed in this new chamber for 3 min and were subsequently presented three tones identical to those used in the training session. On day 3 (48 h after the training), a recent contextual fear memory was tested by placing the mice in the conditioning chamber for 3 min. Freezing, defined as the complete lack of motion for a minimum of 0.75 s, was recorded with a video camera and analyzed by FreezeFrame software (Actimetrics, Evanston, IL). The percent of freezing during each period of the experiment was reported.

Western blot

The hippocampi were dissected on an ice-cold preparation table and homogenized in RIPA buffer (50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF) with 1 mg/mL protease inhibitor cocktail (aprotinin, leupeptin, pepstatin). The protein concentrations were determined using BCA protein Assay kit (Pierce, Rockford, IL). β -Tubulin was used as a reference protein. 20 μ L (1 mg/mL) of total protein per lane were loaded onto precast 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA), and separated by electrophoresis at 90 volts for ~2 hours. Proteins were transferred to a low-fluorescent, hydrophobic polyvinylidene difluoride (PVDF) membrane, with 0.2 μ m pore size (Amersham Hybond-LFP, GE Healthcare, UK), and the membranes were blocked with 4% nonfat milk in TBS-T solution (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.6). Membranes were then incubated with rabbit polyclonal primary antibody anti-BDNF (sc-546 (N-20), 1:200, Santa Cruz), or mouse monoclonal antibody anti- β -tubulin (1:2000, Sigma). The blots were washed in TBS-T (3 times x 10 min) followed by incubation with goat anti-rabbit IgG-HRP conjugates at a dilution of 1:5000. The blots were washed in TBS-T and then developed with SuperSignal (Pierce, Rockford, IL). Immunoblots were scanned with imaging system (Molecular Imager ChemiDoc XRS⁺, Bio-Rad, USA) and the images were analyzed with ImageJ (NIH, USA).

Electrophysiology

Transverse hippocampal slices were prepared as previously described (Salehi et al., 2009; Kleschevnikov et al., 2012a). Adult (3-5 month-old) male mice were 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 CaCl₂, 1.3 mM MgSO₄, 1 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM glucose, osmolarity 310 mOsm, continuously bubbled with 95% O₂-5% CO₂, pH 7.4. The hippocampus was extracted and cut in ice-cold ACSF with a vibratome (Leica 1000) into 350- μ m-thick slices, which 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 recording submerged chamber and superfused with ACSF at a constant rate of 2.5 ml/min at 32°C. Recording electrodes were made of borosilicate glass capillaries (1B150F, World Precision Instruments, Sarasota, FL) and filled with 2M NaCl (resistance 0.3-0.5 M Ω). Monopolar stimulating electrodes were made of Pt/Ir wires of diameter 25.4 μ m (PTT0110, World Precision Instruments, Sarasota, FL) and had 100- μ m-long exposed tips. The stimulating electrode was inserted under visual control perpendicular to the slice surface into the middle molecular layer (MML); the recording electrode was inserted into the granule cell layer of the DG upper blade. The distance between the electrodes was 250-300 μ m. The initial slope of field excitatory postsynaptic potential (fEPSP) was measured at latencies 0.1 - 0.9 ms. Testing stimuli (duration 100 μ s, currents 80-100 μ A) evoked field responses with amplitudes of 70-80% of maximum. LTP was induced by tetanizations consisting of 4 trains of stimuli (0.5 s x 100 Hz), applied with a 10 s interval.

Chemicals

GABA_B receptor antagonists CGP52432 and CGP55845 were purchased from Tocris Cookson Inc (Ellisville, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Statistics

Two-way ANOVA followed by Turkey's *post hoc* test was used to evaluate the data. The data are presented as mean \pm SEM.

Results

GABA_B receptor antagonist treatment rescued cognitive deficits of Ts65Dn mice in hippocampally mediated memory tasks

Balance between excitatory and inhibitory neurotransmission is a necessary pre-requisite for activity-dependent synaptic plasticity and, hence, efficient learning and memory. Previous studies in Ts65Dn mice pointed to enhanced GABAergic signaling as possibly responsible for reduced learning and memory. Because enhancement in GABAergic signaling is due, at least in part, to excessive activation of GABA_B receptors, we tested the possibility that antagonists of the GABA_B receptors could improve learning and memory in mouse models of DS. Thus, we examined what effect if any might result from inhibiting GABA_B receptors by treating Ts65Dn and 2N mice with the high affinity selective GABA_B receptor antagonist CGP55845 (0.5 mg/kg, i.p. injections). Possible drug effects on locomotor activity and general exploratory habits were first evaluated. This was followed by evaluation of the drug effects on working, short-term and long-term memory.

Spontaneous locomotor activity—It was previously observed in mice that GABA_B receptor antagonists, administered at high doses, may increase spontaneous locomotor activity (Colombo et al., 2001). Such changes would influence general exploratory habits and, perhaps, performance of animals in tests for learning and memory. To monitor possible changes in locomotion, the mice were tested in the activity chamber three times: before administration of the drug (baseline), and during the acute (1-2 days) and chronic (3 weeks)

phases of treatment. In all three tests, Ts65Dn mice showed significantly greater locomotor activity than 2N controls (Fig. 1). The most notable difference was observed for distance traveled (Fig. 1A-C) and ambulatory time (Fig. 1E), both of which were approximately twice greater in Ts65Dn than in 2N mice. Average velocity of movement also was significantly greater in Ts65Dn mice (Fig. 1D), while resting time was reduced (Fig. 1F). Neither acute nor chronic treatment with the GABA_B receptor antagonist had an effect on these measures. E.g., total distance traveled, distance travelled at the periphery (zone 1) or distance travelled in the center (zone 2) of the activity chamber showed no difference between the CGP and Veh groups during the acute (Fig. 1B) and chronic (Fig. 1C) phases of treatment in both 2N and Ts65Dn mice. Also, the ratio of the distances traveled in zones 1 and 2 (zone1/zone 2) was not different in Ts65Dn vs. 2N mice ($p = 0.36$), and it was not affected by the treatment in both 2N and Ts65Dn ($p = 0.24-0.98$). Interestingly, the distance moved by Ts65Dn and 2N mice during the first minute was smaller in baseline test (Fig. 1A) than in acute and chronic tests (Fig. 1B, C), but this change was similar for the groups treated with CGP and vehicle. Thus, suppression of GABA_B receptors had no effect on locomotion or exploratory habits of either Ts65Dn or 2N mice.

Working memory—Working memory was evaluated by measuring the rate of spontaneous alternations in T-maze. Consistent with previous findings, Ts65Dn mice showed a severe deficit in this behavioral task (Fig. 2). Thus, the baseline rate of spontaneous alternations was in 2N: $66.5 \pm 2.9\%$, $n = 25$, and in Ts65Dn: $49.9 \pm 2.4\%$, $n = 26$, $p = 0.001$. Treatment with CGP55845 had no effect on performance of Ts65Dn or 2N mice; the alternation rates following the acute and the chronic treatment did not differ from the baseline (Fig. 2). Thus, working memory of Ts65Dn and 2N mice was not affected by the antagonist of the GABA_B receptors.

Short-term memory—Short-term memory was evaluated using two behavioral paradigms: novel place recognition and novel object recognition, both tasks with retention period of 10 min (Fig. 3A, B). Although both these tasks require involvement of the hippocampus, the place recognition paradigm is expectedly more sensitive to hippocampal dysfunction due to the necessity of spatial orientation during the test. The acquisition phase was similar in both tasks: a mouse was placed in the center of the acquisition box and allowed for 10-min to investigate two identical objects. All groups of Ts65Dn and 2N mice, treated with the drug or vehicle, spent on average equal time investigating the objects (Fig. 3C, E). To test for side preference, the discrimination indexes were computed for the acquisition phase. The averaged values of the indexes were not different from 50% for all groups of animals suggesting no side preference during the acquisition phase ($p = 0.4-0.9$). These data suggest that 2N and Ts65Dn mice were equal in attention paid to the objects, and that treatment had no effect on baseline exploratory behavior.

After the retention period, in the place recognition task one of the objects was moved in a new location and the mouse was again allowed to explore for 3-min (Fig. 3A, testing). Total time spent on exploration of objects during this phase was again similar in all groups of animals: 2N Veh 45.5 ± 4.5 s; 2N CGP 46.2 ± 4.1 s; Ts65Dn Veh 42.9 ± 4.6 s and Ts65Dn CGP 41.9 ± 5.6 s ($p = 0.4-0.9$). However, the discrimination index was significantly smaller in Ts65Dn Veh than in 2N Veh group ($p = 0.016$) (Fig. 3D), suggesting an impairment of the short-term memory. Treatment with CGP55845 improved performance of Ts65Dn mice. Indeed, Ts65Dn CGP group performed significantly better than Ts65Dn Veh mice ($p = 0.027$) and did not differ from the 2N CGP littermates ($p = 0.77$) (Fig. 3D). Thus, hippocampus-dependent short-term memory for place recognition was impaired in Ts65Dn mice, and it was restored by the GABA_B receptor antagonist.

In the object recognition task, the testing was performed after replacement of one familiar object with a novel one (Fig. 3*B*). Similar to the previous test, all groups spent equal time exploring the objects ($p = 0.4-0.7$). However, in difference with the place recognition task, Ts65Dn mice showed no deficit in this behavioral test. Thus, the discrimination indexes were: 2N Veh 63.8 ± 3.9 ; Ts65Dn Veh 61.9 ± 4.9 ($p = 0.38$) (Fig. 3*F*). Treatment with CGP55845 had no effect on performance of Ts65Dn ($p = 0.67$) or 2N mice ($p = 0.65$) (Fig. 3*F*). Thus, short-term object recognition memory was spared in Ts65Dn mice, and it was not affected by the treatment.

Long-term memory—To evaluate effects of CGP55845 on long-term memory, we used two tests: novel object recognition with a retention period of 24 h, and contextual fear conditioning with testing of cued and contextual types of memory 24 h and 48 h after the acquisition respectively (Fig. 4).

As was demonstrated previously, novel object recognition tests with prolong (hours-days) retention periods depend on function of the hippocampus and, in part, the DG (Squire et al., 2007; Jessberger et al., 2009). Both acute (1-2 days) and chronic (3 weeks) effects of CGP55845 on novel object recognition have been examined (Fig. 4 *A, B*). Of note, the acute and chronic tests were performed using different cohorts of animals and different sets of objects. Similar to previous, all groups of Ts65Dn and 2N mice, treated with CGP55845 or vehicle, spent on average equal time exploring the objects during both the acquisition ($p = 0.4-0.8$) and the testing ($p = 0.35-0.75$) phases (Fig. 4 *A, B*). Testing was performed 24 h after the acquisition and showed severe impairment of Ts65Dn mice after both the acute and the chronic treatment. Indeed, averaged discrimination indexes for 2N Veh vs. Ts65Dn Veh groups were $58.6 \pm 2.1\%$ vs. $49.9 \pm 3.2\%$ ($p = 0.011$) for the acute test, and $71.5 \pm 3.1\%$ vs. $55.9 \pm 4.2\%$ ($p = 0.006$) for the chronic test respectively. Treatment with CGP55845 improved performance of Ts65Dn mice in both the acute and chronic tests (Fig. 4*A, B*). For the acute test, the discrimination indexes in the 2N CGP vs. Ts65Dn CGP group were: $59.7 \pm 1.7\%$ vs. $60.1 \pm 2.9\%$ ($p = 0.89$), and for the chronic test $67.4 \pm 3.5\%$ vs. $65.5 \pm 3.7\%$ ($p = 0.67$) respectively. Also, Ts65Dn CGP group performed significantly better than the Ts65Dn Veh group in both the acute ($p = 0.013$) and chronic ($p = 0.044$) tests. Thus, both acute and chronic treatment with CGP55845 improved long-term object recognition memory in Ts65Dn mice.

We next used the contextual fear conditioning test allowing for estimation of both hippocampus-independent (cued) and hippocampus-dependent (contextual) memory (Mchugh et al., 2007). After training on day 1, Ts65Dn and 2N mice underwent a test of cued learning on day 2 and contextual learning on day 3 (Fig. 4 *C*). We found that cued memory was normal in Ts65Dn mice. Indeed, total freezing on day 2 was in 2N Veh vs. Ts65Dn Veh group: $24.7 \pm 3.2\%$ vs. $22.1 \pm 3.5\%$ ($p = 0.25$). In contrast, contextual fear memory was significantly impaired in Ts65Dn mice; total freezing on day 3 in 2N Veh vs. Ts65Dn Veh mice: $29.7 \pm 4.6\%$ vs. $17.6 \pm 3.6\%$ ($p = 0.039$). Remarkably, treatment with the GABA_B antagonist CGP55845 improved contextual fear memory in Ts65Dn mice to the level seen in 2N mice. Total freezing on day 3 in 2N CGP vs. Ts65Dn CGP groups was: $24.6 \pm 3.3\%$ vs. $27.9 \pm 6.1\%$ ($p = 0.61$). The difference between Ts65Dn Veh and CGP groups also had a tendency for improvement, but the difference did not reach the level of significance ($p = 0.07$). Thus, suppressing GABA_B receptors improved long-term contextual fear memory in Ts65Dn mice.

GABA_B receptor antagonists increased hippocampal levels of BDNF

Enhancement of cognition by GABA_B receptor antagonists can involve a number of mechanisms. It was reported that GABA_B antagonists increase hippocampal levels of the

brain-derived neurotrophic factor (BDNF) (Heese et al., 2000), which is required for normal cognition (Egan et al., 2003; Nagahara and Tuszyński, 2011). To test whether or not involvement of this mechanism can explain the improvement of cognition in Ts65Dn mice, we examined BDNF levels in the hippocampus of Ts65Dn and 2N mice following administration of vehicle or CGP55845 (0.5 mg/kg, i.p. injections). Hippocampal samples were collected 2 h after the injections. Levels of both pro-BDNF (~32 kDa) and mature BDNF (~15 kDa) were similar in 2N and Ts65Dn mice treated with the vehicle. Treatment with CGP55845 increased levels of BDNF by about 30%, but the effects were similar in 2N and Ts65Dn mice (Fig. 5A, B). Levels of pro-BDNF were not affected by the treatment (Fig. 5, C). Thus, enhancement of BDNF may contribute to the memory-enhancing efficiency of GABA_B receptor antagonists. However, this effect is not specific for DS and, therefore, it cannot explain selective improvement of cognition in Ts65Dn mice. This result suggests that other mechanisms that have selectivity for DS models should be involved in the improvement of cognition following treatment with the GABA_B receptor antagonists.

GABA_B receptor antagonists enhanced LTP in the Ts65Dn DG

Improvement of long-term and short-term memory, with little or no effect on locomotion and working memory, suggests that GABA_B receptor antagonists could act through enhancement of long-term synaptic plasticity. To test this suggestion, we used hippocampal slices to examine effects of GABA_B receptor antagonists on LTP. The experiments were performed in the middle molecular layer of the dentate gyrus, a brain region involved in the novel object recognition (Jessberger et al., 2009) and contextual part of fear conditioning memory (Mchugh et al., 2007). Effects of two concentrations (0.1 μM and 1.0 μM) of the GABA_B receptor antagonist CGP55845 were explored. GABA_B receptor antagonists have ~5-7 times greater potency for postsynaptic than for presynaptic receptors (Pozza et al., 1999). For CGP55845, the reported IC₅₀ values for post- and presynaptic GABA_B receptors in hippocampal slices are 0.11 μM and 0.74 μM respectively (Pozza et al., 1999). Thus, at the lower concentration of CGP55845 used, one would expect to suppress mostly postsynaptic receptors; at the higher concentration, both post- and presynaptic receptors would be affected. We observed that both concentrations of CGP55845 enhanced LTP in Ts65Dn DG (Fig. 6A, B). This result suggests that suppression of postsynaptic GABA_B receptors plays critical role in enhancement of LTP in Ts65Dn DG. Interestingly, only the higher concentration of CGP55845 was effective in 2N slices (Fig. 6B). We tested also the effect of CGP52432, a high-affinity selective GABA_B receptor antagonist with potency lower than CGP55845. At concentration of 1 μM, this drug enhanced LTP in the Ts65Dn, but not in 2N slices (Fig. 6C). Thus, suppression of GABA_B receptors restores LTP in Ts65Dn DG.

Possible mechanisms for LTP enhancement by GABA_B antagonists

Induction of LTP in the DG strongly depends on activation of NMDA receptors. Thus, one mechanism by which GABA_B antagonists could increase LTP is through greater depolarization of neurons during tetanus allowing for increased activation of the NMDA receptors. To explore this possibility, we first examined the effect of CGP52432 (1 μM) on field responses during tetanization (Fig. 7). Suppression of GABA_B receptors significantly increased the tetanus-evoked field responses in both 2N and Ts65Dn slices (Fig. 7A). To compare changes in Ts65Dn and 2N slices, tetanus-evoked responses were averaged for each ten consecutive pulses in the 50-pulse train (marked by horizontal line with triangles in Fig. 7A) and scaled to the averaged magnitude of the first ten responses evoked at the baseline conditions. In the absence of the drug, the responses evoked at the end of the tetanization train were smaller in Ts65Dn than in 2N slices (Fig. 7B, white vs. black bars). The difference between 2N and Ts65Dn slices diminished and became insignificant ($p > 0.2$) in slices treated with CGP52432 (Fig. 7B, light grey vs. dark grey bars). Since the

amplitude of field responses reflects depolarization during tetanus, these data demonstrate that suppression of the GABA_B receptors enhanced tetanus-evoked depolarization in Ts65Dn slices to the level of depolarization in 2N slices. Such changes could possibly allow for increased activation of the NMDA receptors in Ts65Dn DG.

We next examined directly the effect of CGP52432 on the NMDA receptor-mediated component of the tetanus-evoked responses (Fig. 8). The NMDA receptor-mediated component was measured as the difference between the responses recorded before and during application of the selective NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV, 50 μ M). These measurements were carried out before, and then during, application of CGP52432 (1 μ M). Because the first tetanization could affect the responses by inducing LTP, two tetanizations with a 10-min interval were applied, and the responses evoked by the second tetanus were used for analysis. At baseline conditions, the NMDA-receptor mediated responses during the tetanus (marked by horizontal line with triangles in Fig. 8A) were smaller in Ts65Dn slices by about 60% (Fig. 8A). On average, the magnitude of the responses was in 2N: $100 \pm 9.8\%$, $n = 7$; in Ts65Dn: $42.72 \pm 3.5\%$, $n = 5$ ($p = 0.012$). Application of CGP enhanced the NMDA receptor-mediated responses and eliminated the difference between 2N and Ts65Dn slices (2N: 174.5 ± 31.8 , $n = 7$; Ts65Dn: 121.6 ± 28.9 , $n = 5$; $p = 0.69$) (Fig. 8B). These data are evidence that one mechanism by which GABA_B receptor antagonists restore LTP in Ts65Dn DG is through increased depolarization of neurons during tetanus allowing for a more effective activation of the NMDA receptors.

Discussion

An important characteristic of mental retardation in Down syndrome (DS) is impairment of learning and memory. Studies of mouse genetic models have revealed that deficient learning in DS could be a result of reduced synaptic plasticity and that increased efficiency of GABAergic neurotransmission could play critical role in these abnormalities. Here we present the first evidence that antagonists of metabotropic GABA_B receptors effectively improve cognition in a mouse model of DS. Administration of the GABA_B receptor antagonist CGP55845 resulted in improved performance of Ts65Dn mice in the place recognition test with a retention period of 10 min, and in the novel object recognition and contextual fear conditioning tasks with retention periods of 24 h and 48 h respectively. The treatment had no effect on locomotion, working memory and novel object recognition with a retention period 10 min. The treatment increased hippocampal levels of BDNF, similarly in 2N and Ts65Dn mice. Experiments on hippocampal slices showed that suppression of the GABA_B receptors restored LTP and increased the NMDA receptor-mediated component of tetanus-evoked responses in Ts65Dn DG. These results point to antagonists of GABA_B receptors as potential medicines for improving cognition in DS.

Enhancement of inhibitory efficiency in DS model mice was first suggested based on electrophysiological evidence (Kleschevnikov et al., 2004). Subsequent histological, morphological and biochemical studies demonstrated numerous findings consistent with an imbalance of excitation and inhibition that favors the latter in DS model mice. Thus, it was shown that immunoreactivity of several proteins associated with GABAergic synapses, including GABA_A receptor-associated protein (GABARAP), neuroligin 2 and vesicular GABA transporter (VGAT), was increased in Ts65Dn mice (Belichenko et al., 2009b). These changes were accompanied by alterations in the microcircuitry of inhibitory synaptic connections in Ts65Dn DG (Belichenko et al., 2004). Profound changes in the properties of inhibition were observed in the hippocampus and neocortex of neonatal Ts65Dn mice (Chakrabarti et al., 2010; Mitra et al., 2012), and the level of GAD-67 was increased in the Ts65Dn neocortex (Perez-Cremades et al., 2010). Interestingly, in contrast to inhibition,

properties of excitatory glutamatergic neurotransmission appear to be spared in the hippocampus and DG of Ts65Dn mice (Siarey et al., 1997; Kleschevnikov et al., 2004; Costa and Grybko, 2005; Belichenko et al., 2009b).

Increased efficiency of the inhibitory system suggests that one strategy to improve synaptic plasticity and, perhaps, cognition in DS would be to reduce the inhibition. Indeed, non-selective antagonists of ionotropic GABA_A receptors restored LTP (Kleschevnikov et al., 2004; Fernandez et al., 2007) and improved learning and memory (Fernandez and Garner, 2007b; Fernandez et al., 2007; Rueda et al., 2008) in Ts65Dn mice. Improvement of learning in Ts65Dn mice was recently demonstrated with $\alpha 5$ subunit-selective inverse agonists of the GABA_A receptors (Braudeau et al., 2011a; Braudeau et al., 2011b). One such compound is currently tested in a clinical trial (clinical identifier NCT01436955;

<http://www.clinicaltrials.gov>). Finally, improvement of cognition in Ts65Dn mice following environmental enrichment was also linked to a decrease in GABAergic inhibition (Begenisic et al., 2011).

Here we tested another approach aimed at reducing inhibitory efficiency - blockade of metabotropic GABA_B receptors. Postsynaptic GABA_B receptors use as effectors inwardly-rectifying potassium channels containing Kir3.2 subunits (Luscher et al., 1997), which gene, *Kcnj6*, is triplicated in DS. Owing to this genetic alteration, the level of the Kir3.2 protein is increased in Ts65Dn hippocampus by about 50% (Kleschevnikov et al., 2005; Harashima et al., 2006; Kleschevnikov et al., 2012a). As a result, postsynaptic GABA_B/Kir3.2 signaling is significantly enhanced (Best et al., 2007; Cramer et al., 2010; Best et al., 2012; Kleschevnikov et al., 2012a). Importantly, GABA_B receptors and Kir3.2 subunits are co-localized at extrasynaptic locations and around putative glutamatergic synapses (Kulik et al., 2003; Kulik et al., 2006), where their presence may influence the efficiency of the excitatory neurotransmission during repetitive stimuli. Both GABA_B receptors and Kir3.2 subunits are highly expressed in the hippocampus and neocortex (Murer et al., 1997; Margeta-Mitrovic et al., 1999; Charles et al., 2001; Harashima et al., 2006), brain regions critically involved in learning. It was recently shown that *Kcnj6* triploid mice exhibit deficits in hippocampal dependent learning and memory, as well as altered synaptic plasticity (Cooper et al., 2012). Thus, GABA_B receptors may represent a promising target for pharmacotherapy of cognitive abnormality in DS.

To examine effects of GABA_B receptor antagonists on cognition, we used CGP55845, a high-affinity selective GABA_B antagonist exhibiting behavioral effects after i.p. injections (Getova and Bowery, 1998; Getova and Bowery, 2001). Suppression of GABA_B receptors did not affect locomotion in 2N and Ts65Dn mice. This result suggests that the enhanced hyperactivity of Ts65Dn mice is not caused by increased GABA_B/Kir3.2 signaling owing to triplication of the *Kcnj6* gene and, for this matter, cannot be compensated by administration of GABA_B receptor antagonists. Consistent with this observation, Ts1Cje mice, which also have three copies of *Kcnj6*, did not exhibit hyperactivity (Sago et al., 2000).

Treatment with CGP55845 significantly improved performance of Ts65Dn mice in tasks requiring long-term memory. Novel object recognition with retention periods of 24 h depends in part on the hippocampus and DG (Squire et al., 2007; Jessberger et al., 2009). It was previously observed that Ts65Dn mice are severely impaired in this test (Fernandez et al., 2007), although results in other DS models were contradictory (Fernandez and Garner, 2007a; Belichenko et al., 2009a). We confirmed a severe deficit of Ts65Dn mice in this task and found that treatment with CGP55845 fully reversed the deficit. Contextual fear conditioning also showed a severe abnormality in Ts65Dn mice (Costa et al., 2008; Salehi et al., 2009; Faizi et al., 2011). Treatment with CGP55845 reversed deficits in the

hippocampus-dependent contextual memory and allowed Ts65Dn mice to perform at the level of 2N controls.

Testing of short-term memory showed mixed results: a severe deficiency of Ts65Dn mice in place recognition task, but no change in object recognition. Because dependence from the hippocampus is stronger for place than for object recognition (Barker and Warburton, 2011), especially for short retention periods (Jessberger et al., 2009), this result additionally confirms hippocampal dysfunction in DS. Treatment with CGP55845 restored performance of Ts65Dn mice in the place recognition task. Interestingly, although working memory was also abnormal in Ts65Dn mice, it was not affected by the treatment. Thus, Ts65Dn mice mostly exhibited deficiency in hippocampus-dependent types of memory, and suppression of GABA_B receptors restored the short-term and long-term types of memory.

One mechanism by which antagonists of GABA_B receptors might improve cognition is through elevation of hippocampal levels of BDNF (Heese et al., 2000). We found that acute treatment with CGP55845 enhanced levels of both pro-BDNF and mature BDNF. However, this enhancement was similar in 2N and Ts65Dn mice suggesting that the effect has no specificity for DS.

The improvement of short-term and long-term types of memory, but not working memory, suggests that the underlying cellular mechanisms may involve modulation of synaptic plasticity. It was previously demonstrated that suppression of the GABA_B receptors may enhance LTP (Mott and Lewis, 1991; Olpe et al., 1993; Staubli et al., 1999), and that such enhancement correlated positively with learning (Staubli et al., 1999). Consistent with such a possibility, we observed that CGP55845 and CGP52432, two high affinity selective antagonists of the GABA_B receptors, improved LTP in Ts65Dn slices. Because postsynaptic GABA_B receptors play an essential role in the regulation of NMDA receptor-mediated synaptic responses (Morrisett et al., 1991; Chalifoux and Carter, 2010), we investigated the NMDA receptor-mediated component of field responses during tetanus. This component was significantly smaller in Ts65Dn DG thus providing a plausible link to reduced LTP. The GABA_B antagonist increased the NMDA receptor-mediated component in Ts65Dn slices to the level seen in the untreated 2N slices. Thus, enhanced activation of the NMDA receptors represents one possible mechanism by which GABA_B receptor antagonists increased LTP.

Besides improvement of synaptic plasticity, a number of additional mechanisms can be envisioned to explain the facilitatory effects of GABA_B antagonists on learning and memory. Thus, suppression of presynaptic GABA_B receptors on terminals of somatostatin-containing GABAergic neurons may increase the release of somatostatin (Nyitrai et al., 2003), which positively affects learning and memory (Bennett et al., 1997; Kluge et al., 2008). GABA_B antagonists also increased responsiveness to exogenously applied acetylcholine (Andre et al., 1992), suggesting that suppression of GABA_B receptors could augment cholinergic function that, in turn, may affect learning. Finally, GABA_B receptors on presynaptic terminals of glutamatergic fibers effectively modulate release of glutamate. Suppression of these receptors may enhance efficiency of the excitatory signaling and, thus, also contribute to the effects of the GABA_B receptor antagonists. Role of these receptors in DS remains to be investigated.

It was shown previously that GABA_B receptor antagonists improved performance of normal animals in a number of behavioral tests (Mondadori et al., 1993; Staubli et al., 1999; Getova and Bowery, 2001; Froestl et al., 2004; Helm et al., 2005). In this study, we observed improvement only in Ts65Dn mice, while performance of 2N mice remained unchanged. This result might suggest that, in our experimental conditions, performance of 2N mice was close to biological limits and, for this matter, could not be improved. On the other hand, it is

possible that DS-specific genetic alterations of Ts65Dn mice enhance receptivity of these subjects to the treatment. Thus, antagonists of GABA_B receptors may represent a class of drugs selectively effective in subjects with DS.

Taken together, the data presented in this study suggest that suppression of the GABA_B receptor-mediated signaling is an effective way for improving synaptic plasticity and cognition in mouse models of DS. These findings motivate studies to test therapeutic efficiency of GABA_B receptor antagonists in people with DS.

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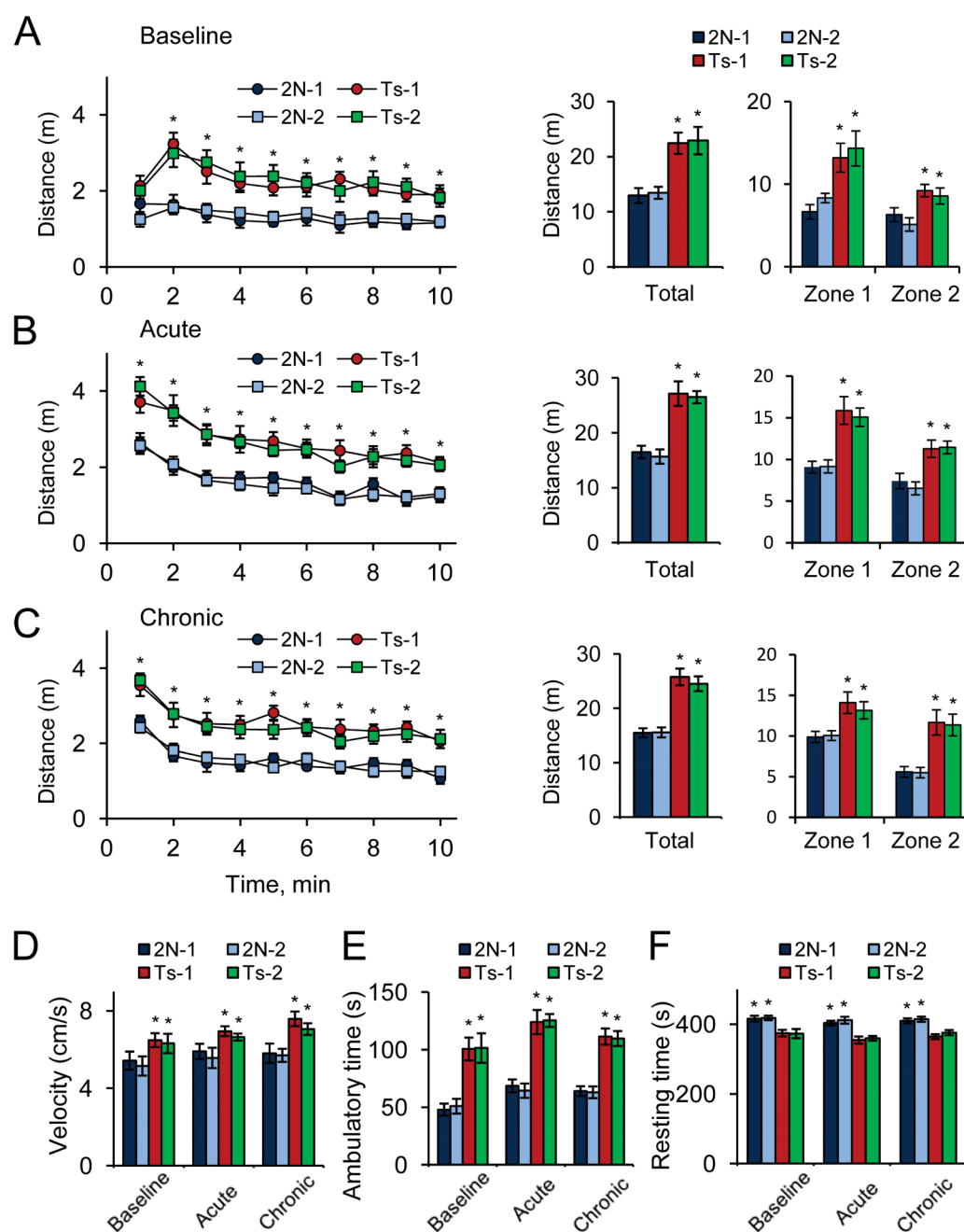
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**Figure 1.**

Spontaneous locomotor activity was enhanced in Ts65Dn mice, but it was not affected by the GABA_B receptor antagonist CGP55845. **A-C**, Distance moved in the activity chamber at baseline (**A**), and during acute (**B**) and chronic (**C**) phases of the treatment, averaged for 1-min periods (left) and for the total 10-min testing period (right graphs). Ts65Dn mice moved significantly greater distances both at the periphery (zone 1) and in the center (zone 2). During the acute (**B**) and chronic (**C**) tests, mice from groups 2N-1 and Ts-1 were treated with vehicle, while mice from groups 2N-2 and Ts-2 with CGP55845. The treatment had no effect on the distance moved by 2N or Ts65Dn mice. **D**, Averaged velocity of movement. **E**, Ambulatory time. **F**, Resting time. All these parameters were abnormal in Ts65Dn mice, but

not affected by the treatment. Mean \pm SEM; n = 12 – 15 per group. *p < 0.03 for 2N vs. Ts65Dn groups.

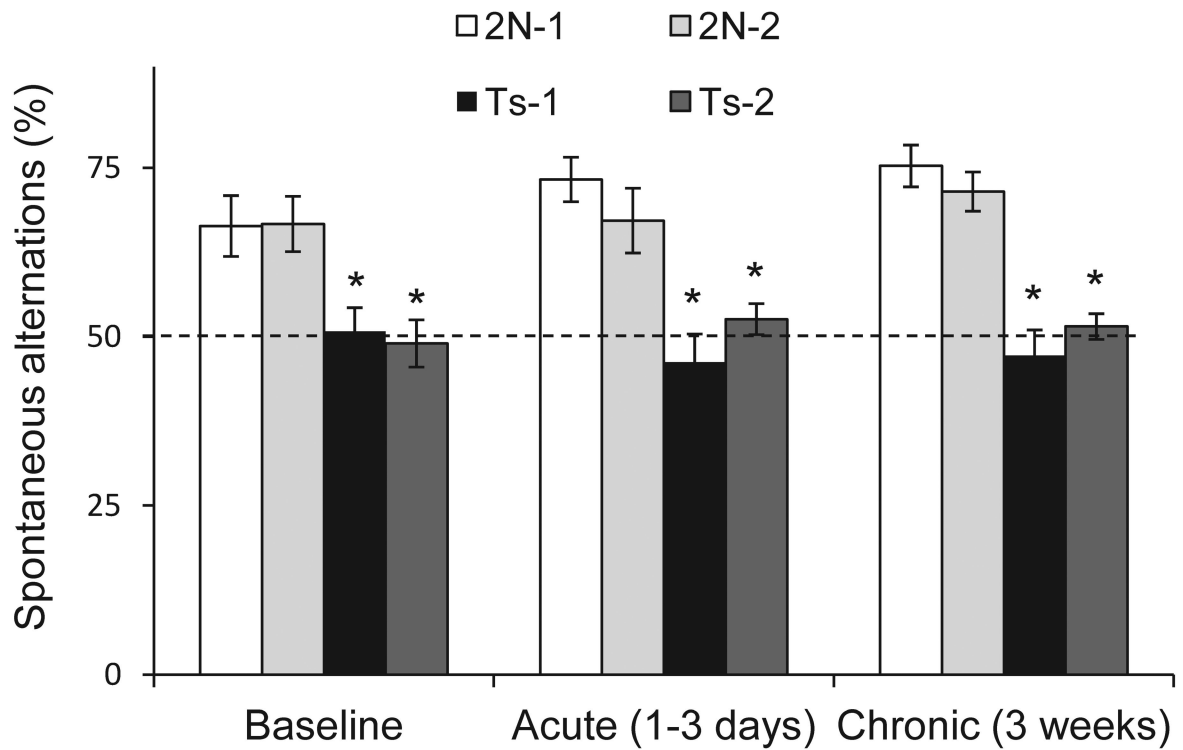
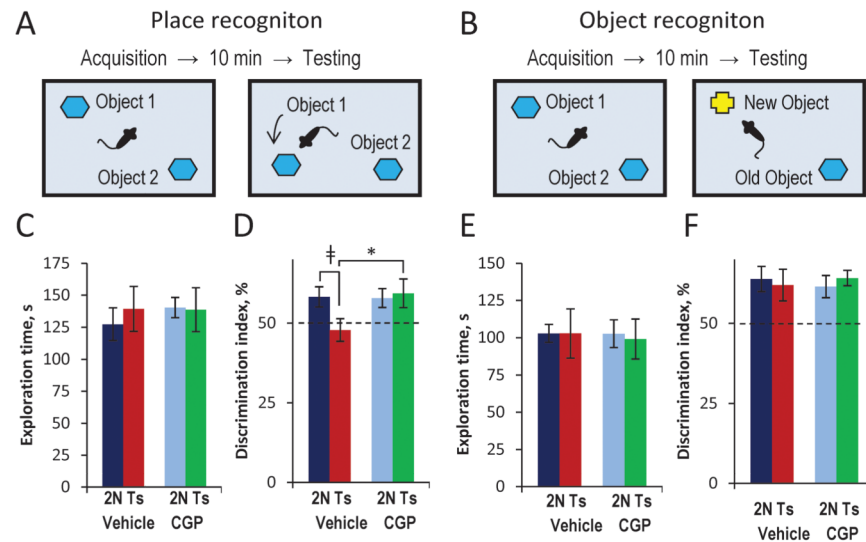


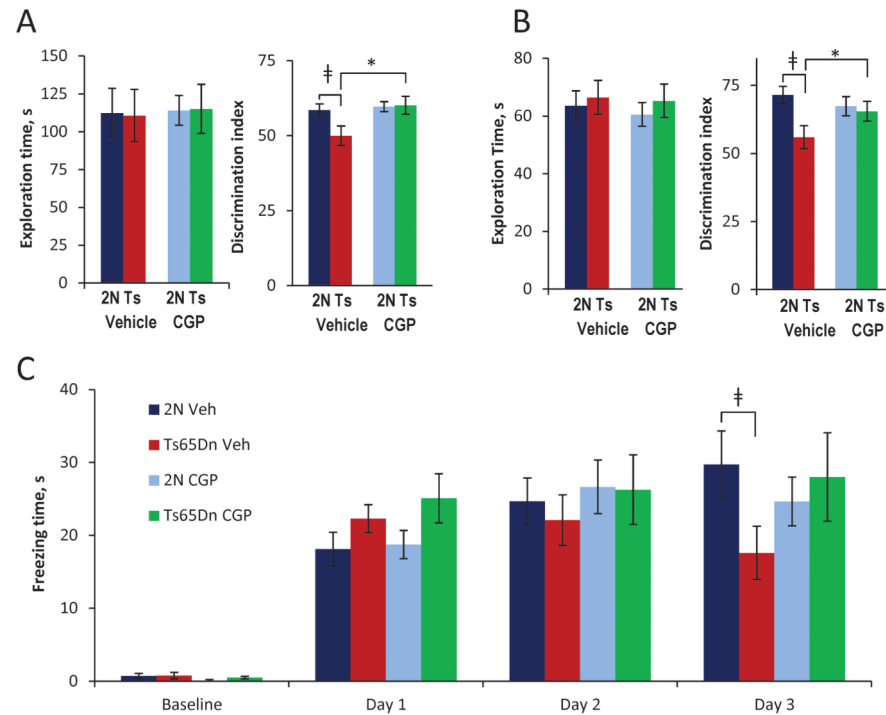
Figure 2.

Performance in T-maze was not affected by treatment with CGP55845. The baseline rate of spontaneous alternations was greater in 2N vs. Ts65Dn mice. Acute and chronic treatment with CGP55845 had no effect on the alternation rate. Groups 2N-1 and Ts-1 were treated during the acute and chronic phase with the vehicle, groups 2N-2 and Ts-2 with CGP55845.

*p < 0.01 for all 2N vs. Ts65Dn groups.

**Figure 3.**

Effects of CGP55845 on short-term memory. **A, B**, Experimental designs for novel place recognition and novel object recognition tasks. **C**, Time spent investigating the objects during the acquisition phase in the place recognition task. There was no difference between any experimental groups. **D**, Discrimination index in the place recognition test. Vehicle-treated Ts65Dn mice were impaired and performed at a chance level (50%). Treatment with CGP55845 restored performance of Ts65Dn to the level of 2N littermates. **E**, Time spent investigating the objects during the acquisition phase in the object recognition task. All groups of mice spent equal time exploring the objects. **F**, Discrimination index for the object recognition test showed no deficiency in Ts65Dn mice and no change following the treatment. $p = 0.016$ for 2N Veh vs. Ts65Dn Veh; * $p = 0.027$ for Ts65Dn Veh vs. Ts65Dn CGP.

**Figure 4.**

GABA_B receptor antagonist improved long-term memory in Ts65Dn mice. **A**, Novel object recognition task with a retention period of 24 hours: acute treatment with CGP55845. Left graph: Exploration time during the acquisition was similar in all groups. Right graph: The discrimination index was smaller in the Ts65Dn Veh vs. 2N Veh group showing impaired long-term memory of Ts65Dn mice. Treatment improved performance of Ts65Dn mice to the level of 2N littermates. **B**, Novel object recognition with a retention period of 24 hours: chronic treatment with CGP55845. Left graph: exploration time during the acquisition phase was again similar in all groups. Right graph: The discrimination index was reduced in the Ts65Dn Veh vs. 2N Veh mice. Ts65Dn mice treated with CGP55845 were not different from 2N littermates. **C**, GABA_B antagonist improved contextual memory in fear conditioning test. All groups of mice showed similar performance during baseline activity before the training and during the training (Day 1). Testing of cued memory (Day 2) also showed no difference between the groups. In contrast, testing of contextual memory (Day 3) revealed a severe deficit in the Ts65Dn vehicle control group. This deficit was not apparent in Ts65Dn mice treated with CGP55845.

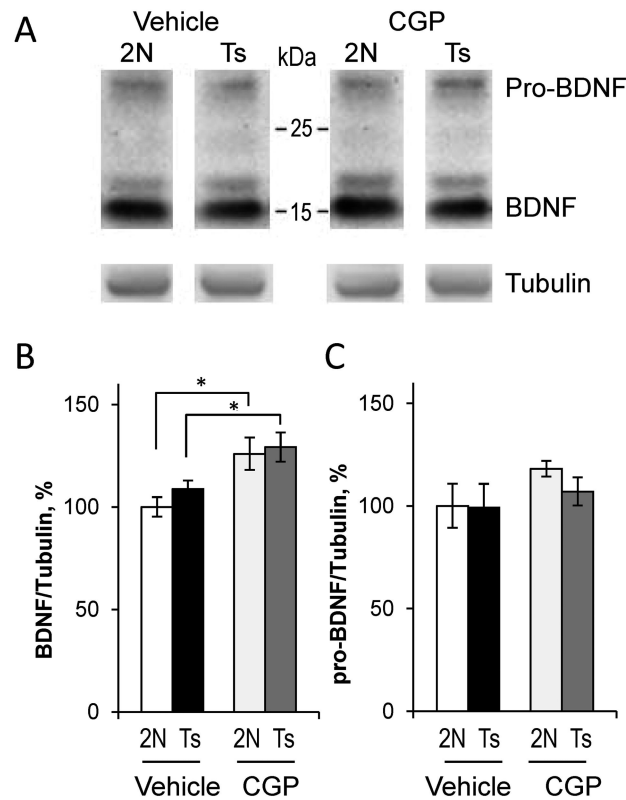
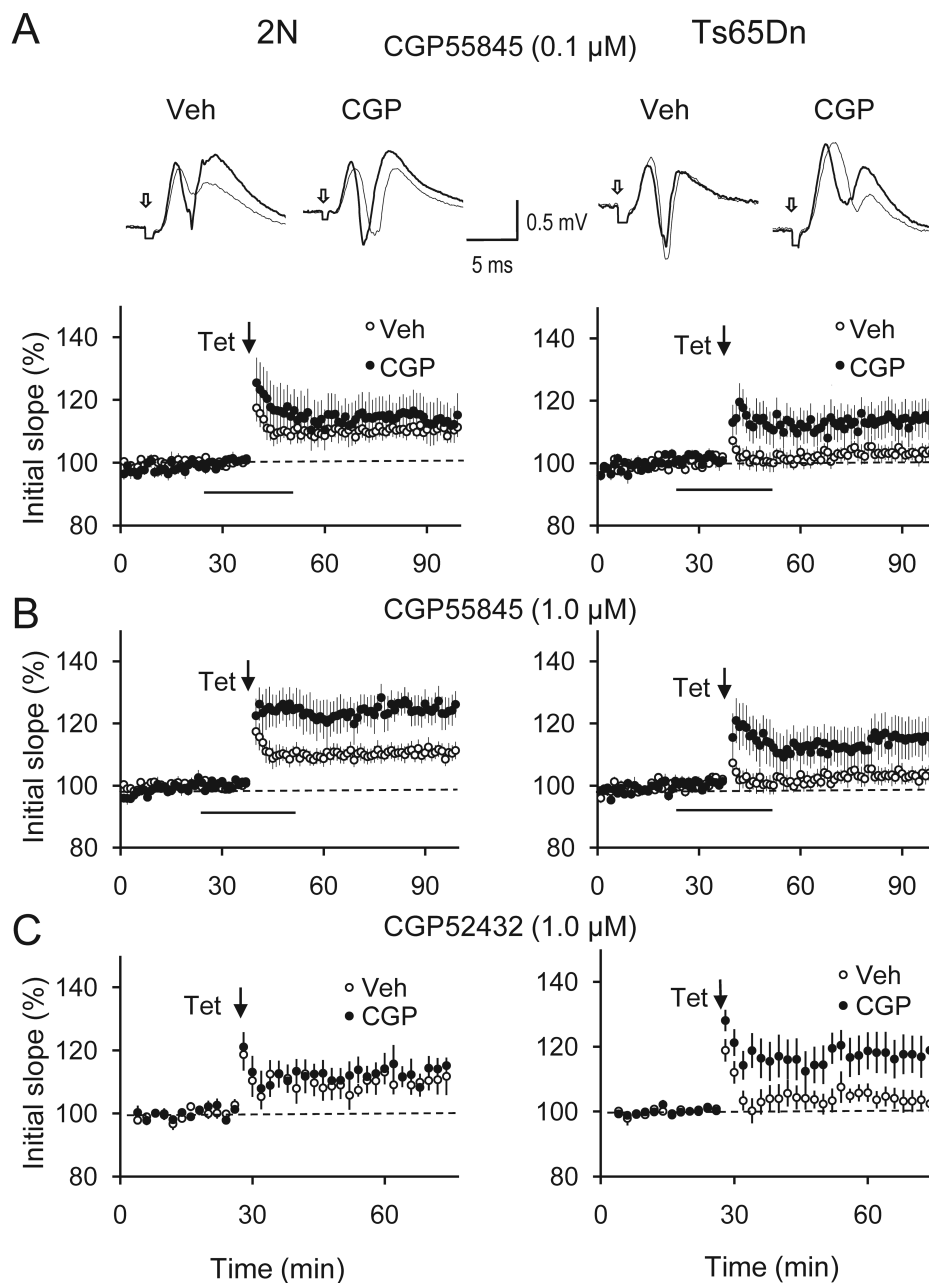


Figure 5. Hippocampal levels of proteins following treatment with CGP55845 or vehicle. **A**, Representative Western blots of hippocampal extracts from 2N and Ts65Dn mice. **B**, Quantification of the data. Baseline levels of both mature BDNF and pro-BDNF were similar in 2N and Ts65Dn mice. Treatment with CGP55845 increased levels of mature BDNF in both 2N and Ts65Dn mice. Tubulin was used as a reference protein. $n = 4$ for all groups. *) $p < 0.03$ for 2N Veh vs. 2N CGP or Ts Veh vs. Ts CGP.

**Figure 6.**

Enhancement of LTP by GABA_B receptor antagonists. **A**, At baseline conditions, tetanization evoked robust LTP in 2N slices (left graph, empty circles), but failed to induce LTP in Ts65Dn slices (right graph, empty circles). Application of CGP55845 (0.1 μ M) significantly improved LTP in Ts65Dn slices (right graph, black circles), but not in 2N slices (left graph, black circles). Solid lines below the graphs mark the application of the drug or vehicle. Top: representative field responses evoked 5 min before and 50 min after the tetanus (thin and thick lines respectively) in the vehicle control (Veh) and CGP groups. **B**, Application of CGP55845 at higher concentration (1 μ M) enhanced LTP in both 2N and Ts65Dn slices. **C**, Another antagonist of the GABA_B receptors, CGP52432 (1 μ M), also

enhanced LTP in the Ts65Dn slices. The drug was present in the perfusing solution during the whole period of the experiment. Mean \pm SEM; n = 8-13.

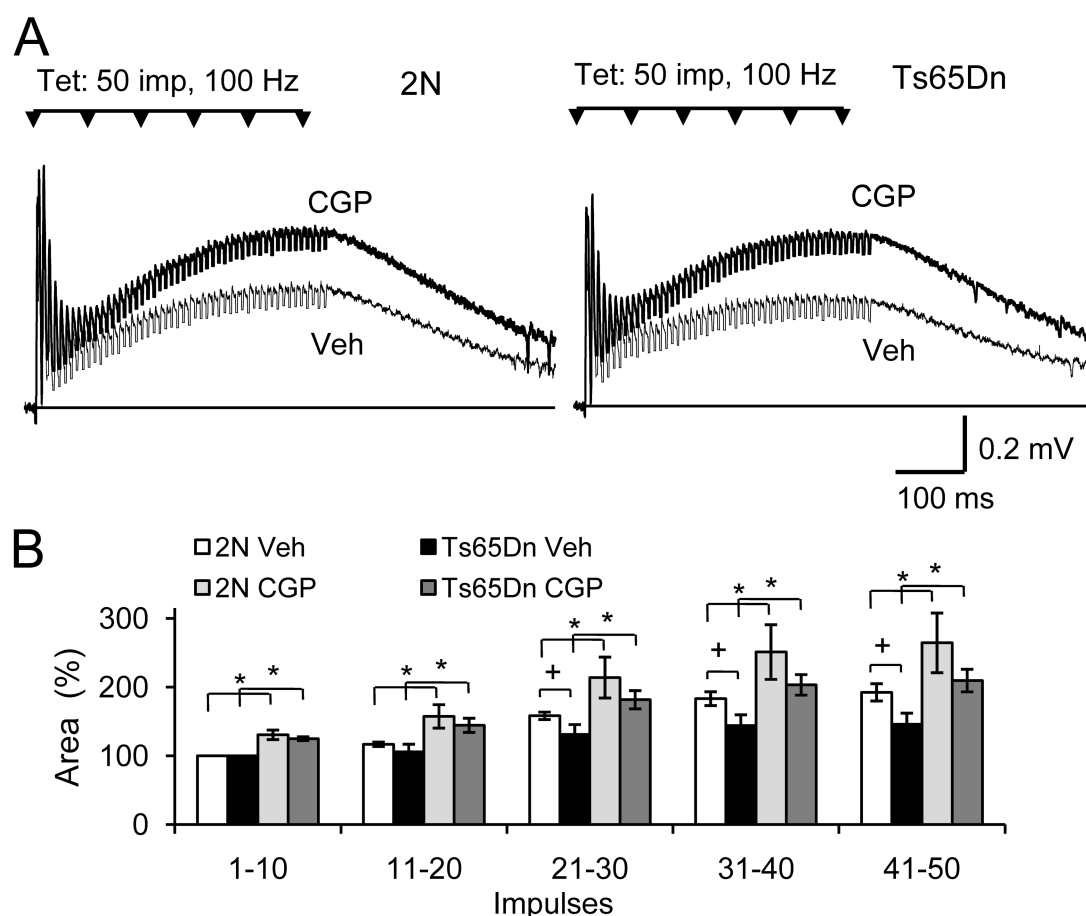


Figure 7.

Suppression of GABA_B receptors increased tetanus-evoked field EPSPs. **A**, Examples of tetanus-evoked field responses during application of vehicle or CGP52432 (1 μ M) in 2N (left) and Ts65Dn (right) slices. Horizontal lines on top mark the time of the tetani. Downward triangles indicate each ten's pulse of the tetanization train. Stimulus artifacts are truncated. **B**, Averaged amplitudes of responses to each ten consecutive pulses of a 50-pulse tetanization (marked by horizontal line in 'A') normalized to the averaged amplitude of first ten responses (1-10) at baseline conditions. Responses evoked during application of vehicle were smaller in Ts65Dn slices at the end of the 50-pulse train (pulses 20-50, white vs. black). Application of CGP enhanced the responses in both 2N and Ts65Dn slices and abolished the difference between 2N and Ts65Dn slices (light grey vs. dark grey). +p < 0.05, 2N Veh vs. Ts65Dn Veh; *p < 0.01, Veh vs. CGP for 2N or Ts65Dn slices.

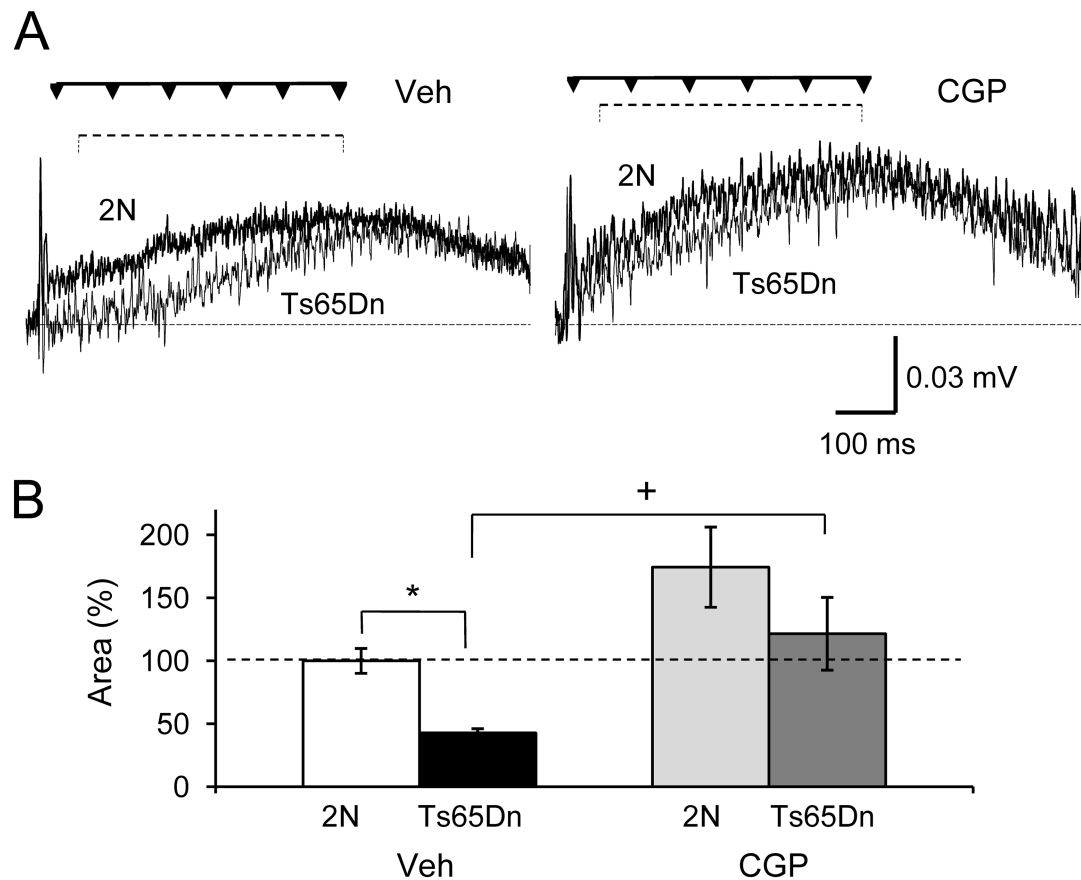


Figure 8.

GABA_B receptor antagonist CGP52432 (1 μ M) increased the NMDA receptor-mediated component of the tetanus-evoked responses. **A**, Examples of the NMDA receptor-mediated responses in 2N and Ts65Dn slices during application of vehicle (left) and CGP (right). **B**, Averaged data. At baseline conditions (Veh), the responses were smaller in Ts65Dn than in 2N slices (graph, black vs. white). Application of CGP52432 increased the responses in both 2N and Ts65Dn slices. Note that the NMDA receptor-mediated responses evoked in Ts65Dn slices during application of CGP52432 were similar to the 2N Veh responses. The NMDA receptor-mediated response was evaluated as the area under the curve at latencies of 30-500 ms (marked by horizontal dashed line). * $p = 0.012$ for 2N Veh vs. Ts65Dn Veh; + $p = 0.029$ Ts65Dn Veh vs. Ts65Dn CGP.