

Pharmacotherapy for Cognitive Impairment in a Mouse Model of Down syndrome

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Recent neuroanatomical and electrophysiological findings from a mouse model of Down syndrome (DS), Ts65Dn, suggest that there is excessive inhibition in the dentate gyrus, a brain region important for learning and memory. This circuit abnormality is predicted to compromise normal mechanisms of synaptic plasticity, and perhaps mnemonic processing. Here, we show that chronic systemic administration of non-competitive GABA_A antagonists, at non – epileptic doses, leads to a persistent, post drug, recovery of cognition in Ts65Dn mice, as well as recovery of deficits in long – term potentiation (LTP). These data suggest that excessive GABAergic inhibition of specific brain circuits is a potential cause of mental retardation in DS, and that GABA_A antagonists may be useful therapeutic tools to facilitate functional changes that can ameliorate cognitive impairment in children and young adults with the disorder.

Ts65Dn mice, like patients with DS, exhibit comprehensive deficits in declarative learning and memory¹. Interestingly, these cognitive deficits are likely not due to gross abnormalities in Ts65Dn neuroanatomy², but rather, appear to

derive from selective decreases in the number of excitatory synapses in the brain³ and corresponding changes in synaptic connectivity^{4, 5}. These findings are supported by *in vitro* studies demonstrating that synapses in the Ts65Dn hippocampus can express normal LTP, but that excessive GABA – mediated inhibition impairs its induction^{6, 7}. Assuming that triplicated hC21 – related genes in Ts65Dn mice shift the optimal balance of excitation and inhibition in the dentate gyrus (and perhaps other brain regions) to a state where excessive inhibition obscures otherwise normal learning and memory, we hypothesized that subtly reducing the inhibitory load in the Ts65Dn brain with GABA_A receptor antagonists might rescue defective cognition.

After establishing that the pattern of cognitive impairments in Ts65Dn mice (3 – 4 months of age) matched those observed in children and young adults with DS (**Supplementary Figure 1** online)⁸, we proceeded to assess whether a non – epileptic dose of the non-competitive GABA_A antagonist picrotoxin (PTX; i.p., 1.0 mg/kg, a dose used extensively in classic rodent studies on memory consolidation)⁹, could improve Ts65Dn object recognition memory. While pilot studies indicated that a single dose, one day before testing, could not rescue Ts65Dn object recognition performance, a chronic 2 – week daily regimen showed obvious beneficial effects (**Supplementary Figure 2** online). We therefore initiated a 4 – week longitudinal, cross – over study. Here, WT and Ts65Dn mice were randomly assigned to groups receiving daily i.p. injections of saline or PTX (1.0 mg/kg), and were submitted to four weekly repetitions of object recognition testing, in which the animals were serially presented with 4

different object sets. At the 2 – week midpoint of this experimental period, WT and Ts65Dn mice that had been receiving saline were randomly segregated into groups that continued to receive daily saline injections, or into groups that began daily injections of PTX. Conversely, WT and Ts65Dn mice that had been chronically administered PTX in the first fortnight of testing, were now switched onto a saline regimen. Alongside saline and PTX, we also evaluated the efficacy of bilobalide (BB; i.p., 5.0 mg/kg)¹⁰, a PTX – like compound that could be safely administered for the whole, 4 – week experimental period.

Not surprisingly, Ts65Dn mice injected with saline during the first 2 – week period of novel object recognition testing, or those receiving saline over the course of the whole experimental period, did not exhibit novelty discriminations significantly above chance (**Figs. 1a – 1d**). In marked contrast, Ts65Dn mice treated with PTX during the first or second fortnight, showed normalized object recognition performance, as did those receiving BB throughout the study (**Figs. 1a – 1d**). Unexpectedly, Ts65Dn mice that had undergone chronic PTX administration during the first 2 – week period of novel object recognition testing, maintained their improved performance when evaluated one and two weeks later (**Figs. 1a – 1d**, and **Supplementary Table 1** online). Importantly, WT and Ts65Dn mice did not differ in total object exploration time, spending invariably ~ 25% of their experimental sessions investigating objects (**Supplementary Table 2** online).

To extend these findings, we next evaluated the effects of pentylentetrazole (PTZ), a non – competitive GABA_A antagonist with a long

history of medical use¹¹, on declarative memory in the novel object recognition test and in a modified spontaneous alternation task. To mimic the most typical route of drug administration in humans, WT and Ts65Dn were administered PTZ (3.0 mg/kg in milk; a non – epileptic dose that can be safely given to rodents for up to a year)¹², via voluntary oral feeding (see **Methods** online). In total, WT and Ts65Dn mice received 17 daily doses of milk or a milk – PTZ cocktail, and were submitted to two repetitions of novel object recognition testing, or to three daily T – maze sessions at the tail end of the treatment regimen. In agreement with previous results, milk – fed Ts65Dn mice showed an inability to discriminate object novelty in the object recognition task. PTZ – treated Ts65Dn mice, on the other hand, exhibited discrimination indices on par with those of WT mice (**Fig. 2a** and **Supplementary Table 1** online). In the spontaneous alternation task, milk – fed Ts65Dn mice also exhibited a similar pattern of impairment as untreated Ts65Dn. However, those orally receiving PTZ showed normal levels of alternation, approximately 70%¹³ (**Fig. 2c – 2d** and **Supplementary Table 3** online). Notably, WT and Ts65Dn mice or those on PTZ did not differ in object exploration time in the object recognition task, or exhibit arm biases in the spontaneous alternation task (**Supplementary Tables 2** and **4** online).

To better define the longevity of Ts65Dn cognitive improvement after GABA_A antagonist administration, we subsequently evaluated Ts65Dn mice in the novel object recognition task, exactly 2 months after the termination of a 17 – day oral PTZ regimen. Consistent with the post – drug recovery in cognition

observed with PTX, Ts65Dn mice having underwent PTZ administration showed normal object recognition performance at this time point (**Fig. 2b**).

The ability of animals to learn and remember is thought to be encoded at the synaptic level, and involves the ability of synapses to undergo long-term changes in synaptic strength. Indeed, recent work has provided compelling evidence that LTP in the hippocampus occurs during learning¹⁴, and is required for memory¹⁵. Accordingly, we assessed LTP in the dentate gyrus, a structure that shows the most pronounced inhibition – related pathology in the Ts65Dn brain⁴. Specifically, we determined whether LTP deficits at perforant path synapses in Ts65Dn mice were rescued by chronic oral PTZ administration at 3 – 4 weeks post – drug treatment, a time window congruent with performance improvement by Ts65Dn mice in the novel object recognition task post – PTX treatment. In agreement with these behavioral findings, we found that PTZ – treated Ts65Dn mice exhibited normalized LTP in the dentate gyrus approximately 1 month after the cessation of drug administration (**Figs. 3a – 3d**). We then assessed the relative permanency of this LTP rescue in Ts65Dn mice, and found that the Ts65Dn dentate gyrus continued to show greater LTP in PTZ – treated mice versus milk – fed ones for up to 3 months post – drug regimen (albeit diminished relative to that of WT mice) (**Figs. 3e – 3h**), in keeping with Ts65Dn behavioral improvement 2 months after PTZ administration.

In summary, we have demonstrated that chronic administration of non-competitive GABA_A antagonists (at non – epileptic doses) ameliorates cognitive deficits in Ts65Dn mice for a period of months extending beyond the window of

drug treatment. Likewise, we have shown that drug – mediated improvements in Ts65Dn learning and memory are accompanied by rescue of impaired LTP, the most prominent synaptic correlate of learning and memory in the hippocampus. These results point to overinhibition, in at least some brain regions, as one possible mechanism that reduces cognitive performance in a mouse model of DS (see **Supplementary Discussion** online), though further experimentation will be necessary in order to more directly test this mechanism, and to elucidate the neuroadaptations that are orchestrated in response to repetitive GABA_A antagonist administration. They also highlight the potential clinical utility of non – competitive GABA_A antagonists in DS (including bilobalide and pentylenetetrazole), providing one window into how cognitive impairment in DS may be pharmacologically mitigated over time (see **Supplementary Discussion** online).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Figures

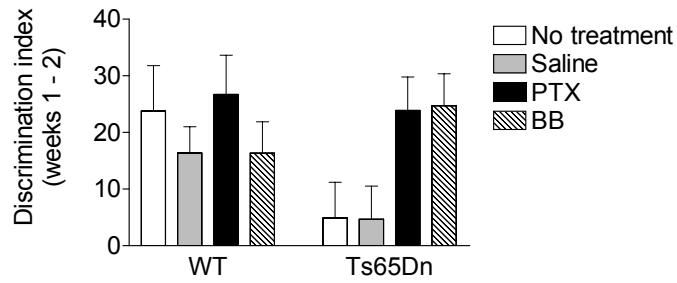
Figure 1. Picrotoxin (PTX) and bilobalide (BB) rescue Ts65Dn performance in the novel object recognition task.

Discrimination Indices (DI's) of WT and Ts65Dn mice involved in a 4 week cross – over study. (a) Animals in the first two weeks were either left untreated, or received daily injections of saline, PTX or BB. While untreated and saline treated Ts65Dn mice do not exhibit a novel object preference during the first fortnight (i.e., $DI > 0$; $t_{17} = 0.7737$, $P > 0.4497$; and $t_{16} = 0.8169$, $P > 0.4260$), PTX and BB treated Ts65Dn mice show an ability to discriminate object novelty ($t_9 = 4.083$, $P < 0.003$; and $t_{15} = 4.390$, $P < 0.001$). (b) Saline treated Ts65Dn mice given PTX during the second period of object recognition testing (Sal → PTX), start out with the same deficits as those continuing to receive saline in the second fortnight (Sal → Sal), suggesting that there is no sampling bias for animals in subsequent treatment groups. (c) During the second two weeks, saline treated Ts65Dn mice switched to PTX discriminate novel objects similarly to WT mice ($t_8 = 3.756$, $P < 0.006$). Surprisingly, PTX treated Ts65Dn mice switched to saline in the second fortnight also maintain their ability to discriminate novel objects versus familiar ones ($t_6 = 3.250$, $P < 0.02$), suggesting a persistent change in brain function. (d) Compilation of WT and Ts65Dn mouse novelty discrimination scores with no treatment or treatment with saline, PTX or BB demonstrating that PTX and BB normalize Ts65Dn object recognition memory ($F_{5, 187} = 5.204$, $P < 0.0002$; all post hoc comparisons with Ts65Dn control, P 's < 0.05 ; all other post hoc comparisons, P 's > 0.05). Note that control observations

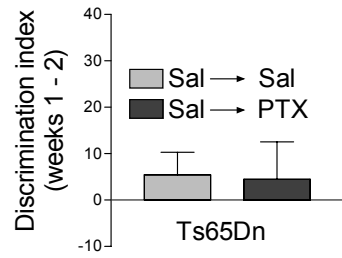
are pooled from untreated and saline treated (PTX naïve) mice, while PTX observations are pooled from mice treated in either the first or second fortnight. Discrimination scores for each condition are tabulated and defined in **Supplementary Data Table 1** online. Error bars represent SEM. All experimental procedures were approved by the Stanford University Institutional Animal Care and Use Committee (IACUC), and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals. See **Supplementary Methods** online for experimental details.

Figure 1.

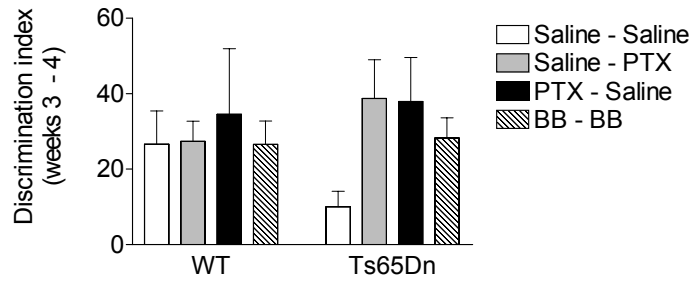
a.



b.



c.



d.

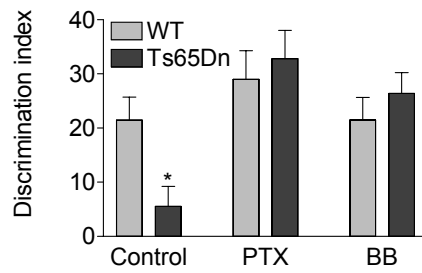


Figure 2. Pentylentetrazole (PTZ) elicits long-lasting cognitive improvement in Ts65Dn mice.

Novelty discrimination scores (DI's) of WT and Ts65Dn mice directly following a ~ 2 week treatment with PTZ (a), or two months post treatment (b). (a) Although Ts65Dn mice on milk do not exhibit a net novelty preference ($t_{17} = 1.099$, $P > 0.28$), those receiving PTZ perform as well as WT mice receiving either milk or PTZ ($F_{3, 71} = 3.356$, $P < 0.03$; all post hoc comparisons with Ts65Dn on milk, P 's < 0.05 ; all other post hoc comparisons, P 's > 0.05). (b) The normalized object recognition memory shown by Ts65Dn mice immediately post treatment, is sustained at two months post drug treatment ($F_{3, 38} = 5.134$, $P < 0.005$; all post hoc comparisons with Ts65Dn previously on milk, P 's < 0.05 ; all other post hoc comparisons, P 's > 0.05). Discrimination scores are tabulated in **Supplementary Data Table 1** online. (c & d) Alternation scores (%'s) of WT and Ts65Dn mice across 3 – 6 sessions of testing in the spontaneous alternation task. In contrast to WT mice, which exhibit optimal alternation percentages (~ 70%), untreated or milk treated Ts65Dn mice exhibit significantly lower alternation percentages ($t_{83} = 5.051$, $P < 0.0001$). However, PTZ normalizes Ts65Dn alternation scores to WT levels ($F_{3, 272} = 5.998$, $P < 0.0006$; all post hoc comparisons with Ts65Dn control, P 's < 0.05 ; all other post hoc comparisons, P 's > 0.05). Alternation scores for each condition are tabulated and defined in **Supplementary Data Table 3** online. Error bars represent SEM.

Figure 2.

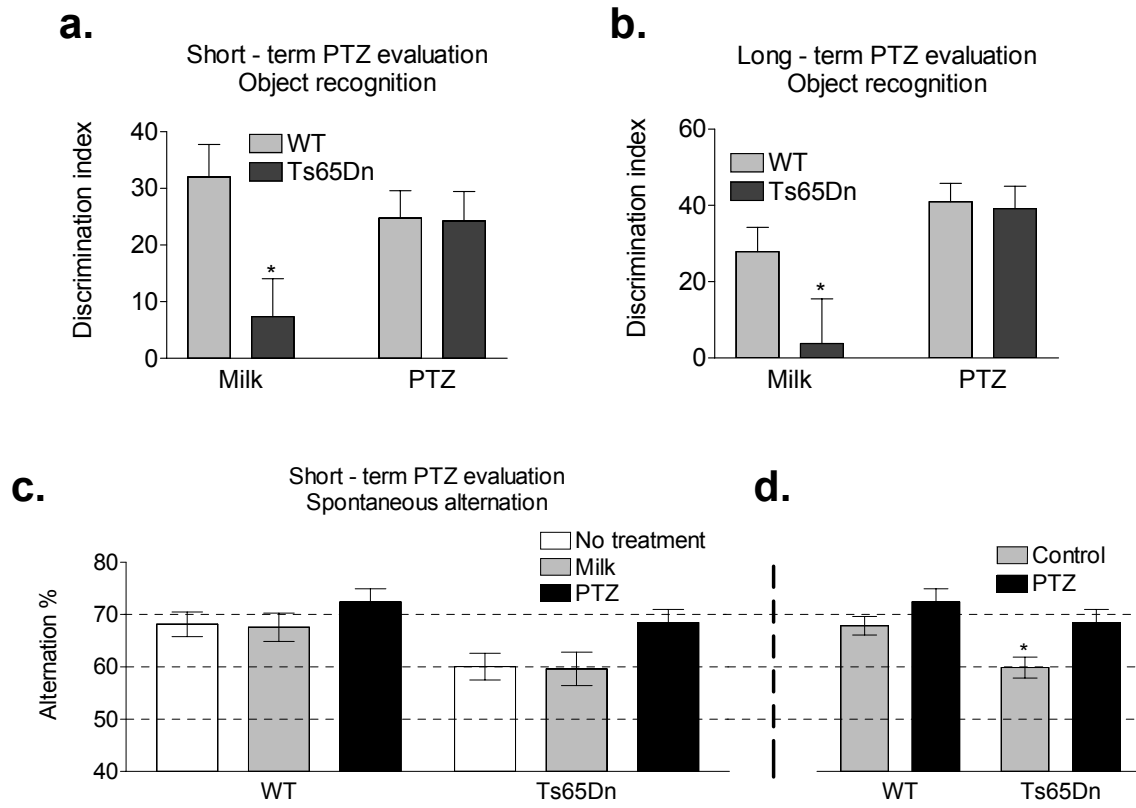
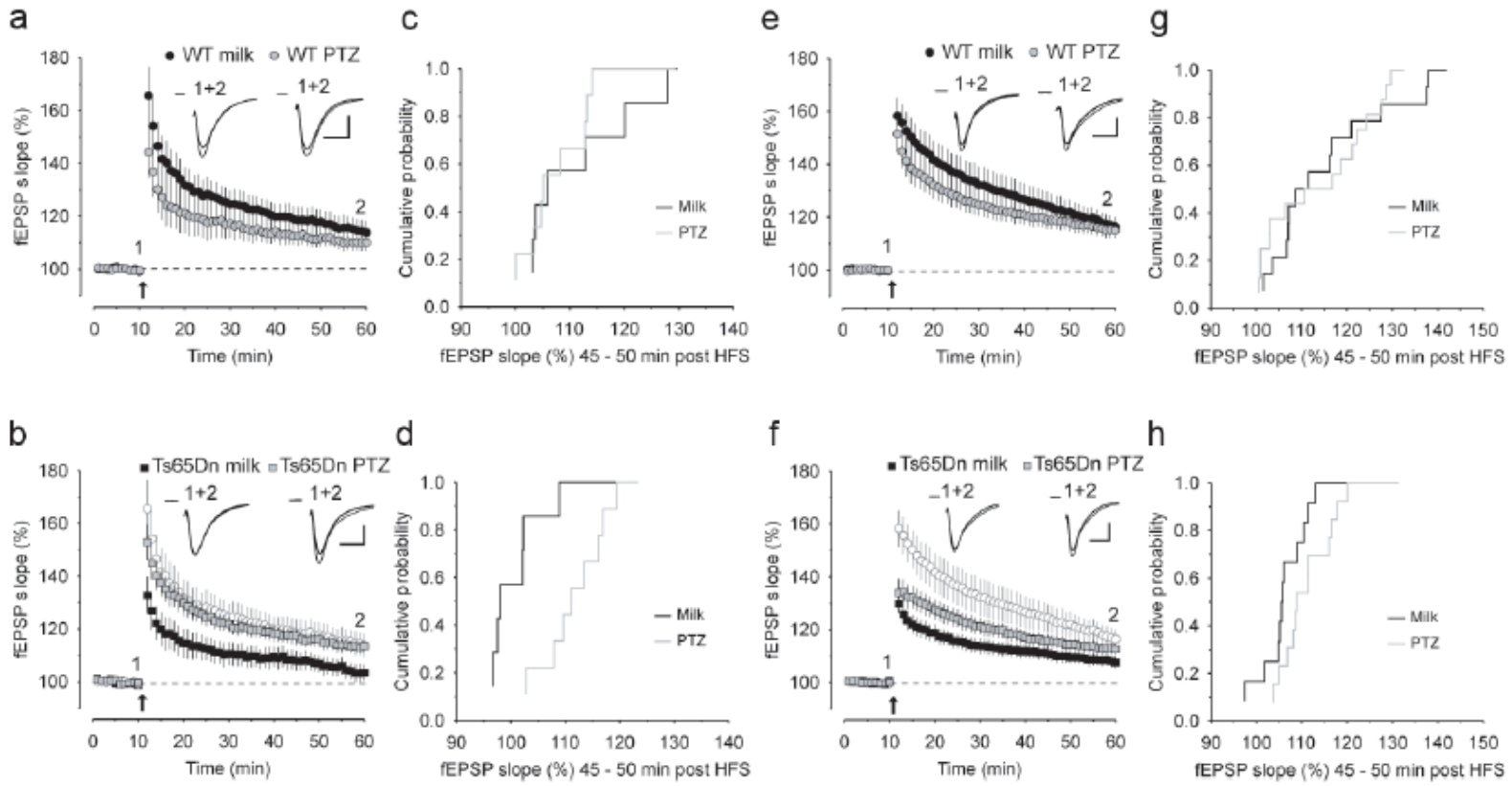


Figure 3. PTZ rescues LTP at medial perforant path-granule cell synapses in Ts65Dn mice.

(a & b) Averaged data for LTP induced in WT (a) or Ts65Dn mice (b) treated with milk (WT Milk LTP: $115 \pm 4.3\%$, filled circles, 2 mice, $n = 7$ slices; Ts65Dn Milk LTP: $104 \pm 3.2\%$, filled squares, 2 mice, $n = 7$ slices) or PTZ (WT PTZ LTP: $110 \pm 2.9\%$, open circles, 3 mice, $n = 9$ slices; Ts65Dn PTZ LTP: $113 \pm 2.1\%$, open squares, 3 mice, $n = 9$ slices), evaluated ~ 1 month after the cessation of drug administration (For comparison, data from milk treated WT mice are also shown in *b*; circles). (c & d) Cumulative probability plots of LTP observed in WT (c) or Ts65Dn mice (d) fed milk (black line), or PTZ (gray line). (e & f) Averaged LTP graph for WT (e) or Ts65Dn mice (f), 2 – 3 months after discontinuation of milk (WT Milk LTP: $117 \pm 3.6\%$, filled circles, 5 mice, $n = 14$ slices; Ts65Dn Milk LTP: $108 \pm 2.1\%$, filled squares, 3 mice, $n = 12$ slices) or PTZ treatment (WT PTZ LTP: $115 \pm 2.9\%$, open circles, 5 mice, $n = 16$ slices; Ts65Dn PTZ LTP: $113 \pm 2.1\%$, open squares, 4 mice, $n = 13$ slices; For comparison, data from milk treated WT mice are also shown in *f*; circles). (g & h) Cumulative probability plots of average LTP for WT (g) or Ts65Dn mice (h) previously fed milk (black line) or PTZ (grey line). Sample traces in graphs *a*, *b*, *e*, and *f* are averaged from 10 consecutive fEPSPs taken at the indicated time points. Accompanying scale bars are 1 mV, 5 ms. Values are expressed as mean \pm SEM.

Figure 3.



Methods

Supplementary Data Figure 1: Ts65Dn mice exhibit declarative memory problems, but intact procedural learning.

Supplementary Data Figure 2: Chronic but not acute administration of picrotoxin (PTX) normalizes Ts65Dn performance in the novel object recognition task.

Supplementary Data Table 1: Tabulated discrimination indices (DI's) in the novel object recognition task.

Supplementary Data Table 2: Picrotoxin (PTX), bilobalide (BB) and pentylentetrazole (PTZ) do not affect exploration times in the novel object recognition task.

Supplementary Data Table 3: Tabulated alternation percentages (%) in the spontaneous alternation task.

Supplementary Data Table 4: Pentylentetrazole (PTZ) does not affect arm entries in the spontaneous alternation task.

Supplementary Discussion: Potential utility of non-competitive GABA_A receptor antagonists for the treatment of cognitive impairment in Down syndrome.

Supplementary Reference list

Methods

General Strategy. To determine the relationship between excessive inhibition in the Ts65Dn brain and defective Ts65Dn cognition, we designed a line of experimentation that satisfied four criteria:

(a) that the cognitive tasks we used would be representative of the cognitive problems observed in children and young adults with DS; patients with DS exhibit particular neuropathology in the medial temporal lobe (comprised of the hippocampus proper and the *parahippocampal region*), and accordingly, exhibit particular deficits in declarative memory processing.

(b) that the cognitive tests we used would be non-aversive; Ts65Dn mice are naturally more emotionally labile than WT mice^{1, 2}, and their performance in cognitive tests has been demonstrated to be disproportionately influenced by stress³.

(c) that the drugs we used would be agents capable of globally reducing GABAergic inhibition – i.e., non – competitive GABA_A receptor antagonists that interfere with GABA – associated chloride channels.

(d) that the drugs we used could be clinically relevant – i.e., that they have a record of safe human use when given at low doses and can be administered orally.

To satisfy (a) and (b), we used exploratory paradigms in Ts65Dn mice capable of detecting hippocampal – and parahippocampal dysfunction, namely novel object recognition, and spontaneous alternation.

To satisfy (c) and (d), we used the quintessential non-competitive GABA_A antagonists, picrotoxin (PTX) and pentylentetrazole (PTZ), and bilobalide (BB), a picrotoxin – like compound that impairs chloride ionophore conductance. Due to its clinical history, we were particularly interested in the effects of PTZ on Ts65Dn learning and memory.

Animals. Segmental trisomy 16 (Ts65Dn) mice were obtained by mating female carriers of the 17¹⁶ chromosome (B6EiC3H – a/ATs65Dn) with (C57BL/6JEi x C3H/HeJ)F1 (JAX # JR1875) males. Ts65Dn mice were thus maintained on the B6/C3H background.

WT and Ts65Dn mice (Jax West Laboratories, Davis, CA and The Center for Research and Treatment of Down Syndrome at Stanford Breeding Facility, Palo Alto, CA), were housed with littermates in standard Plexiglas cages and kept on a 12 h/12 h light-dark cycle (lights on 0700 h). Food and water were available *ad libitum*. The temperature (22 ± 2 °C) and relative humidity (55 – 60%) were controlled. Mice arriving from Jax Laboratories were acclimated to these controlled housing conditions for at least two week before inclusion in any behavioral studies. All experimental procedures were approved by the Stanford University Institutional Animal Care and Use Committee (IACUC), and were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping. All WT and Ts65Dn mice were genotyped using real-time quantitative PCR with *App* – and *Apob* – specific TaqMan probes (Applied Biosystems)⁴. Since mice on a C3H background carry the retinal degeneration (*rd*) allele, WT and Ts65Dn mice were also genotyped for *rd* before being submitted to experimentation. *Rd*^{-/-} homozygotes were excluded from behavioral study.

Drug Administration. Picrotoxin (PTX; 1.0 mg/kg) and Bilobalide (BB; 5.0 mg/kg) were dissolved in sterile 0.9% sodium chloride (NaCl) solution and sonicated. WT and Ts65Dn mice were administered freshly prepared drug intraperitoneally (i.p., in a volume of 10.0 ml/kg) during the light cycle at times corresponding to animal handling. For chronic drug administration, the mice were given daily injections of PTX, BB, or saline in their home cages, inclusive of the days of behavioral training, where drug administration was conducted right after the training session.

Pentylentetrazole (PTZ; 3.0 mg/kg) was dissolved in 1% low fat chocolate milk (Berkeley Farms). For the purposes of drug delivery, WT and Ts65Dn mice were conditioned to drinking chocolate milk in their home cages from small Petri dishes, and then in cylindrical feeding tubes via voluntary intake for 4 days. The cylindrical feeding tubes were open top enclosures (12 cm in width, 17 cm high), whose width provided a restricted space for the mice that still allowed for comfortable movement. Subsequent to this conditioning, the animals were placed daily into the feeding tubes, and presented with small eppendorf caps of milk or a milk – PTZ cocktail (in a volume of 2.5 ml/kg). Milk consumption

typically occurred in ~ 5 min, at which point the mice were returned to their home cages. In total, WT and Ts65Dn mice received 17 servings of milk or PTZ, inclusive of the days of behavioral training, where drug administration was conducted right after the training session.

All drugs were purchased from Sigma Chemical Co (St. Louis, MO).

Novel Object Recognition. The novel object recognition task is based on the innate tendency of rodents to differentially explore novel objects over familiar ones. The task can be used repeatedly to evaluate rodent memory over time, and across various drug treatment regimens. Mice are trained and tested once per week, each experimental session separated by a 1 – week interval, and are serially presented with new object sets. In this scheme, each mouse is considered a naïve subject, and each week’s performance is considered an independent observation⁵.

Ten cohorts of WT and Ts65Dn mice (~ 6 mice/genotype/cohort) were submitted to daily handling sessions, and were given an opportunity to habituate to a black acrylic, open field arena (48 cm X 38 cm wide X 27 cm), where they were exposed to two identical objects (simple protocol), or to two different objects (complex protocol), during a 15 – min training session. Objects were made from various non-porous materials (porcelain, metal, glass, etc.), and had various color schemes. All were generally consistent in height and volume, and were symmetrical on a horizontal plane. They were positioned in two corners of the apparatus. A 15 – min testing session was conducted 24 h after training. Here,

the mice were presented with the object they had explored the previous day, and a new item (the objects being alternatively positioned in one corner or another in a balanced fashion within a given week, and from one week to another). Memory was operationally defined as the proportion of time animals spent investigating the novel object minus the proportion spent investigating the familiar one

[Discrimination Index, $DI = (\text{Novel Object Exploration Time} / \text{Total Exploration Time}) - (\text{Familiar Object Exploration Time} / \text{Total Exploration Time}) \times 100$], where exploration constituted any investigative behavior (i.e., head orientation, sniffing occurring within < 1.0 cm) or deliberate contact that occurred with each object.

To control for odor cues, the open field arena and the objects were thoroughly cleaned with 90% ethanol, dried, and ventilated for a few minutes between mice.

** Please note that all of the novel object recognition studies discussed in the main text, were conducted with the **complex** protocol using two different familiar objects during the training phase.

Spontaneous Alternation. Spontaneous alternation is based on the natural tendency of rodents to consecutively alternate between left and right arm choices during exploration in a T – maze apparatus. Notably, the maximum alternation rate exhibited by mice in the task is 70%, setting the ceiling for normal alternation ability. Conversely, the floor for alternation performance is 50%, representing random chance.

Four cohorts of WT and Ts65Dn mice (~ 10 mice/genotype/cohort) were submitted to daily handling, and evaluated in the spontaneous alternation task.

Here, the mice were placed in the start chamber (25 cm X 13 cm X 20 cm) of a transparent, acrylic T – maze, located at the far end of the stem arm (120 cm), and were subjected to daily habituation periods, during which time the area and its surroundings become a familiar “home base.” A divider panel separated the start chamber from the rest of the T – maze apparatus, comprised of an alleyway (95 cm) that conveyed to a choice point at the intersection of the stem arm, and the left and right arms of the maze (each 37 cm X 13 cm X 20 cm). Once acclimated, the mice were given daily free exploration sessions, where they were briefly confined to the start chamber (for 30 s), and then permitted access to the rest of the maze via removal of a sliding door in the divider panel (for a 7 min trial). Alternation attempts during the free alternation session, occurred when the mice entered one of the lateral arms, re – entered the stem arm, and then proceeded back to the choice point, where they could now cross into the left or right arm of the T – maze. Entry into a lateral arm opposite the one previously chosen was defined as an alternation, whereas entry into the previously visited arm was not. Alternation performance, thus, was operationally defined by the percentage of time the mice alternated upon arriving to the choice point of the apparatus (i.e., the number of alternations observed / the number of alternation attempts). To control for odor cues, the T – maze apparatus was cleaned with 90% ethanol, dried, and ventilated for a few minutes between mice.

Rotorod. The rotorod is a classic test to measure motor learning in rodents. Mice are repeatedly placed on an elevated rotating treadmill, and their latency to fall

from the drum of the treadmill is recorded. Typically, the ability of mice to stay atop the treadmill increases as they acquire more rotorod sessions.

Two cohorts of WT and Ts65Dn mice (6 mice/genotype/cohort) were submitted to daily handling sessions, and evaluated in the rotorod test using Med Associates single station rat rota-rods (shaft d = 7.0 cm; width = 8.9 cm). Here, the mice were required to ambulate on the drum of a treadmill, revolving at an accelerating speed (4.0 – 40 RPM), to avoid falling 26.7 cm to a plastic surface below. Each mouse was given 6 acute trials on the rotorod apparatus (separated by 60 – 120 s), followed 24 h later by 2 additional trials. Motor performance was operationally defined by the latency to fall from the treadmill, automatically recorded by photobeams on the plastic floor.

Electrophysiology. Hippocampal slices (400 μ m thick) were prepared from 6-9 month old WT and Ts65Dn mice. Transverse slices from the mid to temporal pole of both hippocampi were cut in ice cold sucrose solution containing (in mM): Sucrose 238, KCl 2.5, NaH₂PO₄ 1.3, NaHCO₃ 26.2, CaCl₂ 1, MgSO₄ 2 and D-glucose 11 (saturated with 95% O₂ / 5% CO₂). Slices were transferred to a holding chamber filled with external solution consisting (in mM): NaCl 119, KCl 2.5, NaH₂PO₄ 1, NaHCO₃ 26.2, CaCl₂ 2.5, MgSO₄ 1.3 and D-glucose 11 (saturated with 95% O₂ / 5% CO₂). Slices were allowed to equilibrate at 28 – 30 °C for at least 1.5 h before being transferred to a recording chamber and superfused (2 ml/min) with external solution warmed to 28 – 30 °C.

Dendritic Field EPSPs (fEPSPs) were recorded in the middle third of the molecular layer with a patch electrode filled with 1 M NaCl solution containing 10 mM HEPES (pH adjusted to 7.4 with NaOH). Basal fEPSPs were evoked at 0.05 Hz with a monopolar stimulating electrode positioned in the middle third of the molecular layer ~ 400 μ m from the recording electrode. Slices were stimulated for at least 5 min prior to data acquisition. During this “warm up” period, stimulation intensity was adjusted to evoke stable baseline fEPSPs that were 70 – 80% of the maximally evoked fEPSP. Activation of medial perforant path synapses was confirmed by paired-pulse stimulation (50 ms inter – stimulus interval) which elicited a paired-pulse depression (PPD) of the fEPSP. PPD was observed for all groups and no obvious differences were detected between them. Long-term potentiation (LTP) was induced by a high frequency stimulus (HFS) consisting of four 100 Hz, 0.5 s trains each separated by 20 s.

Field recordings were filtered at 1 KHz and digitized at 5 KHz with an A/D board (National Instruments) driven by custom acquisition software designed to run on IGOR pro. LTP graphs were generated by averaging the slope of the fEPSPs in 1 min bins, and expressing data as a percentage of the averaged 10 min baseline collected before LTP induction. Cumulative probability plots and LTP values were constructed by averaging the percent of LTP observed between 45 – 50 min following HFS.

Data Analysis. All behavioral experiments were videotaped via a digital camera, and scored offline by observers blind to treatment, but not genotype; Ts65Dn mice exhibit salient craniofacial features, and gait patterns during locomotion, that clearly distinguish them from WT mice. For validation studies, data were analyzed using Student's unpaired *t* test or two – way ANOVA where appropriate. For studies with PTX and BB, data were analyzed by one – way ANOVA, supplemented with Newman – Keuls test for post-hoc comparisons between groups. For studies with PTZ, data were analyzed by one – way ANOVA, supplemented with Fisher's LSD post – hoc tests. Lastly, for electrophysiological studies, data were analyzed by Mann – Whitney *U* tests. Statistical significance was set at $p < 0.05$.

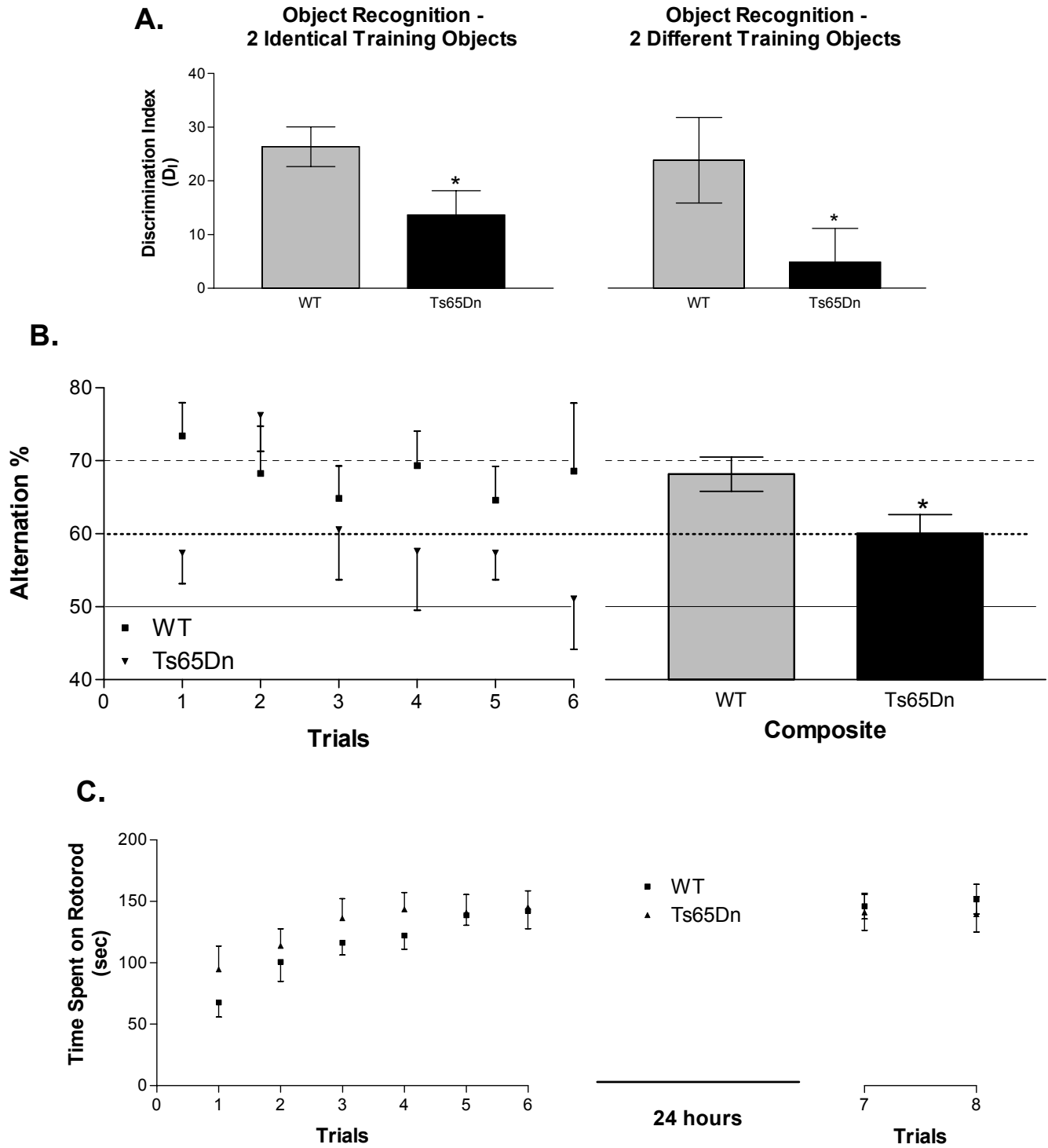
Supplementary Data Figure 1. Ts65Dn mice exhibit declarative memory problems, but intact procedural learning.

Patients with DS exhibit selective deficits in tasks requiring explicit learning and memory (and thus the integrity of structures in the medial temporal lobe)⁶, but not in those requiring procedural or skill learning (and thus the integrity of the cerebellum)⁷. Additionally, their difficulty in performing declarative memory tests scales with increasing task difficulty. To assess whether declarative memory is altered in Ts65Dn mice, we initially conducted two different novel object recognition assays: one using a simple protocol involving a pair of identical objects during the familiarization phase, versus another using a more challenging protocol involving two different familiar objects. In both task variants, the familiarization or “training” phase (15 min; a bulk of time sufficient to saturate and equate mnemonic encoding of objects)^{8, 9} was separated from the testing phase (also 15 min) by 24 hours, a time frame typically used to evaluate rodent long-term memory. Consistent with neuropsychological studies in human DS reporting more pronounced declarative memory deficits in DS patients with increasing task demands, Ts65Dn mice exhibited significantly impaired novel object recognition in the simple task, but a complete inability to discriminate between novel and familiar objects in the complex one (**Supp. Fig. 1A**).

We next evaluated another domain of declarative memory, namely spatial navigation, using spontaneous alternation, a spatial working memory task that is ethologically relevant to most mammalian species, including humans¹⁰. In keeping with previous studies¹¹, Ts65Dn mice exhibited an impaired ability to

freely alternate in the T-maze, a defect that was maintained across a number of experimental sessions (**Supp. Fig. 1B**). This defect was well-defined in Ts65Dn mice, which showed a rate of alternation halfway between 50% chance and the maximum alternation percentage usually achieved by wild-type (WT) mice in a T-maze, 70%^{12, 13}.

In contrast to their deficits in declarative memory tasks, Ts65Dn mice performed as well as WT mice in an implicit memory test. Specifically, in agreement with previous investigations¹⁴, Ts65Dn mice normally acquired and maintained 24h -learning of an accelerating rotorod task (**Supp. Fig. 1C**). These results suggest that procedural learning in Ts65Dn mice, unlike explicit learning, is intact. Notably, despite exhibiting cerebellar pathology, Ts65Dn mice show no significant cerebellar-related cognitive deficits.



Supplementary Figure 1 Legend: (A) WT and Ts65Dn performance in the simple (2 identical objects) and complex (2 different objects) novel object

recognition tasks. Ts65Dn mice exhibit significantly impaired novel object recognition performance in the simple task (left panel) relative to WT mice (DI = 26.35 ± 3.687 , $n = 36$ for WT mice; DI = 13.64 ± 4.491 , $n = 35$ for Ts65Dn mice; $t_{69} = 2.193$, $P < 0.02$), but show a net novelty preference (i.e., DI > 0; $t_{34} = 3.038$, $P < 0.005$). In contrast to their ability to recognize new objects in the simple object recognition task, Ts65Dn mice exhibit a pronounced inability to recognize object novelty in the complex task (involving the presentation of two different objects; right panel) (DI = 23.81 ± 7.969 , $n = 17$ for WT mice; DI = 4.856 ± 6.276 , $n = 18$ for Ts65Dn mice; $t_{33} = 1.880$, $P < 0.04$), demonstrating no net preference between novel and familiar objects (No preference = 0, $t_{17} = 0.7737$, $P > 0.44$).

(B) WT and Ts65Dn performance in the spontaneous alternation task. WT and Ts65Dn alternation percentages do not change across several daily sessions of free exploration in the T-maze [$F_{5, 90} = 1.091$, $P > 0.37$], but are significantly different from each other [$F_{1, 90} = 5.497$, $P < 0.03$]. Indeed, Ts65Dn mice exhibit a well-defined, constitutive alternation deficit (alternation = $68.18 \pm 2.352\%$, $n = 54$ for WT mice; alternation = $60.05 \pm 2.582\%$, $n = 48$ for Ts65Dn mice; $t_{100} = 2.333$, $P < 0.01$), alternating half as well as WT mice, between chance level at 50% and the optimal alternation rate typically observed in mice, 70% [chance = 50%, $t_{47} = 3.891$, $P < 0.0003$; optimal alternation rate = 70%, $t_{47} = 3.854$, $P < 0.0004$].

(C) WT and Ts65Dn performance on an accelerating rotorod. WT and Ts65Dn (12 mice per genotype) exhibit motor learning in the rotorod task over

several trials [$F_{7, 175} = 5.328, P < 0.0001$], and exhibit learning curves that are not significantly different from one another [$F_{1, 175} = 1.587, P > 0.20$].

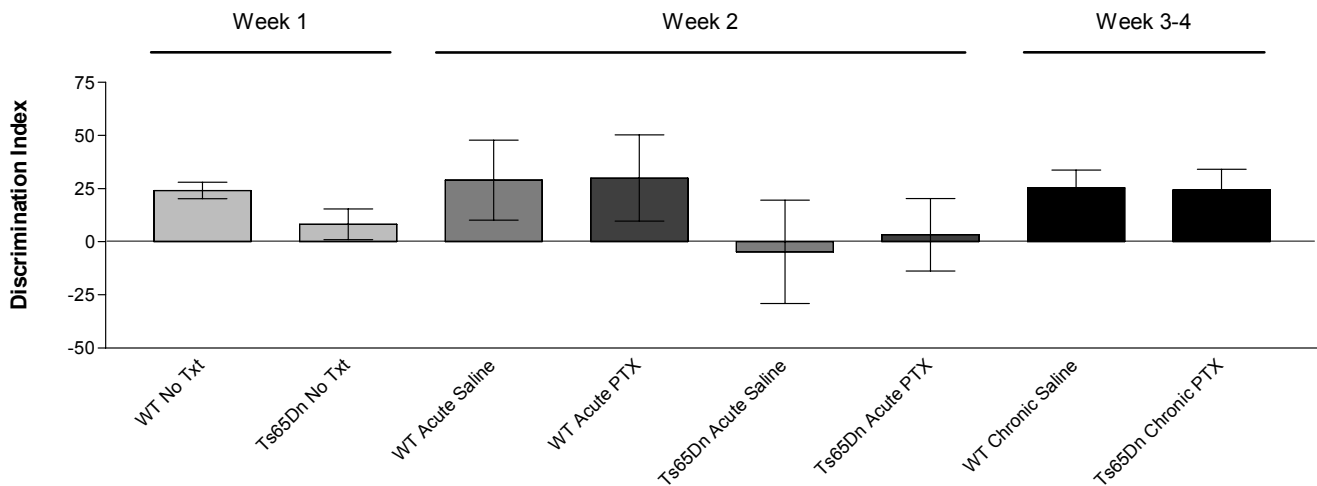
n values reflect the number of observations per treatment. Values are expressed as mean \pm SEM.

Supplementary Data Figure 2. Chronic but not acute administration of picrotoxin (PTX) normalizes Ts65Dn performance in the novel object recognition task.

As an initial test of our hypothesis that excessive inhibition in the brains of Ts65Dn mice impairs declarative learning and memory, we assessed whether the administration of a non-competitive GABA_A receptor antagonist could rescue Ts65Dn performance in the novel object recognition task (see **Supp. Fig. 1A**). In these preliminary studies, we employed the quintessential non-competitive GABA_A receptor antagonist picrotoxin (PTX). This compound has excellent bio-availability and due to its mechanism of action at GABA_A receptors (i.e., direct binding of GABA_A receptor-associated chloride channels and disruption of chloride conductance), is predicted to globally suppress GABA-mediated inhibition in the brain. In the current experiment, one cohort of WT and Ts65Dn mice was evaluated in the object recognition task across 4 sequential weeks. In the first week of the experiment, we tested the ability of WT and Ts65Dn mice to discriminate a novel object from a familiar one using the simple version of the object recognition task (see **Supp. Fig. 1A**). Here, untreated WT mice exhibited significantly higher DI scores than untreated Ts65Dn mice [DI = 24.10 ± 3.920 , $n = 6$, for WT mice; DI = 8.150 ± 7.229 , $n = 6$, for Ts65Dn mice; $t_{10} = 1.940$, $P < 0.04$]. In the second week, we examined whether an acute administration of PTX had any pro-cognitive effects in Ts65Dn mice. Here, WT and Ts65Dn mice that had been tested the previous week were randomly assigned to control and drug treatment groups ($n = 3$ for each genotype/treatment condition). Animals received

an acute injection of saline or PTX (1.0 mg/kg) immediately after object recognition training, and the effects of these treatments were evaluated 24-h later during object recognition testing. Surprisingly, WT and Ts65Dn mice exhibited performances, on average, similar to those in Week 1, suggesting that acute PTX is unable to rescue memory deficits in Ts65Dn mice.

Supplementary Figure 2.



Given the absence of any improvement, we continued to use the same set of animals to explore whether chronic administration of PTX could ameliorate Ts65Dn object recognition performance. Here, Ts65Dn mice received daily injections of PTX (1.0 mg/kg) for two weeks, while WT mice received daily injections of saline. Both groups of mice were evaluated in the object recognition task at weekly intervals during the treatment period (ie., during weeks 3 and 4). Subsequently, we found that with the PTX regimen Ts65Dn mice exhibited improved novelty discrimination scores on par with those of WT mice [DI = 25.42

± 8.297 , $n = 12$, for WT mice on saline; $DI = 24.37 \pm 9.791$, $n = 12$, for Ts65Dn mice on PTX; $t_{22} = 0.08181$, $P > 0.46$].

Supplementary Data Table 1: Tabulated discrimination indices (DI's) in the novel object recognition task.

Based on our preliminary data suggesting that PTX could improve Ts65Dn object recognition when given for an extended period of time (**Supp. Fig. 2**), we designed a more rigorous 4 week cross-over study to more directly assess whether multiple non-competitive GABA_A receptor antagonists could restore normal cognitive function in Ts65Dn mice. Discrimination scores used to create bar graphs presented in **Figs. 1** and **2** in the main text are presented below, providing both DI values and the number of observations (*n*) made for each data set. In these experiments, memory in the novel object recognition task is operationally defined as the proportion of time animals spend investigating the novel object minus the proportion spent investigating the familiar one [Discrimination Index, $DI = (\text{Novel Object Exploration Time} / \text{Total Exploration Time}) - (\text{Familiar Object Exploration Time} / \text{Total Exploration Time}) \times 100$]. Net novelty preference is calculated as a DI statistically greater than zero (0).

Supplementary Data Table 1 cont.

Novel Object Recognition, Discrimination Indices (DI's)

	DI Scores
PTX Experiment	
WT Control	21.46 ± 4.256, <i>n</i> = 37
Ts65Dn Control	5.566 ± 3.652, <i>n</i> = 41
WT PTX	28.97 ± 5.325, <i>n</i> = 23
Ts65Dn PTX	32.82 ± 5.226, <i>n</i> = 26
WT BB	21.47 ± 4.184, <i>n</i> = 36
Ts65Dn BB	26.39 ± 3.854, <i>n</i> = 30
One-way ANOVA	$F(5, 187) = 5.204, P < 0.0002$
PTZ Experiment, Short-Term Evaluation	
WT Milk	32.03 ± 5.705, <i>n</i> = 18
Ts65Dn Milk	7.350 ± 6.688, <i>n</i> = 18
WT PTZ	24.76 ± 4.833, <i>n</i> = 20
Ts65Dn PTZ	24.26 ± 5.194, <i>n</i> = 19
One-way ANOVA	$F(3, 71) = 3.356, P < 0.03$
PTZ Experiment, Long-Term Evaluation	
WT Milk	27.88 ± 6.349, <i>n</i> = 10
Ts65Dn Milk	3.800 ± 11.68, <i>n</i> = 8
WT PTZ	40.96 ± 4.810, <i>n</i> = 10
Ts65Dn PTZ	39.18 ± 5.829, <i>n</i> = 14
One-way ANOVA	$F(3, 38) = 5.134, P < 0.005$

Supplementary Data Table 2: Picrotoxin (PTX), bilobalide (BB) and pentylenetetrazole (PTZ) do not affect exploration times in the novel object recognition task.

As discussed in the main text, PTX, BB, and PTZ were able to rescue Ts65Dn object recognition memory when delivered along a chronic regimen. Considering the rich literature that exists concerning the anxiogenic potential of GABA_A antagonists in rodents and in humans (i.e., the ability of these compounds to increase anxiety-related behavior), we wanted to make sure that the effects of these compounds were strictly related to mnemonic processing, and not related to changes in the anxiety state of Ts65Dn mice during cognitive testing. In the object recognition task, increases in anxiety generally lead to decreases in object exploration, often referred to as object *neophobia*. However, our data indicate that WT and Ts65Dn object exploration times remain unaffected by drug treatment during the novel object recognition training or testing sessions.

Supplementary Data Table 2 cont.

	Novel Object Recognition, Total Object Exploration Time (sec)	
	Object Recognition Training Sessions	Object Recognition Testing Sessions
PTX Experiment		
WT Saline	228.4 ± 12.40 s	232.7 ± 19.24 s
Ts65Dn Saline	227.5 ± 16.23 s	242.3 ± 18.22 s
WT PTX	219.2 ± 23.28 s	210.0 ± 19.89 s
Ts65Dn PTX	206.4 ± 22.75 s	205.3 ± 19.36 s
WT BB	232.6 ± 13.10 s	211.2 ± 13.21 s
Ts65Dn BB	216.9 ± 17.98 s	197.5 ± 14.82 s
One-way ANOVA	$F(5, 157) = 0.2950, P > 0.91$	$F(5, 157) = 1.067, P > 0.38$
PTZ Experiment, Short-Term Evaluation		
WT Milk	260.5 ± 17.81 s	279.0 ± 24.03 s
Ts65Dn Milk	260.7 ± 21.62 s	286.8 ± 18.83 s
WT PTZ	227.5 ± 19.05 s	258.9 ± 14.37 s
Ts65Dn PTZ	264.0 ± 26.89 s	260.9 ± 21.16 s
One-way ANOVA	$F(3, 71) = 0.6591, P > 0.57$	$F(3, 71) = 0.4835, P > 0.69$
PTZ Experiment, Long-Term Evaluation		
WT Milk	213.8 ± 26.13 s	218.5 ± 33.68 s
Ts65Dn Milk	198.1 ± 28.09 s	192.3 ± 26.47 s
WT PTZ	177.5 ± 16.28 s	183.6 ± 24.63 s
Ts65Dn PTZ	232.6 ± 26.94 s	247.2 ± 20.78 s
One-way ANOVA	$F(3, 38) = 0.9136, P > 0.44$	$F(3, 38) = 1.322, P > 0.28$

Supplementary Data Table 3: Tabulated alternation percentages (%) in the spontaneous alternation task.

- Memory in the spontaneous alternation task is operationally defined as the number of successful alternations made in the T-maze divided by the number of alternation attempts, and is expressed as a percentage (%)

Spontaneous Alternation	
PTZ Experiment, Short-Term Evaluation	
	Alternation Percentages (%)
WT Control	67.88 ± 1.781%, <i>n</i> = 109
Ts65Dn Control	59.86 ± 2.007%, <i>n</i> = 84
WT PTZ	72.40 ± 2.501%, <i>n</i> = 51
Ts65Dn PTZ	68.45 ± 2.524%, <i>n</i> = 32
One-way ANOVA	$F(3, 272) = 5.998, P < 0.0006$

Supplementary Data Table 4: Pentylentetrazole (PTZ) does not affect arm entries in the spontaneous alternation task.

As reported in the main text, repetitive administration of PTZ was able to normalize spontaneous alternation in Ts65Dn mice. Considering the design of the testing situation (see **Methods**), we wanted to make sure that the alternation deficits exhibited by Ts65Dn mice derived from genuine problems with spatial navigation, and not from left or right arm biases that would inflate alternation errors. However, our data indicate that within each genotype/treatment condition, WT and Ts65Dn mice showed similar numbers of left, right, and stem arm entries, ruling out this possibility, and singling out Ts65Dn deficits in the spontaneous alternation task as being purely “cognitive” in nature.

PTZ Experiment	Spontaneous Alternation, Arm Entries			One-way ANOVA
	Left Arm	Right Arm	Stem Arm	
WT Control	7.75 ± 0.33	7.79 ± 0.38	8.00 ± 0.32	$F(2, 324) = 0.1479, P > 0.86$
Ts65Dn Control	8.70 ± 0.42	9.51 ± 0.47	8.96 ± 0.39	$F(2, 249) = 0.9262, P > 0.39$
WT PTZ	7.80 ± 0.49	7.63 ± 0.53	7.94 ± 0.46	$F(2, 150) = 0.1017, P > 0.90$
Ts65Dn PTZ	9.59 ± 0.72	8.88 ± 0.60	9.47 ± 0.66	$F(2, 93) = 0.3357, P > 0.71$

Supplementary Discussion: Potential utility of non-competitive GABA_A receptor antagonists for the treatment of cognitive impairment in Down syndrome.

Our results support the hypothesis that excessive GABA-mediated inhibition in the Ts65Dn brain actively interferes with declarative memory in Ts65Dn mice. An important question raised by our findings is how non-competitive GABA_A antagonists are working mechanistically to overturn this imbalance, thus permitting normal cognition. In principle, the administration of these drugs is likely to change the equilibrium between excitation and inhibition ubiquitously throughout the brain, perhaps by causing subtle, but stable changes in synaptic weights as illustrated by the reemergence of elicited LTP in the dentate gyrus of chronically treated Ts65Dn mice. Yet, it is predicted that some neuronal circuits will be more sensitive to the actions of GABA_A antagonists than others. One such circuit, explored here, is the perforant pathway¹⁵. The medial perforant path, a circuit that conveys information from layer II of the entorhinal cortex to granule cells in the dentate gyrus, is under tight control by GABAergic inhibition¹⁵. This creates a strict filter for information flow from the neocortex to the hippocampus, and suggests that systemic administration of non-competitive GABA_A antagonists (at low doses) might alter cognition by disproportionately influencing dentate gyrus function. Remarkably, chronic administration of non-epileptic doses of GABA_A antagonists leads to sustained improvements in Ts65Dn cognition that extend considerably beyond the window of drug treatment. These changes are observed at the behavioral level and at the synaptic level

within the dentate gyrus, implying that repetitive drug application may cause a slight, but stable rewiring of microcircuitry within the Ts65Dn dentate gyrus, and perhaps other areas, lasting for several months. The requirement for lengthened treatment to elicit these changes, is reminiscent of the mode of actions of other drugs that bring about therapeutic effects in psychiatric disorders only after continued administration, such as selective serotonergic reuptake inhibitors (SSRI's) in unipolar depression or D₂ receptor antagonists in schizophrenia.

Might picrotoxin, bilobalide and/or pentylentetrazole have clinical utility in the treatment of DS? All three drugs are non-competitive GABA_A receptor antagonists functioning within the pore of the GABA_A receptor chloride channel, albeit with different affinities¹⁶⁻²². Moreover, all are documented to readily cross the blood brain barrier²³⁻²⁵. Unfortunately, picrotoxin is of limited use in this application, as the therapeutic window between its cognitive enhancing properties and its pro-convulsive ones is very narrow. Conversely, bilobalide is likely to be of therapeutic value, but has not been approved by the FDA. In contrast, pentylentetrazole, based on its clinical history in humans, is a reasonable candidate for the treatment of cognitive impairment in DS. Numerous studies, designed to explore its pro-cognitive use in geriatric patients and institutionalized individuals, reveal that pentylentetrazole is remarkably well-tolerated at low doses (similar to those used in the current study), precipitating no major adverse effects even after years of administration²⁶⁻²⁷. Although not examined in depth, patients receiving pentylentetrazole were also reported to maintain basic personality structure and reaction patterns, and in some cases, to

exhibit an improved ability to reintegrate themselves into society, or to live more independently. Unfortunately, pentylenetetrazole's pro-cognitive properties at low doses have not been rigorously explored and have been overshadowed in the last three decades by its pro-convulsant properties at higher doses, as exemplified by its inclusion as a pharmacological tool in the study of epilepsy²⁸.

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