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# Dysfunctional hippocampal inhibition in the Ts65Dn mouse model of Down syndrome

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## Abstract

GABAergic dysfunction is implicated in hippocampal deficits of the Ts65Dn mouse model of Down syndrome (DS). Since Ts65Dn mice overexpress G-protein coupled inward-rectifying potassium (GIRK2) containing channels, we sought to evaluate whether increased GABAergic function disrupts the functioning of hippocampal circuitry. After confirming that $GABA_B$/GIRK current density is significantly elevated in Ts65Dn CA1 pyramidal neurons, we compared monosynaptic inhibitory inputs in CA1 pyramidal neurons in response to proximal (stratum radiatum; SR) and distal (stratum lacunosum moleculare; SLM) stimulation of diploid and Ts65Dn acute hippocampal slices. Synaptic $GABA_B$ and $GABA_A$ mediated currents evoked by SR stimulation were generally unaffected in Ts65Dn CA1 neurons. However, the $GABA_B$/$GABA_A$ ratios evoked by stimulation within the SLM of Ts65Dn hippocampus were significantly larger in magnitude, consistent with increased $GABA_B$/GIRK currents after SLM stimulation. These results indicate that GIRK overexpression in Ts65Dn has functional consequences which affect the balance between $GABA_B$ and $GABA_A$ inhibition of CA1 pyramidal neurons, most likely in a pathway specific manner, and may contribute to cognitive deficits reported in these mice.

### Keywords

Down syndrome; Ts65Dn; $GABA_B$; GIRK; Hippocampus; GABAergic modulation; mental retardation

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## INTRODUCTION

Down syndrome (DS) is the most common nonheritable cause of mental retardation and results from the presence of an extra chromosome 21. The overexpression of genes from this chromosome are considered to drive the DS phenotype (Antonarakis, et al., 2004, Lejeune, et al., 1959). One such gene found within the purported DS critical region (DSCR) is *Kcnj6* (*Girk2*), which encodes the G-protein coupled inward rectifying $K^+$ channel subunit 2 (GIRK2). The DS mouse model, Ts65Dn, overexpresses GIRK2 throughout the brain and, in particular, the hippocampus (Harashima, et al., 2006). This overexpression leads to a significant increase in the efficacy of agonist induced GIRK current density in cultured hippocampal neurons (Best, et al., 2007). GIRK channel overexpression may be involved in the documented changes in neuronal cell numbers since chronic treatment of Ts65Dn mice with a GIRK channel antagonist increased neurogenesis of hippocampal neurons to levels comparable with diploid mice (Kobayashi, et al., 2003).

Hippocampal GIRK channel expression is graded and reflects the laminar nature of the hippocampus. Within the CA1 region, the most intense GIRK1 and GIRK2 immunoreactivity is found within the stratum lacunosum-moleculare (SLM), decreasing through the distal and proximal portions of the stratum radiatum (SR) to the pyramidal layer (Drake, et al., 1997, Liao, et al., 1996). $GABA_B$ receptor subunit expression parallels GIRK channel expression where the immunohistochemical signal within the SLM is more intense than in the SR, the pyramidal cell layer or in stratum oriens (Harashima, et al., 2006, Lopez-Bendito, et al., 2004, Sloviter, et al., 1999). This expression pattern suggests that synaptically evoked $GABA_B$/GIRK current would be larger in the SLM than the SR (Pham, et al., 1998) and thus may serve as a primary inhibitory modulator of the direct, perforant path inputs onto CA1 pyramidal neurons. This pathway is likely to play a key role in learning/memory since it strongly activated during sensorimotor and cognitive tasks and is a principal mediator of hippocampal place field memory (Brun, et al., 2002, Sybirska, et al., 2000).

Synaptic GABA release elicits both fast and slow inhibitory postsynaptic currents (IPSC) mediated by $GABA_A$ and $GABA_B$ receptors, respectively. The slow IPSC is sensitive to pertussis toxin and is associated with a $K^+$ conductance which is attenuated by GIRK channel specific blockers and is absent in GIRK2 knockout mice (Dutar and Nicoll, 1988, Huang, et al., 2005, Luscher, et al., 1997, Thalmann, 1987). GIRK channels are activated by agonists at Gi/o coupled receptors such as the $GABA_B$ receptor (Sodickson and Bean, 1996). The slower kinetics of their activation is consistent with GIRK channels as effectors for the $GABA_B$ induced slow IPSC.

Given that $GABA_B$ receptor and GIRK channel expression patterns mirror each other within the hippocampus and that GIRK channels are overexpressed in Ts65Dn hippocampus, we hypothesized that $GABA_B$/GIRK mediated slow IPSCs would be increased and this would affect the manner by which proximal and distal hippocampal circuitries are integrated. This could lead to abnormal inhibitory hippocampal function and may contribute to cognitive deficits in DS. To test this hypothesis we measured monosynaptic slow and fast IPSCs in

CA1 pyramidal neurons generated by stimulation within the SLM and SR. The summation of $GABA_B$ and $GABA_A$ receptor mediated currents in Ts65Dn hippocampus showed diminished dynamic range in response to varying stimulation frequencies in SLM but not in SR. In addition, the ratio between the charge transfer of slow and fast IPSCs in Ts65Dn was significantly elevated only in response to SLM stimulation. Together our results suggest that GIRK2 overexpression in the Ts65Dn hippocampus functionally alters the integration of synaptic inputs in CA1 pyramidal neurons in a pathway specific manner.

## METHODS

### Animals

Ts65Dn and control diploid littermates were bred to have the mixed genetic background C57BL/6JEi×C3H/HeSnJ as used in our previous studies (Harashima, et al., 2006, Siarey, et al., 1997). The mice used in this research were genotyped by fluorescence *in situ* hybridization (Chakrabarti, et al., 2010, Harashima, et al., 2006). Mice were maintained under a 12-h light/dark cycle and fed standard laboratory food (following NIH guidelines). All protocols were approved by the Uniformed Services University of the Health Sciences Institutional Animal Care and Use Committee.

### Slice Preparation

Mice, 2-3 weeks old, were anesthetized, decapitated and the brain was rapidly removed and placed in ice cold (~4°C) cutting artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, $CaCl_2$ 2, $NaH_2PO_4$ 1.25, $MgSO_4$ 5, $NaHCO_3$ 26, dglucose 10, bubbled with a mixture of 95% $O_2$/5% $CO_2$. Parasagittal slices, 400-μm thick, were cut on a Lancer Vibratome (Vibratome series 1000; Vibratome, St. Louis, Missouri USA) and transferred to a warmed (~37°C) solution of 50% slicing ACSF and 50% recording ACSF (the same composition as cutting ACSF, but $MgSO_4$ at 1 instead of 5 mM) bubbled with a mixture of 95% $O_2$/5% $CO_2$. After 20 minutes they were transferred to room temperature (~21°C) $O_2$/$CO_2$ bubbled recording ACSF where they were maintained for at least 1 hour before recording.

### Electrophysiology

Slices were placed in a recording chamber on the stage of an upright Zeiss FS-1 microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) and continuously perfused with room temperature ACSF bubbled with a mixture of 95% $O_2$/5% $CO_2$. Using a Photonics IR camera, CA1 pyramidal neurons were identified and a whole-cell patch-clamp configuration was obtained with a borosilicate patch pipette of resistance 3-5 MΩ containing (in mM): K-gluconate 130, KCl 15, HEPES 5, EGTA 1, Mg-ATP 4, Na-GTP 0.3 with pH adjusted to ~7.3 with KOH. A 5 mV hyperpolarizing step from a holding potential of -70 mV was applied to estimate membrane capacitance and resistance at the initiation of whole-cell access and at intervals throughout the recording. Similarly, resting membrane potential (current-clamp: I=0) was measured at the beginning of each recording and throughout the experiment to assess and monitor cell viability. Series and access resistances were monitored throughout experiment to assess stability of recording conditions. Recordings were performed in voltage-clamp configuration and data acquired by way of an Axopatch 200A

or 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 3 kHz (8-pole Bessel filter, NPI, ALA Scientific Instruments, Inc., Westbury, NY, USA), and recorded on a personal computer using Clampex acquisition software (Molecular Devices).

In the presence of 20 μM CNQX and 50 μM D-APV monosynaptic GABAergic currents were elicited by current stimulation with a concentric bipolar platinum electrode placed within the SR and by a second stimulating electrode placed within the SLM opposite the patched CA1 pyramidal neuron. Stimulation electrodes were placed in such a way as to avoid co-stimulation of the same terminals; that in the SR near the border of the pyramidal cell layer and that in the SLM within ~100 μm of the hippocampal fissure (Figure 1A). GABAergic currents were elicited while voltage clamping the neurons to near -40 mV to allow for both $GABA_A$ and $GABA_B$ mediated conductances of $Cl^-$ and $K^+$ respectively to generate outward currents. Multiple stimulation trains at frequencies of 5, 10, 20, 50, or 100 Hz were given at an intersweep interval of 20 seconds. At least three sweeps of each condition were averaged and later used for analysis. An example of Ts65Dn SLM stimuli at 10 Hz is given in Figure 1B.

Monosynaptic $GABA_B$ mediated GIRK currents were pharmacologically isolated from general GABAergic currents by application of 20 μM bicuculline to the existing 20 μM CNQX and 50 μM D-APV containing ACSF. Digitally subtracted $GABA_A$ traces were obtained by subtracting the pharmacologically isolated $GABA_B$ traces (blue traces in Figure 1B) from total GABAergic traces (black traces in Figure 1B). Trace subtraction and comparison were performed only in traces of identical stimulus intensity and holding potentials. In some instances the specific GABA uptake inhibitor (GAT1) NO-711 was used to evaluate GABA accumulation in the cleft. Successful isolation of $GABA_B$ mediated currents was tested through use of 2 μM CGP55845 (a specific $GABA_B$ receptor antagonist) which completely inhibited slow IPSCs within 5 minutes of perfusion (n=16). Likewise slow IPSCs were partially suppressed by GIRK channel blockers tertiapin-Q (100 nM, n=5, 55 ± 0.1% at 15 minutes) and SCH23390 (10 μM, n=6, 54 ± 0.1% at 12 minutes) (Fink, et al., 2007, Jelacic, et al., 2000, Kuzhikandathil and Oxford, 2002). Incomplete block of slow IPSCs by GIRK channel blockers may be due to heterogenous $GABA_B$ postsynaptic currents, partial block, or use dependence (Best, et al., 2007, Kanjhan, et al., 2005, Pham, et al., 1998, Tabata, et al., 2005). Additionally, since SCH23390 is a known dopamine D1 and D5 receptor antagonist as well as an agonist at serotonin $5HT_{1C}$ and $5HT_{2C}$ receptors, the involvement of these receptors may play a role in the partial block (Bourne, 2001, Briggs, et al., 1991, Millan, et al., 2001).

In order to evaluate short-term plasticity of fast IPSCs, peak amplitudes of pharmacologically isolated $GABA_A$ mediated IPSCs (recorded in the presence of 20 μM CNQX, 50 μM D-APV, and 2 μM CGP55845) were normalized to the amplitude of the first peak in a series of 5 stimuli at 5, 10, 20, and 50 Hz.

Action potentials were elicited from CA1 pyramidal neurons at increasing steps (10 pA) while current-clamped at -70 mV. Spike threshold was evaluated by visually identifying the voltage at which maximum acceleration occurred within the rising phase of the first spike of

each neuron. Spike amplitude, half-width, and maximum rise slope were measured using the statistics functions in ClampFit data analysis software (Molecular Devices).

CNQX, D-APV, Bicuculline methiodide, CGP55845, SCH23390, TTX and tertiapin-Q were all purchased from Tocris (Ellisville, Missouri USA). NO-711 was purchased from Sigma-Aldrich (St. Louis, Missouri USA).

### Western blot analysis

Proteins from the hippocampus of P25 pups were obtained by tissue homogenization in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined according to manufacturer's instruction using BCA Protein Assay Kit (Pierce, Rockford, IL). Twenty five micrograms of protein homogenate were loaded per well for electrophoresis after which the proteins were transferred to polyvinylidene difluoride membranes and blocked with 5% milk in Tris buffered saline and 0.1% Tween-20 for 1 hour at room temperature. The blots were incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-$K_{ir}3.2$ (GIRK2, 1:200, Alomone Labs, Jerusalem, Israel), anti-$GABA_A\alpha1$ (1:1000, Chemicon, Temecula, CA) and anti-GAPDH (1:5,000, Chemicon), mouse anti-$GABA_A\beta2/3$ (1:1000, Chemicon) and guinea pig anti-$GABA_BR2$ (1:1000, Chemicon). After washing, blots were incubated for one hour with HRP-conjugated secondary antibodies (1:10,000, Chemicon). Blots were developed by chemiluminescence using SuperSignal Kit (Pierce). Quantification was done by band densitometry using Scion Image software.

### Immunohistochemistry

All the immunohistochemical reactions were performed on 20 μm frozen brain sections from P15 pups. After washing with PBS three times, sections were blocked with 5% normal goat serum in PBS and 0.3% TritonX-100 for 30 minutes, and then incubated in primary antibody, rabbit anti-$K_{ir}3.2$ (1:500, Alomone Labs) and anti-$GABA_A\alpha1$ (1:1000, Chemicon), mouse anti-$GABA_A\beta2/3$ (1:1000, Chemicon) and guinea pig anti-$GABA_BR2$ (1:1000, Chemicon), overnight at 4°C. After three washes in PBS, sections were incubated with secondary antibody, AlexaFluor 488 and AlexaFluor 546 goat anti-rabbit or goat anti-guinea pig (1:200, Invitrogen, Carlsbad, CA), for 1 hour at room temperature, rinsed and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Images were taken on a LSM 510 confocal microscope (Carl Zeiss Inc., Germany).

Unpaired two-tailed t-tests were performed for statistical significance unless otherwise stated. Significance was set at $p<0.05$.

## RESULTS

### Increased $GABA_B$/GIRK current Density in Ts65Dn

Primary hippocampal cultured neurons from Ts65Dn neonatal mice have significantly elevated $GABA_B$ receptor dependent GIRK channel current density (Best, et al., 2007). To verify that this increase is also found in native brain slices, we performed whole-cell patch-clamp recordings of CA1 pyramidal cells from acute hippocampal slices from diploid and

Ts65Dn mice. Application of baclofen (25 μM), a $GABA_B$ receptor agonist, elicited a significantly larger increase in holding current in Ts65Dn neurons than in those from diploid mice (Fig 1C and D; dip n=12, 24.55 ± 0.84 pA; Ts n=6, 40.22 ± 2.67 pA; $p<0.05$; 60% increase) Similarly, current density, calculated for each cell as the baclofen induced current divided by the cell capacitance, was also significantly increased in Ts65Dn (dip n=12, 0.18 ± 0.01 pA/pF; Ts n=6, 0.27 ± 0.01 pA/pF; $p<0.05$; 60% increase). Thus, even when potential differences in cell sizes are accounted for, Ts65Dn CA1 pyramidal neurons experience a significantly greater hyperpolarizing current in response to $GABA_B$ receptor activation than those from diploid mice. These data confirm our previous results and suggest that the genetic overexpression of GIRK2 containing channels drives an increase $GABA_B$ receptor mediated GIRK conductance in Ts65Dn. To determine if distal or proximal dendritic pathways might be preferentially affected by GIRK2 overexpression we elicited GABAergic inputs in CA1 pyramidal cells by electrical stimulation of stratum lacunosum molecular (SLM) or stratum radiatum (SR) respectively.

### Short term plasticity of $GABA_A$ mediated IPSCs

Following pharmacological isolation of $GABA_A$ transmission (2 μM CGP55845, 20 μM CNQX, 50 μM D-APV), five stimuli at 5, 10, 20 and 50 Hz were applied within the SR and SLM of hippocampal slices from diploid and Ts65Dn mice and the ratios between the first and $n^{th}$ peak were plotted as a function of stimulus frequency (see supplementary Table 1). Previous work has shown a decrease in paired-pulse facilitation of IPSCs at 50 Hz in Ts65Dn dentate gyrus suggesting an increased probability of GABA release (Kleschevnikov, et al., 2004). We found no such deficits in facilitation at 50 Hz in the SR (two-way RM ANOVA, dip n=14, Ts n=12, p=0.14) or the SLM (Supplementary Table 1; two-way ANOVA, dip n=14, Ts n=8, $p<0.10$). Furthermore we found no significant differences between Ts65Dn and diploid at 5 or 10 Hz for both the SLM and SR stimulation and at 20 Hz with SLM stimulation. However, 20 Hz stimuli of diploid SR showed significant facilitation compared to Ts65Dn (Supplementary Table 1; two-way ANOVA, dip n=14, Ts n=13, $p<0.01$). Overall, short-term plasticity of $GABA_A$ergic responses were not significantly different in 7 of 8 cases examined (2 pathways, 4 frequencies) suggesting that GABA release probability is generally unchanged in the Ts65Dn CA1 region. Furthermore, $GABA_A$ergic short-term plasticity after SLM stimuli is normal in Ts65Dn hippocampus at all frequencies tested. These results indicate that changes in GABA release are minimal at best and do not explain the increase in stimulation evoked $GABA_B$ currents seen.

### Charge transfer of GABAergic IPSCs

Monosynaptic $GABA_B$ receptor mediated GIRK responses in CA1 pyramidal neurons were evaluated in response to SR and SLM stimuli of Ts65Dn hippocampus. Due to the slow kinetics of $GABA_B$/GIRK IPSCs we evaluated the current integral (charge transfer or area) instead of the peak amplitude of the response. Pharmacologically isolated $GABA_B$ responses to 5, 10, 20, 50 and 100 Hz stimulation in the SR and SLM were normalized to responses at 5 Hz (similar to Scanziani, 2000). This analysis normalizes the response to presumably the least amount of GABA accumulation at synaptic terminals, thus providing a means to evaluate the dynamic range of GABAergic responses. $GABA_B$ charge transfer after SR stimuli was comparable between diploid and Ts65Dn (Figure 2A, two-way ANOVA, dip

n=14, Ts n=12, p=0.10). In contrast, the normalized $GABA_B$ charge transfer from Ts65Dn neurons, was significantly decreased with SLM stimuli (Figure 2B, two-way ANOVA, dip n=15, Ts n=11, $p<0.005$), where 100 Hz was significantly greater in diploid than Ts65Dn (Bonferroni post-hoc test $p<0.05$). This suggests that there is diminished dynamic range in the summation of $GABA_B$ergic transmission in Ts65Dn. This apparent shift towards the upper range (nearer to plateau) of $GABA_B$ is evident only in response to SLM stimuli suggesting inhibitory fibers activated along this pathway may be preferentially affected in Ts65Dn. This effect is unlikely from impaired release of GABA at Ts65Dn synapses as we showed above where there was little change in $GABA_A$ergic short-term plasticity. Alternatively, aberrant accumulation of GABA in Ts65Dn synapses may lead to the premature saturation of synaptic receptors and/or activation extrasynaptic $GABA_B$ receptors resulting in impaired CA1 pyramidal neuron responses to SLM activation.

To address whether GABA accumulation in the synaptic cleft could explain this deficit, we employed the GABA transporter subtype I (GAT1) specific antagonist NO-711 (10 μM) to enhance GABA accumulation. When GABA uptake was inhibited, normalized charge transfer of $GABA_B$ responses evoked by SR and SLM stimulation was similar in diploid and Ts65Dn neurons (Figure 2C-D, two-way ANOVA, SR: dip n=5, Ts n=5, p=0.36; SLM: dip n=6, Ts n=5, p=0.95). Thus, increasing accumulation of GABA in diploid synapses caused them to respond to SR and SLM activation in a manner similar to those in Ts65Dn. Together with our observations that GABA release appears to be normal, this result suggests that deficits in the dynamic range of $GABA_B$ mediated responses to SLM stimuli in Ts65Dn could result from diminished clearance of GABA from the synaptic cleft.

In conjunction with the $GABA_B$/GIRK charge transfer evaluation we subsequently analyzed the charge transfer of pharmacologically isolated $GABA_A$ currents (2 μM CGP55845, 20 μM CNQX, 50 μM D-APV) also normalized to 5 Hz. Responses to SR stimuli revealed no significant differences between diploid and Ts65Dn (Figure 2E, twoway ANOVA, dip n=14, Ts n=13, p=0.46). However normalized charge transfer in response to SLM stimuli did show a significant genotype effect (Figure 2F, two-way ANOVA, dip n=14, Ts n=11, $p<0.05$; post-hoc Bonferroni yields no significance for 20 or 50 Hz, $p>0.05$). Since $GABA_A$ responses at all frequencies were similar between the genotypes following SR stimuli it is unlikely that signal integration of $GABA_A$ responses is disrupted along this pathway in Ts65Dn CA1 pyramidal cells. In contrast, the diminished Ts65Dn $GABA_A$ charge transfer in response to SLM stimuli further suggests that signal processing along this pathway is adversely affected by trisomy.

## GABA receptor expression in diploid and Ts65Dn Hippocampus

Previously we determined that GIRK channel subunits are overexpressed in Ts65Dn hippocampus but whether there was a concomitant change in expression levels of $GABA_B$ receptor was unknown. Here we found that expression of $GABA_BR2$, which can be functionally coupled to GIRK channels, was decreased 19% in Ts65Dn hippocampus (Figure 3A-C., dip n=3, Ts n=2, p=0.002). With respect to $GABA_A$ receptor distribution, previous reports indicate a ~20% decrease in the $GABA_A\beta2/3$ subunit in dentate gyrus of Ts65Dn mice (Belichenko, et al., 2009). However, we found no significant difference in

$GABA_A\alpha1$ or $GABA_A\beta2/3$ receptor expression between diploid and Ts65Dn whole hippocampus (Figure 3A-C., dip n=3, Ts n=3, p=0.22 for $GABA_A\alpha1$ and p=0.51 for $GABA_A\beta2/3$). The reduction in $GABA_BR2$ receptor expression may be a compensatory result of the overexpression of GIRK channels or functional $GABA_B$/GIRK complexes.

### Relative contribution of inhibition by $GABA_A$ and $GABA_B$ with SR and SLM stimulation

Previous studies have uncovered frank GABAergic deficits in Ts65Dn hippocampus but the relative contributions of $GABA_A$ and $GABA_B$-mediated currents have not been determined (Fernandez, et al., 2007, Kleschevnikov, et al., 2004). Furthermore, the ratio of $GABA_A$ to $GABA_B$ may impact intrinsic properties and kinetics of synaptic integration of neurons. Therefore, we evaluated the relative contribution each GABA receptor subtype makes toward inhibition within each individual CA1 pyramidal neuron by comparing the ratio of $GABA_B$ to $GABA_A$ mediated responses for SR and SLM stimuli (Figure 4A). When comparing area ratios for the frequencies within each pathway and for each genotype there was no significant difference, therefore the data were combined, (ANOVA $p>0.05$; individual frequency values are shown in Figure 4B inset).

We found that for stimuli delivered to the SR of diploid slices the $GABA_B$ charge transfer was on average ~60% of the $GABA_A$ charge transfer while in the SLM it approached 90%, a difference that was highly significant (two-way ANOVA: SR n=13-14, SLM n=15, $p<0.001$; Figure 4B). This may indicate that SLM stimuli elicit a greater $GABA_B$/GIRK response than does SR stimuli. A result consistent with the immunohistochemical expression profile of $GABA_B$ receptors and GIRK channel subunits within the CA1 subfield (Figure 3) (Drake, et al., 1997, Harashima, et al., 2006, Liao, et al., 1996, Lopez-Bendito, et al., 2004, Pham, et al., 1998, Sloviter, et al., 1999), and previous data suggesting more $GABA_B$ current after SLM stimuli than compared to SR (Pham, et al., 1998). Ts65Dn showed a similar difference in the relative $GABA_B$ charge transfer between SR and SLM. Within the SR, the Ts65Dn $GABA_B/GABA_A$ ratio averaged ~50% while that in the SLM ~120% (Figure 4B). This difference between Ts65Dn SR and SLM responses again was highly significant when examined for the combined range of stimulus frequencies (Figure 4B, two-way ANOVA: SR n=12, SLM n=11, $p<0.0001$), and reflects the potential for GIRK overexpression to preferentially impact pathways traversing the SLM.

The $GABA_B/GABA_A$ ratio was also highly significant for SLM stimuli between Ts65Dn and diploid (Figure 4B, two-way ANOVA, $p<0.0005$) but not for SR stimuli (Figure 4B, two-way ANOVA, p=0.25). In conjunction with the genetic GIRK overexpression and relatively unchanged expression of $GABA_A$ subunits (Figure 3C) in the Ts65Dn hippocampus as well as our finding of a functional overexpression of $GABA_B$/GIRK in Ts65Dn CA1 pyramidal neurons (Figure 1) these results suggest that the altered $GABA_B/GABA_A$ ratio in Ts65Dn arises primarily from aberrant $GABA_B$ signaling.

### Intrinsic CA1 Properties

Changes in the intrinsic properties of CA1 pyramidal cells in Ts65Dn mice could potentially compensate for alterations in GABAergic signaling. Therefore, the intrinsic excitability of CA1 pyramidal neurons was evaluated by examining spike threshold and count in response

to increasing steps of depolarizing current (supplementary Figure 1a). No significant differences in spike threshold or shape (half width or maximum rise slope) between Ts65Dn (n=28) and diploid (n=23) neurons were found (Supplementary Table 2). Likewise, the number of spikes per current step was similar between both genotypes (Supplementary Figure 1B; two-way ANOVA, p=0.98). These findings are in contrast to previous reports from full trisomy 16 mouse cultured hippocampal neurons (Galdzicki, et al., 1993).

Evaluation of passive membrane properties of CA1 pyramidal neurons (Supplementary Table 3) show that Ts65Dn cell mean electrical capacitance is 22% smaller than mean capacitance in diploid mice indicating that the estimated cell surface of Ts65Dn neurons is smaller. Resting membrane potentials, as expected with an overexpressed potassium channel, is hyperpolarized by ~ 2mV in Ts65Dn (Supplementary Table 3) as previously found in cultured hippocampal neurons (Best, et al., 2007). These changes in Ts65Dn intrinsic properties do not indicate a compensatory increase in excitability that might have compensated for excessive $GABA_B$ mediated inhibitory drive.

## DISCUSSION

DS mouse models, such as the Ts65Dn mouse, display impairments in hippocampal synaptic plasticity; long-term potentiation (LTP) is decreased and long-term depression (LTD) is enhanced (Costa and Grybko, 2005, Fernandez, et al., 2007, Kleschevnikov, et al., 2004, Siarey, et al., 1999, Siarey, et al., 2006, Siarey, et al., 1997). Recent studies have shown that pharmacological intervention at $GABA_A$ receptors may, in part, treat abnormal hippocampal plasticity to the point where plasticity is normal under certain conditions (Costa and Grybko, 2005, Fernandez, et al., 2007, Kleschevnikov, et al., 2004). In addition, re-establishing diploid levels of transcriptional factors Olig1/Olig2 leads to restoration of interneuron density and function within Ts65Dn hippocampus and interneuron density in the cortex (Chakrabarti, et al., 2010), and $GABA_A$ antagonists lessen cognitive and hippocampal LTP defects in Ts65Dn (Fernandez et al., 2007). The results from these studies are promising in that simple attenuation of inhibitory neural transmission can improve cognitive performance. It would be important to determine whether targeting $GABA_B$ receptors and/or GIRK2 channel as well would have a synergistically beneficial effect.

Despite the promising reports of the beneficial effects of attenuating inhibition on DS phenotypes related to cognition, basal responses to inhibitory stimuli in Ts65Dn hippocampal neurons are surprisingly under-investigated. We have previously found that Ts65Dn mice overexpress at least one downstream effector of $GABA_B$ signaling—GIRK channels— and that $GABA_B$ activated GIRK currents are significantly elevated in Ts65Dn cultured neurons (Best, et al., 2007, Harashima, et al., 2006). However, the mechanism by which $GABA_A$ block improves function is not understood, nor is there a clear relationship between extra gene copies and $GABA_A$ergic dysfunction in DS (genes encoding $GABA_A$ receptor subunits are not on human chromosome 21, but on 4, 5, 15 and X (Russek, 1999)).

Our present analysis of inhibitory responses of Ts65Dn hippocampal CA1 pyramidal neurons show that with SLM stimulation, the ratio of monosynaptic $GABA_B$/GIRK to $GABA_A$ currents are significantly elevated compared to diploid whereas this ratio appears to

be normal with SR stimulation. We also find that the $GABA_B$/GIRK signal integration is disrupted in Ts65Dn at CA1 in response to SLM stimulation. The only changes in pharmacologically isolated $GABA_A$ergic signaling we detected were the increased short-term facilitation for SR stimuli at 20 Hz indicating that there is little or no change in GABA release probability. It is interesting to note that in the dentate gyrus $GABA_A$ergic presynaptic transmission is affected in Ts65Dn mouse (Kleschevnikov, et al., 2004), however our results suggest that alterations within GABAergic transmission are not uniform across the hippocampal network.

When considering our results in light of genetically driven overexpression of GIRK2 containing channels and our current observation of enhanced $GABA_B$ currents in Ts65Dn CA1 pyramidal neurons (Figure 1), it suggests that the differences between Ts65Dn and diploid within CA1 principally result from an increase in functional $GABA_B$/GIRK complexes. Other downstream consequences of $GABA_B$ receptor signaling outside of GIRK channel activation, such as consequences of inhibiting adenylyl cyclase activity or associations that $GABA_B$ receptors and GIRK channel subunits have with proteins that effect membrane properties may also contribute to these changes (Fowler, et al., 2007, Hibino, et al., 2000, Jelacic, et al., 2000, Lavine, et al., 2002, Sans, et al., 2001). $GABA_B$ receptors mediate inhibition of adenylyl cyclase (Wojcik and Neff, 1984), which is consistent with dramatically reduced cAMP levels in Ts65Dn hippocampus (Dierssen, et al., 1996, Siarey, et al., 2006). GIRK2 interacts with a member of the synaptic associated protein family SAP97(DLG1) (Hibino, et al., 2000), a protein involved in the trafficking of GluR1 receptors from the endoplasmic reticulum to the plasma membrane (Sans, et al., 2001) and recently implicated in synaptic plasticity (Nash, et al., 2010). Similarly, other aspects of synaptic transmission may be altered in Ts65Dn including those involved in excitatory signaling and neuromodulation (Chung, et al., 2009, Siarey, et al., 2006). Further studies where these pathways are left active will be necessary to determine their contributions.

Our demonstration of an increase in $GABA_B$/GIRK current in Ts65Dn CA1 pyramidal neurons corroborates our previous findings of increased conductance from cultured neurons as well as the gene dosage induced overexpression of GIRK channels (Best, et al., 2007, Harashima, et al., 2006). Here we did not find an accompanying increase in $GABA_B$ receptor expression, in fact we found a ~20% decrease. This result suggests that the GIRK channel overexpression may induce compensatory mechanisms that reduce $GABA_B$ receptor levels. However, given that an increased conductance level still exists in Ts65Dn CA1 pyramidal neurons, the degree of compensation seems to be insufficient. Alternatively, activation of GIRK channels by other neuromodulators may offset the benefit of reduced $GABA_B$ receptor expression levels or a similar offset may result from a change in the stoichiometric ratio between receptor and channel coupling or an increase in single channel conductance levels. However, we did not observe this in cultured Ts65Dn hippocampal neurons (Best, et al., 2007). Given the enhanced $GABA_B$ receptor mediated currents in these neurons (Figure 1), it is unlikely that other neurotransmitters coupled to GIRK channels are making up for reduced $GABA_B$ receptor expression levels.

The observed close linkage between GIRK expression levels and $GABA_B$ mediated current magnitudes suggests that the density of these channels may be a limiting factor in $GABA_B$ transmission at SR and SLM synapses (see patterns of GIRK2 expression in Ts65Dn and diploid hippocampus Fig. 7A,B Harashima, et al., 2006). While this conclusion is supported by our results, we cannot rule out contributions from other pathway specific changes such as alterations in efficiency or stoichiometry in receptor and channel coupling. Our current data does not provide sufficient detail to discriminate changes at this level. However, in our previous study in cultured Ts65Dn hippocampal neurons we found no evidence for alterations in receptor–channel interactions (Best, et al., 2007). Such localized changes could thus potentially occur and contribute to $GABA_B$ signaling differences between SLM and SR in Ts65Dn in the intact network *in vivo*.

Morphological properties of synapses within the hippocampus are also drastically altered in Ts65Dn mice. Among the reported changes are an increase in the synaptic opposition lengths for symmetric, presumed inhibitory, synapses (Belichenko, et al., 2009). The increased size of inhibitory synapses may make it more difficult for GABA transporters to clear the synaptic cleft of neurotransmitter following release and could explain our findings which suggest clearance is deficient in Ts65Dn mice (Figure 2). However, along with the increase in synapse size there is a subsequent upregulation of GAT1, a GABA transporter, in Ts65Dn hippocampus which could compensate for the increased synaptic size. Further investigations are needed to conclusively identify whether GABA clearance is indeed altered as our findings suggest.

In addition to its impact on signal transmission through its coupling to $GABA_B$ receptors, GIRK2 channel expression levels can alter neuronal resting potentials and consequently impact LTP (Cramer, et al., 2010). In GIRK2 knockout mice, CA1 pyramidal cells have significantly more depolarized resting potentials than controls and hippocampal slices from these mice showed enhanced LTP (Cramer, et al., 2010). In contrast, in DS mouse models such as the Ts65Dn mouse, which overexpresses GIRK2 (Harashima, et al., 2006), CA1 pyramidal cell resting potentials are more hyperpolarized, LTP is decreased and LTD is enhanced when compared to diploid controls. (Costa and Grybko, 2005, Fernandez, et al., 2007, Kleschevnikov, et al., 2004, Siarey, et al., 1999, Siarey, et al., 2006, Siarey, et al., 1997). Given that within the CA1 region, the most intense GIRK2 immunoreactivity is found within the SLM (Drake, et al., 1997, Liao, et al., 1996) and that GIRK channel conductance is much greater in the dendritic arbor than at somatic localizations (Chen and Johnston, 2005), the subcellular localization of GIRK channels might have a larger effect on membrane potentials in the dendrites rather than soma, where the surface to volume ratio is greater. Thus, Ts65Dn dendrites are likely more hyperpolarized than what we observed at the soma, particularly since ambient GABA concentrations are expected to be elevated in Ts65Dn CA1 as evidenced by the increase in sIPSC frequency (Chakrabarti, et al., 2010). Consequently, the impacts of GIRK overexpression are likely exacerbated in distal dendrites of CA1 pyramidal cells such as sites within the SLM.

### Possible disruptions in hippocampal network timing

The timing of pre- and postsynaptic neuron activity can determine the relative efficacy of synaptic connections. In particular, proper gating of information flow through the hippocampus is dependent upon the timing of inputs along the two principal pathways into the CA1 pyramidal neurons (Ang, et al., 2005, Jarsky, et al., 2005). For example, stimulation of the perforant pathway has been shown to block information flow through the Schaffer Collateral (SC) pathway (Dvorak-Carbone and Schuman, 1999) minimizing interference between the two circuits. This information blocking property of the perforant pathway is dependent on $GABA_B$ receptor activation and is most efficacious when timed with the peak of hyperpolarizing currents (Dvorak-Carbone and Schuman, 1999) suggestive of a role for GIRK channels in shunting excitatory input from SC. When the perforant pathway was potentiated by an LTP inducing protocol, the ability to block SC driven spikes was enhanced, likewise, LTD protocols within the SLM decreased the efficacy to block spikes (Remondes and Schuman, 2002). Furthermore, stimulation of PP fibers was also able to impede the potentiation of SC-CA1 synapses by LTP protocols (Remondes and Schuman, 2002). Thus, in addition to the potentially detrimental effects of excessive inhibition within the PP itself, the gating of SC inputs to CA1 pyramidal neurons is also likely to be adversely impacted in Ts65Dn. Such changes would be expected to further disrupt cognitive behaviors dependent on the proper segregation of information flow through these primary hippocampal pathways and contribute to observed deficits in spatial memory in Ts65Dn mice (Demas, et al., 1998). Furthermore, because interneuron activity strongly regulates spike timing and rhythmic activity (Bacci and Huguenard, 2006, Wang and Buzsaki, 1996), any change in response to GABAergic signaling would be expected to further disrupt the control of timing and rhythmicity by overpopulated interneurons in Ts65Dn mouse (Chakrabarti, et al., 2010).

Our results showing enhanced $GABA_B$ – mediated inhibition within CA1 with relatively unaltered $GABA_A$ergic transmission, coupled with studies such as (Kleschevnikov, et al., 2004) indicating altered $GABA_A$ergic transmission within the dentate gyrus, suggest that there are region specific GABAergic signaling deficits within the Ts65Dn hippocampus. The increased $GABA_B$/GIRK response in CA1 pyramidal neurons is likely to contribute to abnormal coordinated firing and plasticity (Ehrengruber, et al., 1997, Takigawa and Alzheimer, 2003) and, ultimately, information flow through this region of the hippocampus. Thus, therapies that target both GABAergic pathways may produce a synergistically beneficial effect on hippocampal mediated cognitive processing.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Highlights

- $GABA_B$/GIRK current density is elevated in CA1 neurons of Ts65Dn mouse model of DS
- GABAergic dysfunction is implicated in hippocampal deficits in a mouse model of DS
- $GABA_B/GABA_A$ current ratio evoked by SLM were larger in CA1 Ts65Dn hippocampus
- Balance between $GABA_B$ & $GABA_A$ inhibition is impaired in CA1 Ts65Dn pyramidal cells
- GIRK2 represents potential therapeutic target for cognitive treatment of DS

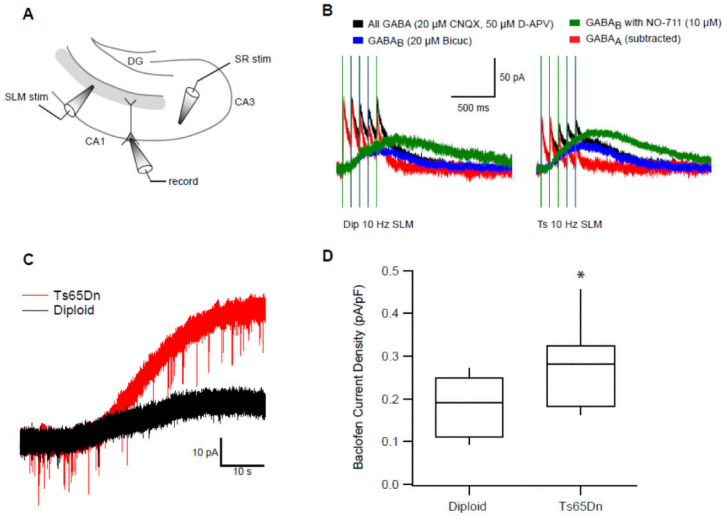


**Figure 1. GABAergic responses in Ts65Dn and diploid CA1 pyramidal neurons**

**(A)** Diagram depicting recording setup. Stimulating electrodes were placed in the stratum radiatum and stratum lacunosum moleculare on opposite sides of the CA1 pyramidal cell from which whole-cell recordings were performed. **(B)** Diploid and Ts65Dn neuron responses to SLM stimulation at 10 Hz. $GABA_A$ (red), $GABA_B$ (blue) and $GABA_B$ with NO-711 (green) recordings. **(C)** Bath application of 25μM baclofen evokes a larger hyperpolarizing current in Ts65Dn CA1 pyramidal cells (n=6, red) compared to diploid controls (n=12, black). Mean membrane capacitance values were: dip 141 ± 2 pF, Ts 148 ± 3 pF (not significantly different, p = 0.57). **(D)** Group data of current density reveals that this effect is statistically significant (*p<0.05, Student's two tailed t-test).

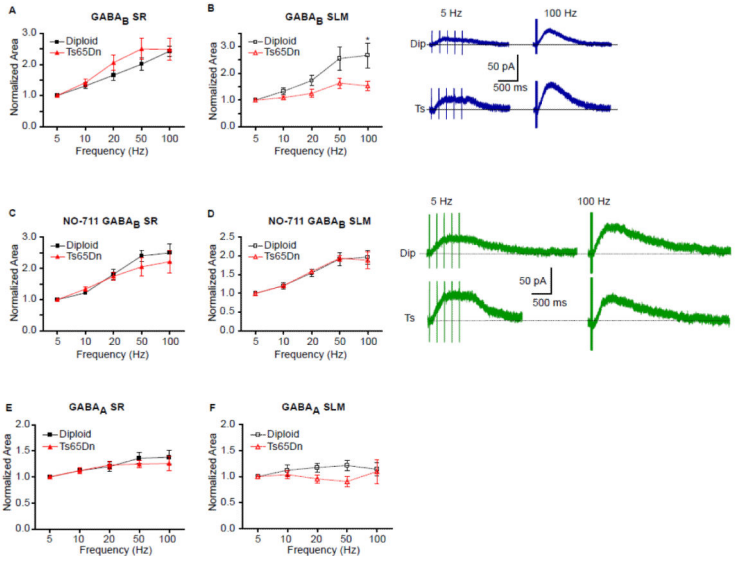


**Figure 2. IPSC charge transfer normalized to stimuli at 5 Hz**

**(A)** Normalized $GABA_B$ charge transfer (area) from SR stimuli is similar between diploid and Ts65Dn. **(B)** For SLM stimuli, the normalized $GABA_B$ area from Ts65Dn neurons is significantly decreased (two-way ANOVA, dip n=15, Ts n=11, $p<0.005$) at 100 Hz being significantly less (Bonferroni *$p<0.05$) in Ts65Dn than diploid. Example CA1 recordings in response to SLM stimuli on right. **(C, D)** Following application of 10 μM NO-711 the normalized $GABA_B$ area for either SR or SLM stimuli did not differ between Ts65Dn and control. Example CA1 recordings in response to SLM stimuli on right. **(E)** Normalized $GABA_A$ charge transfer in response to SR stimuli is similar between diploid and Ts65Dn but not in response to SLM stimuli (**F**; two-way ANOVA, dip n=14, Ts n=11, $p<0.05$; Bonferroni post-hoc was not significant for any frequency).

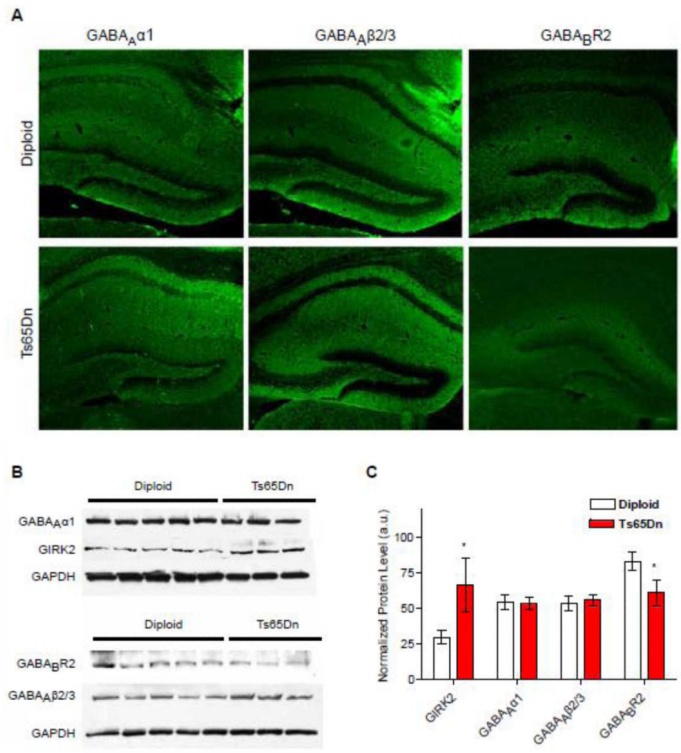


**Figure 3. Decrease in GABA$_B$R2 protein expression in Ts65Dn hippocampus**

**A.** Representative images of GABA$_A$α1, GABA$_A$β2/3, GABA$_B$R2 and GIRK2 immunohistochemistry on the hippocampus of diploid and Ts65Dn mouse at P15. **(B)** Representative Western Blot of the total protein level for GABA$_A$α1, GABA$_A$β2/3 and GABA$_B$R2 from the hippocampus of P15 Ts65Dn/diploid littermate pairs. GAPDH protein is used as an internal control. **(C)** Quantification of band densitometries shows no significant change in the GABA$_A$α1 or GABA$_A$β2/3 protein level. However, a significant decrease in GABA$_B$R2 was observed in the hippocampus of Ts65Dn mouse compared to the diploid littermates (n = 5-8 mice; *p<0.05). Significant (n = 5-8 mice; *p<0.01) differences in GIRK2 protein confirms previous results (Harashima *et al.*, 2006b).

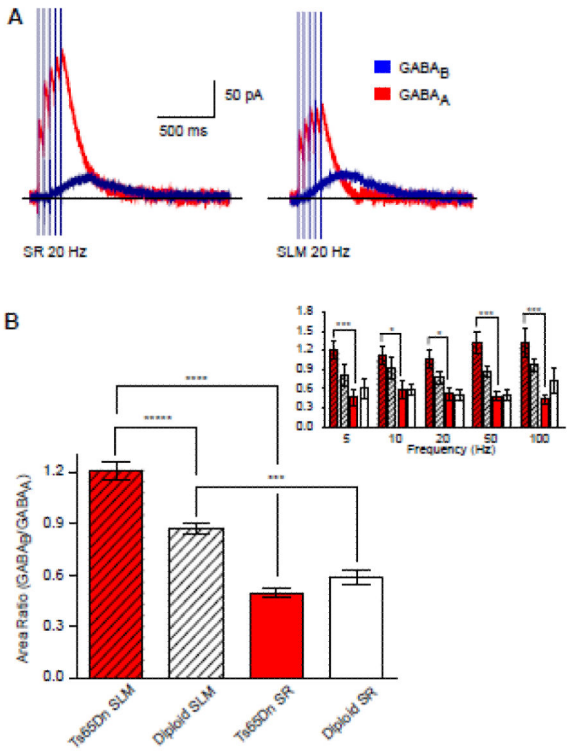


**Figure 4. In Ts65Dn SLM stimuli produce a greater GABA$_B$/GABA$_A$ charge transfer ratio than diploid**

**(A)** Examples of GABA$_B$ (blue) and GABA$_A$ (red, digitally subtracted) responses to SR and SLM stimuli at 20Hz for Ts65Dn mouse. **(B)** The charge transfer ratio of GABA$_B$ and GABA$_A$ receptor mediated currents (the ratio of total area under the GABA$_B$ or GABA$_A$ current curve for the entire train of five synaptic stimuli) is significantly greater for SLM (hatched bars) than SR (solid bars) stimuli in both diploid (white bars; two-way ANOVA: SR n=13-14, SLM n=15, ***p<0.001) and Ts65Dn neurons (red bars; two-way ANOVA: SR n=12, SLM n=11, ****p<0.0001). There is no difference between diploid and Ts65Dn for SR stimuli, however, Ts65Dn SLM shows significantly higher charge ratios than diploid SLM (two-way ANOVA, *****p<0.0005). The inset shows the ratios for each frequency delivered. Bonferroni post-hoc analysis showed no significant differences between diploid SR and SLM for any single frequency. However, in Ts65Dn, Bonferroni post-hoc analysis indicates that at each frequency SLM stimuli produced a significantly greater GABA$_B$/GABA$_A$ ratio than SR stimuli (*p<0.05; ***p<0.001).