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Decreasing the expression of $GABA_A$ α5-subunit-containing receptors partially improves cognitive, electrophysiological and morphological hippocampal defects in the Ts65Dn model of Down syndrome

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ABSTRACT

Trisomy 21 or Down syndrome (DS) is the most common cause of intellectual disability of a genetic origin. The Ts65Dn (TS) mouse, which is the most commonly used and best-characterized mouse model of DS, displays many of the cognitive, neuromorphological and biochemical anomalies that are found in the human condition. One of the mechanisms that has been proposed to be responsible for the cognitive deficits in this mouse model is impaired GABA-mediated inhibition. Because of the well-known modulatory role of $GABA_A$ α5-subunit-containing receptors in cognitive processes, these receptors are considered to be potential targets for improving the intellectual disability in DS. The chronic administration of $GABA_A$ α5-negative allosteric modulators has been shown to be procognitive without anxiogenic or proconvulsant side effects. In the present study, we use a genetic approach to evaluate the contribution of $GABA_A$ α5-subunit-containing receptors to the cognitive, electrophysiological and neuromorphological deficits in TS mice. We show that reducing the expression of $GABA_A$ α5 receptors by deleting one or two copies of the *Gabra5* gene in TS mice partially ameliorated the cognitive impairments, improved LTP, enhanced neural differentiation and maturation and normalized the density of the GABAergic synapse markers. Reducing the gene dosage of *Gabra5* in TS mice did not induce motor alterations and anxiety or affect the viability of the mice. Our results provide further evidence of the role of $GABA_A$ α5 receptor-mediated inhibition in cognitive impairment in the TS mouse model of DS.

INTRODUCTION

It is well established that the $GABA_A$ receptor plays an important role in learning and memory processes. Non-selective positive allosteric modulators (PAMs) of the $GABA_A$ receptor, such as benzodiazepines, disrupt learning and memory processes [1-3], while non-selective antagonists and negative allosteric modulators (NAMs) improve cognitive processes [4-7]. In addition, non-selective $GABA_A$ NAMs, such as DMCM, increase long term potentiation (LTP) [8], whereas nonselective $GABA_A$ positive allosteric modulators (PAMs) (e.g., diazepam) impair LTP [9].

Among the different $GABA_A$ receptor subtypes, the $GABA_A$ α5-subunit-containing receptors, which are predominantly expressed in the hippocampus [10-13], are mainly localized extra-synaptically and generate long-lasting tonic currents [14-17]. These receptors have been demonstrated to play an important modulatory role in learning and memory processes.

Studies have demonstrated that homozygous (-/-) knockout mice lacking the *Gabra5* gene show an improvement in cognitive processes [18, 19], thereby providing genetic evidence supporting that this receptor subtype plays a role in cognition. In addition, α5(H105R) knock-in mice showed a decreased expression of the $GABA_A$ α5 receptor in the hippocampus, which induced an enhancement in hippocampus-dependent learning and memory [20] and fear conditioning [21]. These findings, together with the restricted expression pattern of the $GABA_A$ α5 receptors, makes these receptors attractive targets for the pharmacological enhancement of learning and memory [22, 23]. Several $GABA_A$ α5 NAMs, including L-655708, α5IA, MRK-016 and RO4938581, have been shown to improve learning and memory in preclinical studies [22, 24, 25]. In addition, α5IA was shown to restore ethanol-induced cognitive impairments in healthy volunteers [26].

Down syndrome (DS) is the most common cause of an intellectual disability of a genetic origin [27, 28]. To understand the neurobiological basis of the cognitive impairments found in DS and develop therapeutic strategies to reduce these alterations, several murine models of DS have been developed. Among these models is the Ts65Dn (TS) mouse, which bears a partial trisomy of a segment of MMU16, which extends from the *Mrp139* to the *Znf295* genes and contains approximately 92 genes that are orthologous to the human chromosome 21 (HSA21) genes [29]. Additionally, TS mice carry a trisomy of ~10 Mb of MMU17 containing 60 genes that are nonhomologous to HSA21 [30, 31]; thus, these genes are not relevant to DS and may confound phenotypic consequences. Although the TS mouse is not the ideal model from a genetic point of view, it recapitulates many fundamental features of DS, including cognitive deficits and alterations in brain morphology and function [32-34]. Several other segmental trisomic models of different segments of MMU16, 17 and 10 have been created [see 31, 32, 34]. A comparison of phenotypes in TS mice with those of other partial trisomic models suggests that the set of genes triplicated in this model contributes to several DS phenotypes, including cognitive and neuroanatomical impairments [34]. For these reasons, most previous studies on DS structural and functional alterations have been performed on the TS mouse.

Although many of the neuromorphological alterations that are present in the TS mouse are likely partially responsible for their cognitive deficits, including the changes in the size, morphology and cellular density of different brain areas and the alterations in pre- and post-natal neurogenesis, dendritic structures and the morphology of synapses and spines [see 32, 34], abnormal synaptic plasticity (reduced hippocampal LTP and increased LTD) has been proposed to be a key mechanism underlying the intellectual disabilities in TS mice [35-37].

In addition, several studies have demonstrated that in this murine model of DS, there is altered synaptic inhibition, which is mediated by the $GABA_A$ receptor [36-40]**.** The administration of non-selective $GABA_A$ antagonists to TS mice rescued the deficits in LTP and the hippocampal-mediated memory impairments in the TS mice [41-43]. However, these drugs are not adequate candidates for the treatment of learning impairments in the DS population due to their anxiogenic and proconvulsant effects.

The identification of the different functional roles of $GABA_A$ receptor subtypes suggests that receptor subtype-selective compounds could overcome the limitations of non-selective $GABA_A$ receptor modulators. The administration of the α5-selective NAMs α5IA and RO4938581 rescued the cognitive deficits in TS mice [44, 45]. In addition, the chronic administration of RO4938581 rescued the deficits in hippocampal synaptic plasticity, enhanced neurogenesis in the dentate gyrus and the granular cell density, and normalized the density of hippocampal GABAergic boutons in TS mice [40, 45]. Importantly, none of these compounds are proconvulsant or anxiogenic [40].

Although *Gabra5*, which is the gene that encodes the α5 subunit of the $GABA_A$ receptor, is not localized in HSA21 or in the segment of MMU16 that is triplicated in the TS mouse and there is no evidence of alterations in the density of this type of receptor in this model of DS [40, 45], the impaired GABA-mediated inhibition could be mediated via the $GABA_A$α5 subtype, which is abundant in the hippocampus. To test this hypothesis, in this study, we used a genetic approach to specifically reduce the inhibition that is mediated by these receptors in the TS mouse by deleting one or two copies of the *Gabra5* gene. We crossed female TS and euploid (control, CO) mice with male *Gabra5* knockout mice and performed a behavioral, electrophysiological and neuromorphological characterization of the progeny (TS and CO mice carrying 2, 1 or 0 functional copies of the *Gabra5* gene).

MATERIAL AND METHODS

Animals

This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

Ts(17<16>)65Dn (TS) mice were generated by repeated backcrossing of B6EiC3Sn a/A-Ts(17<16>)65Dn females with C57BL/6Ei x C3H/HeSNJ (B6EiC3Sn) F1 hybrid (CO) males at the animal facilities of the University of Cantabria.

Because the *Gabra5* (α5 subunit of the $GABA_A$ receptor) gene is located in MMU7 and its orthologous human gene is located in HSA15, the TS progenitors carry two alleles of this gene. TS females were crossed with heterozygous males carrying a mutated copy of the *Gabra5* gene (see **figure 1a**). *Gabra5* KO mice were generated as previously described (gl-α5-KO, Rodgers et al., 2015) and provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland).

From these crossings, a first generation (F1) was obtained, including the following four mouse genotypes: TS with two copies of *Gabra5* (TS +/+), TS with a single functional copy of *Gabra5* (TS +/-), CO with two copies of *Gabra5* (CO +/+) and CO mice with a single functional copy of *Gabra5* (CO +/-).

Female TS +/- mice were then crossed with male CO +/- mice to obtain TS and CO mice (F2) with two functional copies, one functional copy or no functional copies of *Gabra5* (i.e., TS +/+, CO +/+, TS +/-, CO +/-, TS -/- and CO -/- mice, see **figure 1a**).

To determine the presence of the trisomy, animals were karyotyped using real-time quantitative PCR (qPCR) as previously described [46]. Because the C3H/HeSnJ mice carry a recessive mutation that leads to retinal degeneration (Rd) [47], all animals were genotyped by standard PCR to screen for mice carrying this gene.

To determine the number of functional copies of the *Gabra5* gene carried by each animal, their genomic DNA was amplified by standard PCR following the protocol described by Rodgers et al. [48].

Mice were housed in groups of two or three in clear Plexiglas cages (20 x 22 x 20 cm) under standard laboratory conditions with a temperature of 22 ± 2 ºC, 12-hour light/dark cycle and free access to food and water. The light/dark cycle was inverted to allow for the behavioral studies to be conducted during the active period of the mice.

In this study, two cohorts of male mice were used (see **Supplementary table 1**). The first cohort was used to determine the effect of the gene dosage of *Gabra5* on cognition, behavior and neuromorphology in the TS and CO mice. The second

cohort of animals was used to assess the $GABA_A$ α5 receptor density in the different *Gabra5* genotype groups by autoradiography.

The behavioral experiments were performed with a total of 66 4- to 5-month-old mice (8-13 animals per group; see **Supplementary table 1**). After the completion of these studies, the mice were sacrificed at the age of 5-6 months; 6 animals from each group were used for the histological experiments. In addition, seven 6-month-old animals per group were used for the autoradiography experiments (2-7 animals per group). The researchers were blinded to the genotype and karyotype throughout the entire assessment.

Viability of the different groups of mice and somatometry

The viability of the different groups of mice born from the crossings of female TS +/- and male +/- mice was assessed by quantifying the number of animals with each karyotype and genotype born in each litter. In total, 100 male mice, born in 28 litters, were analyzed.

The effect of the karyotype and the genotype on the weight of the animals was evaluated once a month from weaning to the age of 5 months in 10 mice from each experimental group.

Quantification of $GABA_A$ α5 receptors: *in vitro* autoradiography

To evaluate whether the gene dosage of *Gabra5* led to changes in the number of $GABA_A$ α5 receptors, the occupancy of $GABA_A$ α5 receptors was quantified using a specific radioligand.

Mice were sacrificed by cervical dislocation, and their brains were rapidly removed and stored at -20 °C. The brains were subsequently cut into 10 µm-thick coronal sections with a cryostat and mounted on HistoBond glass slides (Marienfeld, Lauda-Königshofen, Germany).

To perform the autoradiographic marking, a tritiated radioligand with a high affinity for $GABA_A$α5 receptors, [^{3}H] RO 15-4513 (synthesized in the isotope laboratory of F. Hoffmann-La Roche, Basel, Switzerland), was used following the protocol described by Sur et al. [49].

The slides were preincubated in a Ringer buffer with a pH of 7.4 (Sigma Aldrich, St Louis, MO, USA) at room temperature for 20 min, followed by an incubation with 0.94 µl of [^{3}H] RO 15-4513 (specific activity: 47.2 Ci/mmol) in 400 ml of the Ringer buffer (pH 7.4) for 1 hour. This procedure was performed in duplicate for each animal. Non-specific binding was defined in a single sample per mouse under the same conditions described previously, but 10 µM of flunitrazepam (Sigma Aldrich) were added. The slides were then washed twice for 2 min in the Ringer buffer at 4 °C, followed by four immersions of the samples in distilled water at 4 °C. The samples were dried by a cold air current for 3 hours, and they were then exposed to tritium sensitive photographic films (Biomax MR Kodak, Madrid,

Spain) for 3 months at 4 ℃ in hermetic imaging plates (Kodak X-Omatic cassetteTM, USA).

The autoradiograms were scanned and quantified by optical densitometry using the image analysis software Scion Image (Scion Corporation, Maryland, USA).

The values of the receptor densities were obtained by exposing the films to different tritium-sensitive standards (Autoradiographic [3H]-micro-scaleTM y [14C]-micro-scale, Amersham, U.K.). The densitometric quantification of these impressions with the corresponding concentrations of the isotope allowed to obtain a calibration curve that was used to perform the first transformation of the gray tissue densities into their equivalent nCi/mg. After subtracting the background values and the non-specific binding, the obtained experimental values were transformed into the fmol/mg of the tissue according to the specific activity of [^{3}H] RO 15-4513 (47.2 Ci/mmol). Finally, the density of the binding sites (Bmax) was calculated and expressed as the fmol/mg of the tissue according to the radioligand KD (0.1 nM).

Cognitive studies

1. *Morris water maze*
   The Morris Water Maze test was performed to evaluate spatial learning using the platform relocation protocol [50-53] that was described by Corrales et al. [54]. Briefly, the animals were tested in 12 consecutive daily sessions consisting of eight acquisition sessions (platform submerged), followed by four cued sessions (platform visible). Each session consisted of 8 trials, and the platform position was changed from session to session. Each trial terminated when the mouse located the platform or 60 s had elapsed. All trials were videotaped with a camera located 2 m above the water level. An AnyMaze computerized tracking system (Stoelting, Wood Dale, IL, USA) was used to analyze the swimming trajectories, escape latency, thigmotaxis and swimming speed of each animal in each trial.

2. *Fear Conditioning*
   The fear conditioning experiment procedure was performed as described by Salehi et al. [55]. Briefly, contextual and tone-cued fear conditioning tests were performed using the Fear Conditioning apparatus (Stoelting) and the AnyMaze Video Tracking System (Stoelting). The mice underwent three days of testing as follows: a training day, a tone-cued in a novel context testing day and a contextual testing day. On the first day, the mice underwent a training session in which each mouse received five tone (70 dB, 2 kHz, 20 sec)-shock (0.5 mA, 50 Hz, 2 sec) pairings. On the second day, each mouse was placed in a novel context for 3 min and was presented with three tone exposures without any shocks. On the final day, each mouse was placed in a context that was similar to that in the training

day for 5 min without any tones or shocks. The freezing behavior of the mice in each condition was quantified on both testing days.

Histological and stereological procedures

The animals were deeply anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with saline, followed by 4% paraformaldehyde. Subsequently, the brains were postfixed in 4% paraformaldehyde overnight at 4 ℃ and transferred to 30% sucrose. The brains were frozen on dry ice and coronally sliced with a cryostat (50-µm thick sections). Series of brain slices, comprising 1 section for every 9 slices, were used for the immunohistochemistry protocol. A 1-in-9 series of coronal sections was randomly selected and subjected to Nissl staining using the Cavalieri method, as previously described [56], to calculate the subgranular zone (SGZ) area and the dentate gyrus (DG) volume.

1. Density of GABAergic synapse markers (VGAT immunofluorescence)

One-in-nine serial 50-µm sections of the mice brains were used to determine the density of a GABAergic synapse marker. The sections were preincubated in PBT/BSA, and dual immunohistochemistry was performed. The GABAergic boutons were labeled using a goat anti-GABA vesicular transporter antibody (VGAT, 1:100 (Santa Cruz Biotechnology, Dallas, TX, USA)), followed by additional labeling with a donkey anti-goat Alexa Fluor® 594-conjugated antibody (1:1,000; Invitrogen, Carlsbad, CA, USA).

Measurements were performed on images obtained using a confocal microscope (Leica SP5). Four sections per animal were used, which constituted the entire hippocampus, and one random area in the hippocampus per section was measured. The image analysis was performed using the ImageJ software as previously described [45], and the percentage of the reference area that was occupied by VGAT-positive boutons was calculated.

VGAT-positive boutons were quantified in the DG inner molecular layer (IML) (**figure 1b**). This area was chosen because it contains an important inhibitory microcircuit that participates in the modulation of the activity of the DG [57, 58]. In this zone, a large number of inhibitory synapses are established between axons from the commissural/associational pathway and the dendrites of proximal granular cells [59, 60]. In addition, the IML is likely the most plastic zone of the ML because it is the first area where the dendritic trees of newborn neurons arrive after neural maturation during adult neurogenesis [61]. The IML is also the only stratum to which the dendritic trees of some of these immature neurons arrive, as after performing their functions, they occasionally do not differentiate further and instead die. Therefore, the IML is an adequate area in which to evaluate inhibitory drive and changes in plasticity that can affect cognitive processes.

2. Cell proliferation in the SGZ of the DG (Ki67 immunofluorescence)

Ki67 immunofluorescence was performed to identify proliferating cells. The sections were preincubated in PBT/BSA (PB containing 0.5% Triton X-100 and 0.1% BSA), and immunohistochemistry was performed as previously described [56]. Briefly, free-floating sections were incubated with a primary rabbit anti-Ki67 antibody (1:750; Abcam, Cambridge, UK) for two days at 4 °C. Subsequently, the slices were incubated overnight at 4 °C with a secondary antibody diluted to 1:1,000 (donkey anti-rabbit-Alexa Fluor® 488; Molecular Probes, Eugene, OR, USA). The sections were counterstained with DAPI and mounted on gelatin-covered slides for the analysis and imaging. The total number of Ki67-positive cells was counted under an optical fluorescence microscope (Zeiss Axioskop 2 Plus, 40x objective) using the optical dissector method as previously described [56].

3. Neuronal differentiation (DCX and CLR immunofluorescence)

Doublecortin (DCX) is a microtubule-associated protein implicated in neural differentiation and migration, and calretinin (CLR) is a calcium binding protein that is briefly expressed before full neuronal maturation. Double labeling of these cells allows the identification of different neurogenic populations: DCX+/CLR- cells correspond to 2b and 3 neurogenic precursors (i.e. cells undergoing late mitosis or in early postmitotic phases), while DCX+/CLR- cells are late postmitotic differentiating cells [62, 63].

One-in-nine series of 50-µm sections were used for the determination of the densities of the cells expressing immature markers (DCX and/or CLR). The sections were initially preincubated in PBT/BSA, and dual immunohistochemistry was subsequently performed. A goat anti-doublecortin antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit anti-calretinin antibody (1:3000; Swant, Switzerland) were used as the primary antibodies. The primary antibodies were labeled with a donkey anti-goat Alexa Fluor® 594-conjugated antibody and a donkey anti-rabbit Alexa Fluor® 488-conjugated antibody (1:1000; Alexa Fluor®-conjugated antibodies purchased from Invitrogen, Carlsbad, CA, USA). The sections were subsequently imaged under a confocal microscope (Leica SP5). The quantification of the DCX- and CLR-expressing cells was performed according to stereological procedures using the physical dissector method. To evaluate the cells expressing the immature markers, six random dissectors per animal were used, comprising sections representative of the entire hippocampus. A series of 11 confocal images was serially recorded in each physical dissector. All immature cells were counted using the physical dissector, which has a square area with one side that lies on the “line” of the SGZ (**figure 1b**). By dividing the number of counted immature cells by the length of the “subgranular” line, a reliable estimate of the cell density by “unit of SGZ” was obtained. The cell density of the immature neurons is presented as either DCX+ (DCX+/CLR-) or CLR+ (DCX+/CLR+ plus DCX-/CLR+).

4. Granule cell counts (DAPI)

The cells in the hippocampal granule cell layer (GCL; **figure 1b**) were counted in sections stained with 4’6-diamidino-2-phenylindole (DAPI, Calbiochem, Billerica,

MA, USA; 1:1,000) for 10 min in phosphate buffer (PB). The cell counts were obtained using a confocal microscope coupled to a physical dissector system as previously described [56]. Six dissector locations in each series were evaluated. Subsequently, a series of confocal images was serially recorded according to the general principles of the physical dissector and the unbiased stereology methods. The confocal images were analyzed using the ImageJ software which generated the total number of cells when the dissector brick was completed. To count the total number of cells in the GCL, a square dissector frame was randomly situated inside the GCL. To obtain the number of cells per unit of volume (cell density), the obtained cell number was divided by the reference volume of the dissector.

Long-term potentiation

LTP recordings were performed in the CA1/CA3 region (**figure 1b**) because $GABA_A\alpha5$ receptors are mainly localized to the extrasynaptic regions of the dendrites of pyramidal neurons of the hippocampal CA1 and CA3 regions, where they mediate tonic inhibition [15, 20, 64, 65]**.**

Due to the low viability of the TS -/- animals (**figure 1c**), it was not possible to obtain the necessary number of mice to perform this experiment, and LTP was not assessed in this group. Six 6-month-old animals were used in each of the other experimental groups (TS +/+, TS +/-, CO +/+, CO +/-, CO -/-). The mice were decapitated, and the brains were rapidly removed. The hippocampi were dissected, and 400-µm slices were generated using a tissue chopper. The slices were allowed to recover for 1 hour in an interface chamber at room temperature in artificial cerebral spinal fluid (ACSF) containing 120 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 10 mM D-glucose saturated with 95% O_2 and 5% CO_2. The field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum using a glass micropipette (1–4 MΩ) containing 2 M NaCl, and the Schaffer collaterals were stimulated using insulated bipolar platinum/iridium electrodes located >500 µm from the recording electrode. The stimulus strength was adjusted to evoke fEPSPs equal to 50% of the relative maximum amplitude without a superimposed population spike. After the stable baseline recordings (100-µs pulse duration, 0.033 Hz), long-term potentiation (LTP) was induced by TBS (10 trains of 5 pulses at 100 Hz and intervals of 200 ms). The duration of the stimulation pulses was doubled during tetanus. The fEPSPs were amplified, bandpass filtered (1 Hz-1 kHz) and stored in a computer using the Spike2 software program (Cambridge Electronic Design, Cambridge, UK). For the analysis, the fEPSP slopes were expressed as a percentage of the recorded baseline values. The results from several slices were expressed as the mean value ± SEM.

Statistical analysis

The data obtained from the MWM test experiments were analyzed using a two-way repeated measures ANOVA ('session x karyotype x genotype' or 'trial x karyotype x genotype'). The LTP data were analyzed using an RM MANOVA ('time x karyotype x genotype').The remaining data were analyzed using a two-way ('karyotype' x 'genotype') ANOVA. The mean values of each experimental group were compared *post hoc* using Bonferroni tests. The percentage of animals born in each experimental group (viability study) was analyzed using Chi-squared tests. All analyses were performed using SPSS for Windows version 22.0. (Armonk, New York, USA).

RESULTS

Viability

The number of animals born with each genotype significantly differed ($\chi^2_{(d.f.=5)}$=20.26, p=0.0011; **figure 1c**) from the predicted Mendelian distribution (12.5% for the CO +/+, TS +/+, CO -/- and TS -/- animals and 25% for the CO +/- mice and TS +/- mice). Although the +/- animals doubled the percentage of the +/+ and -/- mice in both genotypes, the TS mice carrying the different gene dosages of the *Gabra5* gene showed a marked reduced viability as has been previously demonstrated in numerous studies. In addition, while the percentage of animals with the CO +/+ genotype did not differ from that of the CO -/- animals (in both cases, they represented 50% of the number of the CO +/- animals ($\chi^2_{(d.f.=2)}$=0.16, p=0.91)), the TS -/- animals showed a reduced viability (only approximately 3% of the animals born belonged to this genotype and karyotype) from that expected, although this effect did not reach statistical significance ($\chi^2_{(d.f.=2)}$=0.84, p=0.65). Therefore, the karyotype, but not the genotype, affected the viability of the mice.

Effects of reducing Gabra5 dosage on cognitive performance

1. *Morris water maze (MWM)*

Consistently with numerous reports, the TS mice had more difficulties in learning the platform position than the CO mice as they displayed larger latencies to escape from the MWM than the CO mice (ANOVA 'karyotype': $F_{(1,60)}$=105.28, p<0.001; **figure 2a**). The reduction in the *Gabra5* gene dosage had a significant effect in both the TS and CO mice however, this effect was more evident in the TS mice (ANOVA 'genotype': $F_{(2,60)}$=6.89, p=0.002; 'genotype x treatment': $F_{(2,60)}$= 2.80, p=0.069). The TS mice that had a single functional copy of this gene showed an improved performance compared with mice that had both copies, and this improvement was more evident in the TS animals without a functional copy of this gene. In the case of the CO mice, both groups of KO mice displayed a slight improvement in their performance in this test compared with the CO +/+ animals; however, no differences were evident between the CO +/- and the CO -/- groups. It is possible that a floor effect prevented further improvements in the CO -/- group.

These improvements in the performance in the MWM may be partially due to a recovery in their procedural learning. **Figure 2b** shows the time that the animals spent in the periphery of the pool. Reducing the gene dosage of *Gabra5* in the TS mice dose-dependently improved their searching strategy (i.e., it reduced their thigmotactic behavior) (ANOVA 'karyotype': $F_{(1,60)}$=177.01, p<0.001; 'genotype': $F_{(2,60)}$=4.30, p=0.18; 'karyotype x genotype': $F_{(2,60)}$= 1.99, p=0.14). This effect was not found in the CO mice.

The enhancement in the spatial and procedural learning after reducing the gene dosage of *Gabra5* is not due to motor or motivational effects since no significant

differences were found between the 6 groups of animals in the latency to reach the platform during the cued sessions (ANOVA 'karyotype': $F_{(1,60)}$=2.99, p=0.089; 'genotype': $F_{(2,60)}$=1.93, p=0.15; 'karyotype x genotype': $F_{(2,60)}$= 0.29, p=0.74; data not shown) or the swimming speed during the entire experiment (ANOVA 'karyotype': $F_{(1,60)}$=0.74, p=0.39; 'genotype': $F_{(2,60)}$=0.48, p=0.61; 'karyotype x genotype': $F_{(2,60)}$= 0.22, p=0.80; data not shown). These results are consistent with a lack of effects on sensorimotor abilities (**Supplementary table 2**), motor coordination in the Rotarod (**Supplementary figure 1**) or the weight of animals that carry different numbers of copies of this gene (**Supplementary figure 4**).

In addition, the improvements in cognitive abilities found in TS animals homozygous or heterozygous for the *Gabra5* gene are not likely to be due to an amelioration of their attentional deficits because the hyperactivity of these animals was not corrected in the open field (**Supplementary figure 2**), plus maze (**Supplementary figure 3**) or hole board tests (**Supplementary table 3**).

2. Conditioned Fear

The TS mice showed a deficit in their ability to remember the association between a tone and an aversive stimulus. While these animals presented a larger number of freezing episodes (ANOVA 'karyotype': $F_{(1,60)}$=5.70, p=0.020; **figure 2c**), these episodes were of a shorter duration ($F_{(1,60)}$=7.13, p=0.010; **figure 2d**), which is considered the most relevant index reflecting the memory of the association between the conditioned stimulus (the tone) and the unconditioned stimulus (the electric shock). The *Gabra5* gene dosage did not exert any effect on the number of freezing episodes (ANOVA 'genotype': $F_{(2,60)}$=1.23, p=0.29; 'karyotype x genotype': $F_{(2,60)}$=2.00, p=0.14) or the time spent freezing (ANOVA 'genotype': $F_{(2,60)}$=0.14, p=0.86; 'karyotype x genotype': $F_{(2,60)}$=3.13, p=0.51) in the TS or CO mice.

During the context conditioning session, the TS mice displayed a marked difficulty in remembering the association between the context and the aversive stimulus. Although the statistical analysis revealed that the number of freezing episodes did not differ significantly between the TS and CO mice (ANOVA 'karyotype': $F_{(1,60)}$=0.98; p=0.32, **figure 2c**), this effect was likely due to the marked increase in these episodes in the TS +/- and TS +/+ mice. In addition, the *post hoc* analysis revealed that the TS +/+ mice displayed a much smaller number of these episodes than the CO +/+ mice (p<0.001). The TS mice also spent a shorter amount of time freezing during this session than the CO mice (ANOVA 'karyotype': $F_{(1,60)}$=7.88; p=0.007, **figure 2d).** The deficit in the context fear conditioning completely disappeared in the TS +/- and TS -/- mice because these two groups of animals increased the number of freezing episodes (ANOVA 'genotype': $F_{(2,60)}$=5.86, p=0.005) and spent more time freezing ($F_{(2,60)}$=7.69, p=0.001) than the TS +/+ mice. In fact, the heterozygous and homozygous TS mice did not differ from the CO mice with the three genotypes in any of these variables (**figures 2c and 2d**). Despite the effect of the genetic manipulation in the TS animals, reducing one or both functional copies of the *Gabra5* gene in the

CO mice did not exert any benefit in the context conditioning (ANOVA 'karyotype x genotype': freezing episodes, $F_{(2,60)}$=9.29, p<0.001; time freezing $F_{(2,60)}$=4.21, p=0.020).

Differences in the anxiety displayed by these animals are not likely to play a role in these effects, as no differences were found in the motor or cognitive components of anxiety in the 6 groups of animals in the plus maze (**Supplementary figure 2**) or open field (**Supplementary figure 3**) tests.

Putative mechanisms implicated in the changes in cognitive performance induced by reduced *Gabra5* dosage:

1. α5-containing $GABA_A$ receptors density

No significant differences were found in the amount of α5-containing $GABA_A$ receptors between the TS +/+ and CO +/+ mice in any of the areas analyzed ('ANOVA karyotype': anterior CA1 $F_{(1,37)}$=1.78, p=0.19; anterior CA3 $F_{(1,37)}$=2.23, p=0.14; anterior DG $F_{(1,37)}$=0.10, p=0.75; posterior CA1 $F_{(1,37)}$=0.34, p=0.56; anterior Hc $F_{(1,37)}$=1.99, p=0.16; mean of all structures $F_{(1,37)}$=1.54, p=0.22; **figure 3**).

However, the TS +/- mice displayed fewer α5-$GABA_A$ receptors than the CO +/- mice in all structures ('ANOVA karyotype x genotype': anterior CA1 $F_{(2,37)}$=6.46, p=0.005; anterior CA3 $F_{(2,37)}$=6.31, p=0.005; anterior DG $F_{(2,37)}$=3.68, p=0.038; posterior CA1 $F_{(2,37)}$=4.96, p=0.014; anterior Hc $F_{(2,37)}$=9.16, p=0.001; mean of all structures $F_{(2,37)}$=8.86, p=0.001). These results may be due to an interaction between the *Gabra5* gene and other genes or gene products that are overexpressed in the TS mice.

Reducing the number of functional copies of the *Gabra5* gene dose-dependently lowered the number of α5 $GABA_A$ receptors in both the TS and CO mice ('ANOVA genotype': CA1 $F_{(2,37)}$=190.29, p<0.001; anterior CA3 $F_{(2,37)}$=54.72, p<0.001; anterior DG $F_{(2,37)}$=81.93, p<0.001; posterior CA1 $F_{(2,37)}$=54.34, p<0.001; anterior Hc $F_{(2,37)}$=167.34, p<0.001; mean of all structures $F_{(2,37)}$=144.73, p<0.001). This effect was particularly evident in the anterior CA1 area (**figure 3**).

2. Changes in inhibitory- excitatory balance: density of GABAergic synapse markers (VGAT immunofluorescence)

As previously described by several groups, the TS mice displayed an increased area occupied by a marker of GABAergic synapses VGAT (ANOVA 'karyotype': $F_{(1,30)}$=4.70, p=0.037; **figure 4**). Reducing the number of functional copies of the *Gabra5* gene in the TS and CO mice dose-dependently reduced the area occupied by the VGAT+ boutons (ANOVA 'genotype': $F_{(2,30)}$=13.82, p<0.001); however, this effect was more evident in the TS than that in the CO mice ('karyotype x genotype' $F_{(2,30)}$=3.63, p=0.037).

3. Neurogenesis: cell proliferation, differentiation and survival

3.1. Cell proliferation in the SGZ of the DG (Ki67 immunofluorescence)

The TS mice displayed a reduced number of proliferating cells in the SGZ of the hippocampus (ANOVA 'karyotype': $F_{(1,30)}$=21.10, p<0.001; **figure 5**). The reduction in the number of copies of the *Gabra5* gene tended to increase the density of Ki67+ cells in the TS, but not in CO, mice; however, this effect did not reach statistical significance (ANOVA 'genotype': $F_{(2,30)}$=0.36, p=0.70; 'karyotype x genotype' $F_{(2,30)}$=1.35; p=0.27). Therefore, this effect is unlikely to account for the improvements in the cognitive performance in the two learning and memory tests described above that were found in the TS mice with a reduced *Gabra5* gene dosage.

3.2. Neuronal differentiation (DCX and CLR immunofluorescence)

The TS mice presented a lower density of cells during the initial stages of differentiation (DCX+/CLR- cells: ANOVA 'karyotype': $F_{(1,30)}$=14.23, p=0.001; **figure 6**). In the later stages (DCX+/CLR+), the density of this population did not significantly differ between the TS and CO mice when all genotypes were considered (ANOVA 'karyotype': $F_{(1,30)}$=2.21, p=0.14), which was likely due to the increased density found in the heterozygous and homozygous TS animals. However, the *post hoc* analysis revealed that the TS +/+ mice presented a lower density of DCX+/CLR+ cells than the CO +/+ mice. Reducing one or both copies of the *Gabra5* gene significantly increased the density of both populations of immature cells in the TS and CO mice (ANOVA 'genotype': DCX+/CLR- $F_{(2,30)}$=3.45, p=0.043; DCX+/CLR+ $F_{(2,30)}$=3.44, p=0.043; ANOVA 'karyotype x genotype': DCX+/CLR- $F_{(2,30)}$=0.41, p=0.66; DCX+/CLR+ $F_{(2,30)}$=0.28, p=0.75; **figure 6**). These results suggest that reducing the function of the *Gabra5* gene accelerates or increases the maturation of newly born cells.

3.3. Granule cell counts (DAPI)

The density of mature cells in the GCL of the hippocampus was reduced in the TS mice (ANOVA 'karyotype': $F_{(1,30)}$= 55.08, p<0.001; **figure 7**). However, the ANOVA revealed a non-significant effect of genotype in the TS or CO mice ('genotype': $F_{(2,30)}$=1.65, p=0.20; 'karyotype x genotype' $F_{(2,30)}$=1.67, p=0.20); the *post hoc* comparisons between the TS animals with two copies of the *Gabra5* gene and those with one or no functional copy of this gene revealed that reducing its dosage leads to a significant increase in the number of DAPI+ cells in the TS, but not in the CO, mice.

4. Synaptic plasticity: Long Term Potentiation

Figure 8 shows that the induction of LTP was reduced in the TS mice compared with that in the CO mice (ANOVA 'karyotype': $F_{(1,25)}$=4.11, p=0.049). This effect

was not too strong due to the positive effect of reducing the number of copies of the *Gabra5* gene in the TS animals (see below). However, when the TS +/+ mice were compared with the CO +/+ mice, the former presented a stronger reduction in LTP (ANOVA 'karyotype': $F_{(1,25)}$=12.65, p=0.002).

When all groups were analyzed together, the RM MANOVA revealed that the genetic manipulation had no significant effect in the TS or CO mice (ANOVA 'genotype': $F_{(2,25)}$=2.29, p=0.11), and this result was due to the positive outcome in the LTP induction after reducing the *Gabra5* gene dosage in the TS, but not the CO, mice (ANOVA 'karyotype x genotype': $F_{(2,25)}$=4.38, p=0.043; **Figure 8**).

The groups of CO mice carrying two, one or no functional copies of the *Gabra5* gene did not differ in the amount of LTP generated after TBS ('ANOVA' genotype': $F_{(2,15)}$=0.20, p=0.81). However, knocking out a copy of this gene in the TS mice led to a robust enhancement in LTP ($F_{(1,10)}$=16.49, p=0.001). In fact, the TS +/- animals displayed a completely normalized LTP as their slopes did not significantly differ from those of the CO +/+ mice ($F_{(1,10)}$=0.00, p=0.99).

No significant differences were found between the 5 groups of mice in the basal values before the administration of TBS (ANOVA 'karyotype': $F_{(1,25)}$=0.023, p=0.88, 'genotype': $F_{(2,25)}$=0.46, p=0.63; 'karyotype x genotype': $F_{(2,\ 25)}$=1.47, p=0.23; **Figure 8**).

DISCUSSION

This study shows that genetically reducing the α5 $GABA_A$-mediated inhibition ameliorated the cognitive deficits in TS mice. This genetic approach confirms previous findings regarding the role of the GABA-mediated inhibition in the cognitive deficits in TS mice and the procognitive effects of reducing this inhibition by targeting the $GABA_A$ α5 receptors. We characterized the behavioral, cognitive and neuromorphological effects of reducing the dosage of the *Gabra5* gene in trisomic mice and their euploid littermates.

In agreement with previous studies [66, 67], TS mice exhibited reduced viability with respect to CO mice. Although statistical analysis revealed no significant deviation from the expected Mendelian distribution in the percentage of animals with different dosages of the *Gabra5* gene that were born, the extremely low viability of TS -/- animals (3%) suggests that this genetic manipulation might affect the viability of trisomic mice. An explanation for this finding could be that the interaction of *Gabra5* with other triplicated genes aggravated the cardiovascular dysfunctions displayed by a larger number of TS mice that reduce their perinatal survival.

Previous work has shown that knocking out the *Gabra5* gene in wildtype mice improved their performance in different hippocampal-dependent tasks [18, 19]. In the TS mice, modifying the number of functional copies of the *Gabra5* gene attenuated the well-known spatial learning and memory impairments found in the MWM. This effect was also evident in the CO mice, although it was much more pronounced in the trisomic mice likely because of the larger impairment found in these animals and/or a floor effect in the CO mice. The improvements in performance in the MWM were not due to improvements in motivation or motor abilities since no significant differences were found in the latency to reach the platform during the cued trials or the animals' swimming speed. These effects are consistent with the lack of an effect of the *Gabra5* gene dosage on motor coordination in mice of either karyotype as described in the **Supplementary Results** section.

In the case of the TS mice, the enhancement in the procedural learning may have played an important role in the procognitive effects found in the MWM because the heterozygous and homozygous *Gabra5* TS mice presented less thigmotactic behavior.

Additionally, in the fear conditioning test, the TS mice showed impairments in both the cued and context conditioning. The reduction in the *Gabra5* gene dosage completely rescued the context conditioning in the TS mice but did not have any effect on the cued conditioning. In rodents, the $GABA_A$ α5 receptors are mainly located in the hippocampus [10-13], and context conditioning is known to rely on the proper functioning of this structure, while the amygdala and other structures mediate cued conditioning [68, 69], which may explain these results. Studies performed in *Gabra5* KO mice in which their performance in hippocampal-independent tasks, such as delay fear conditioning and two-way avoidance, was unaltered [18-21] further support this hypothesis.

The enhancements in the cognitive performance of the heterozygous and homozygous TS and CO mice in the MWM and in the TS animals in the fear conditioning test are likely due to a reduction in the $GABA_A$α5 receptor-mediated inhibition.

However, reducing the *Gabra5* gene dosage in TS animals only partially ameliorated their cognitive deficits. In the Morris water maze test, the rescue of the cognitively altered phenotype was not complete and might have been mainly mediated by decreased thigmotaxis. In the fear conditioning test, normalization of performance was only achieved in the contextual memory test after reducing the *Gabra5* gene dosage, but this genetic manipulation did not have any effect on the memory abilities displayed by the different groups of TS mice in the cued conditioning test. Moreover, as shown in the **Supplementary Material**, reducing $GABA_A$α5 receptor expression did not rescue the hyperactivity or the motor coordination deficits of TS animals. Therefore, reduction of $GABA_A$α5 receptor-mediated inhibition only partially improved specific cognitive domains (i.e., hippocampal-dependent spatial learning and context conditioning).

Our autoradiographic results confirmed that reducing the number of functional copies of the *Gabra5* gene dose-dependently lowered the density of α5 $GABA_A$ receptors in mice with both genotypes. However, the effect of removing a single copy of *Gabra5* was larger in the TS +/- mice than that in the CO +/- mice in all structures analyzed. These results may be due to an interaction between the *Gabra5* gene and other genes or gene products that are overexpressed in the TS mice. The greater reduction in the density of this type of receptor in the TS +/- mice than that in the CO +/- animals may be partially responsible for the greater cognitive benefits observed after knocking out this gene in the trisomic mice compared with those in the control mice.

The density of the α5 $GABA_A$ receptors in the TS +/+ mice was similar to that in the CO +/+ mice. These results are consistent with other studies that showed that the TS and CO mice do not differ in the number of α5 $GABA_A$ receptors [45, 70] and with the fact that the *Gabra5* gene is located in MMU7, and its orthologous human gene is located in HSA15; thus, this gene is not triplicated in the TS mouse or DS individuals. Therefore, the impairments in the neuronal plasticity and related cognitive problems in the TS mice cannot be attributed to the numbers of these receptors. However, it is possible that the enhanced GABA release in the hippocampus in TS mice results in a higher activity of these and other $GABA_A$ receptor subtypes.

Altogether, these results suggest that although the density of this type of receptor is not responsible for the altered learning abilities in the TS animals, manipulations that reduce the impaired $GABA_A$-mediated inhibition in these mice have procognitive effects. In fact, several studies have demonstrated the procognitive effects of the administration of different α5 selective and non-selective NAMs in this model of DS [for a review see 40]. Pharmacologically modulating the $GABA_A$ α5 receptors with different NAMs improved spatial learning and memory and reduced the thigmotactic behavior, thereby improving navigation strategies [44, 45].

The smaller beneficial effect of knocking out *Gabra5* on the cognitive abilities in the CO mice compared with that in the TS mice described in this study is consistent with a study by Ballard et al. [25], who did not find improvement in working memory in control rats in the DMTP task or the MWM after a chronic RO4938581 treatment. Additionally, the NAM α5IA did not improve the cognitive performance of CO animals during the acquisition sessions in the MWM [37]. In contrast, RO4938581 improved the performance of unimpaired monkeys in the object retrieval task. These animals were exposed to the task infrequently to prevent asymptotic performance, thus allowing a window for improvement [25]. It is also possible that the precognitive effects of reducing $GABA_A$α5 activity, genetically or pharmacologically, is more evident in those animals that have altered inhibition than in those that have an adequate balance between inhibitory and excitatory circuit influences.

In the present study, the TS +/+ mice presented an enhanced density of the area occupied by a marker of GABAergic synaptic boutons, VGAT, in the DG IML. Although several studies have shown that TS mice display impaired neuronal plasticity that is partially due to altered GABAergic signaling [36-41, 71], the imbalance between excitatory and inhibitory activity is not uniform in the trisomic brain. An increase in $GABA_A$-mediated transmission has been found in the DG of TS mice [36, 71] but not in the CA3 [72, 73] or CA1 [74, 75] hippocampal subfields, and tonic GABAergic inhibition is less efficient in the cerebellum of TS mice [76].

In addition, studies evaluating GABAergic synaptic density in different areas of the TS brain suggest that the number of these synapses is differentially affected depending on the brain region analyzed. Electron microscopy studies in the temporal cortex and hippocampus of adult TS mice found a normal density of symmetric (inhibitory) synapses [77-79]. Similarly, immunohistochemical evaluation of GABAergic terminals confirmed normal density in the TS mouse DG. In contrast, an increased GABAergic synaptic density was found in the IML, granular layer and DG of TS mice [38, 45, 80]. The reasons for these discrepancies are not fully understood, but compensatory mechanisms after altered brain development occurs might account for these regional changes.

The changes in the area occupied by VGAT+ boutons found in the present study and in previous studies [45, 80] are likely to be restricted to the area analyzed, and cannot be considered a generalized modification of synaptic inhibition. Future studies should also evaluate the effect of modifying $GABA_A$α5 function and/or expression on GABAergic synaptic markers in other hippocampal areas.

Recently it has been proposed that GABA might be excitatory rather than inhibitory in TS mice challenging the well-established concept of excessive GABA-mediated inhibition underlying cognition impairment in this mouse model of DS [81]. However, these data remains to be reproduced.

Other murine models of DS with different sets of triplicated genes display excitatory-inhibitory balance anomalies similar to those found in TS mice. The Ts1Cje mouse presents abnormalities in the morphology of inhibitory synapses in the hippocampus and cortex [79, 82, 83]. The Dp16 model exhibits an increase in the expression in the cortex and hippocampus of the GAD67 and VGAT

proteins, implicated in inhibitory transmission [84]. These results suggest that the overexpression of one or several of the genes triplicated in the three models could be responsible for the altered inhibition found in DS mouse models. Among these, the *Synj1* [85], *Olig1* and *Olig2* [74] and the *Dyrk1A* [80, 84] gene have been proposed to play a role in the excitatory/inhibitory imbalance found in trisomic animals.

In the heterozygous TS +/- mice, the density of VGAT+ boutons was completely normalized, and this effect was more pronounced in the TS -/- mice. In the CO mice, the reduction in the number of functional copies of the *Gabra5* gene also dose-dependently reduced the area that was occupied by VGAT+ boutons. This reduction in the inhibitory influence of GABAergic synapses could be one of the mechanisms underlying the cognitive enhancement found in mice of both karyotypes in the MWM and the fear conditioning test in the TS mice. Martínez-Cué et al. [45] also demonstrated that a chronic administration of a α5 selective NAM reduced the area that was occupied by VGAT+ boutons in TS animals, thereby reducing the over-inhibition.

Previous studies in *Gabra5* KO mice suggested that the absence of α5 subunit is not compensated by an upregulation of α1, α2, or α3 subunits and that the pharmacology of hippocampal benzodiazepine sites remaining was unchanged [18]. Our data does not exclude that reduction of α5 subunits did not induce compensatory effects in TS mice but restoration of LTP in this mouse is in line with the role of this receptor subtype in controlling synaptic plasticity. Reduction of extrasynaptic $GABA_A$α5 receptors in TS mice may have also impacted on the density and/or properties of their GABAergic synapses. It has been demonstrated that alterations in receptor assembly or subunit expression levels can indirectly alter synaptic localization and function [86]. In addition, tonic $GABA_A$-mediated membrane depolarization has been shown to promote synapse formation [87].

Because $GABA_A$ receptors regulate the proliferation, migration, differentiation and integration of new neurons [88-92], the decrease in the $GABA_A$ mediated-inhibition in the TS mouse after knocking out the *Gabra5* gene could play a role in the procognitive effects found in the TS animals by reducing functional and/or neuromorphological anomalies in their hippocampi. Tonic depolarizing $GABA_A$ responses by GABAergic Parvalbumin interneurons negatively regulate adult neurogenesis in the dentate gyrus (DG) of the hippocampus [93-95].

Although the present neuromorphological studies showed a tendency in which the density of proliferating (Ki67+) cells in the hippocampi of TS +/- and TS -/- mice was increased, this tendency did not reach statistical significance. Therefore, this effect is unlikely to account for the improvements in cognitive performance found in the heterozygous and homozygous TS mice. However, the reduced density of cells during late mitotic and early postmitotic stages (DCX+/CLR- cells) and during late postmitotic stages (DCX+/CLR+ cells) in the TS+/+/+ mice was completely rescued after knocking out both copies of the *Gabra5* gene, which suggests that reducing the function of this gene accelerates or increases the maturation of newly born cells. When the density of the mature granule cells in the GCL of the hippocampus was evaluated, it was found that

reducing the dosage of the *Gabra5* gene led to a significant increase in the number of DAPI+ cells in the TS, but not in the CO, mice, which could also play a role in the described cognitive enhancing effects.

Proliferating, immature neurons in different stages of differentiation and mature granule cells appear to have different electrophysiological properties and roles in learning and memory processes [95-99]. Therefore, the enhanced maturation of newly born cells that was found after reducing the *Gabra5* gene dosage in this study may be one of the mechanisms responsible for the enhancement in cognition that was induced by this genetic manipulation. However, the present results are not entirely consistent with our previous results in which after the chronic administration of RO4938581 to TS mice, a complete rescue of proliferating and mature cells was found, although the effect of this compound on the differentiating cells was not evaluated [45]. Therefore, the mechanisms by which the pharmacological and genetic reduction of the over-inhibition in TS mice induce the procognitive effects appear to be slightly different. Another explanation is that the genetic approach may lead to an adaptation or compensatory changes early in development, which is less visible in adulthood.

There are numerous reports of the role of adult hippocampal neurogenesis in the stablishment of LTP and in learning and memory processes [100-103]; therefore, enhancing neurogenesis could enhance LTP and the cognitive abilities of TS mice. In agreement with numerous reports demonstrating that LTP is altered in the hippocampal CA1/CA3 and DG regions in TS mice due to altered GABA-mediated activity [36, 37, 41, 82], the present study also showed a deteriorated LTP in the CA1/CA3 region of TS +/+ animals.

A comparative evaluation of synaptic plasticity deficits in other DS mouse models has shown that LTP in the CA3/CA1 region was decreased in Ts1Cje, Dp16 and triple trisomic mice (Dp10/Dp16/Dp17), unchanged in Ts1Rhr and Dp10 mice and even significantly increased in Dp17 mice [104-107]. In the DG, LTP was impaired in Ts1Cje, Ts1Rhr and triple trisomic (Dp10/Dp16/Dp17) mice [82, 108]. Because Dp16 and triple trisomic mice (Dp10/Dp16/Dp17) show behavioral and synaptic plasticity deficits comparable to those found in TS mice [106-108], and Dp10 and Dp17 mice show normal (or even enhanced) performances [107], it is possible that the set of MMU16 syntenic genes overexpressed in TS and in Dp16 mice plays an important role in this altered synaptic plasticity.

In the present study, the impairment to LTP presented by TS +/+ mice was completely rescued after reducing the *Gabra5* gene dosage. The $GABA_A$ α5-subunit-containing receptors are predominantly localized extra-synaptically in the dendrites of hippocampal CA1 pyramidal neurons [10-13] and generate long-lasting tonic currents [14-17]. However, these receptors have also been detected at GABAergic synapses on the same dendrites of hippocampal pyramidal neurons and could, therefore, mediate phasic GABAergic inhibition as well [65]. Because tonic currents drive neuronal migration and maturation, axon growth, and synaptic plasticity [109-112], the reduction of the density of $GABA_A$α5-subunit-containing receptors in heterozygous or homozygous *Gabra5* TS mice could be a mechanism partially responsible for the observed improvements in neurogenesis and LTP, thereby enhancing cognition in these animals.

However, reducing the number of functional copies of the *Gabra5* gene did not have any significant effect on LTP in the CO mice, which is consistent with other studies that failed to find changes in LTP in *Gabra5* KO mice [18, 20], except for under certain stimulation conditions [19].

The differential effects of reducing the number of copies of the *Gabra5* gene in the TS and CO mice on LTP may be one of the mechanisms implicated in the improved cognition that is found in the TS mice but is not as evident in the CO mice in the MWM and was absent in the fear conditioning test. These results are consistent with the normalization of LTP displayed by the TS animals after a chronic administration of RO4938581 without a significant effect in the CO animals [45]. However, other authors have reported that the chronic administration of α5IA or RO4938581 increases hippocampal LTP in normal rodents [25, 113]. As mentioned above, the discrepancies between the results of these reports and the present study may be due to the different backgrounds of the CO mice.

Finally, the TS and CO animals that differently expressed the *Gabra5* gene did not show alterations in anxiety or motor abilities (see **Supplementary Results** and **Discussion**). These results provide further support for the selectivity of the α5 receptor in cognitive functions and its role as a therapeutic target for learning and memory altered states.

In summary, this study shows that reducing the number of functional copies of the *Gabra5* gene in TS mice had effects that were similar to those observed following the chronic administration of an α5-selective NAM as follows: it induced a partial procognitive effect, improved LTP, enhanced neural differentiation and maturation and normalized the density of the GABAergic synapse markers. These effects were gene dose-dependent and were more evident in the TS mice than in the CO mice. In addition, modifying the gene dosage of *Gabra5* did not induce motor alterations and anxiety or reduced the viability of the mice. Although the present work and the results from pharmacological studies support that reducing the $GABA_A$ mediated-inhibition is a good target for improving cognitive alterations in DS, the fact that all these studies have been performed in animal models of DS limit their translational value to the human condition. Thus far, there is no confirmation that there is an enhanced inhibition in the brains of individuals with DS. Thus, studies in humans are necessary to demonstrate the potential translational value of these findings.

REFERENCES

1. Lister RG (1985) The amnesic action of benzodiazepines in man. Neurosci Biobehav Rev 9:87-94.

2. Cole SO (1986) Effects of benzodiazepines on acquisition and performance: a critical assessment. Neurosci Biobehav Rev 10:265-272.

3. Ghoneim MM, Mewaldt SP (1990) Benzodiazepines and human memory: a review. Anesthesiology 72:926-938.

4. Jensen S, Kirkegaard L, Anderson BN (1987) Randomized clinical investigation of Ro 15-1788, a benzodiazepine antagonist, in reversing the central effects of flunitrazepam. Eur J Anaesthesiol 4:113-118.

5. Venault P, Chapouthier G, Simiand J, Dodd RH, Rossier J (1987) Enhancement of performance by methyl beta-carboline-3-carboxylate, in learning and memory tasks. Brain Res Bull 19:365-370.

6. Sarter M, Bruno JP, Berntson GG (2001) Psychotogenic properties of benzodiazepine receptor inverse agonists. Psychopharmacology Berl 156:1-13.

7. Venault P, Chapouthier G (2007) From the behavioral pharmacology of beta-carbolines to seizures, anxiety, and memory. ScientificWorldJournal 7:204-223.

8. Seabrook GR, Easter A, Dawson GR, Bowery BJ (1997) Modulation of long-term potentiation in CA1 region of mouse hippocampal brain slices by GABAA receptor benzodiazepine site ligands. Neuropharmacology 36:823-830.

9. del Cerro S, Jung M, Lynch G (1992) Benzodiazepines block long-term potentiation in slices of hippocampus and piriform cortex. Neuroscience 49:1-6.

10. Laurie DJ, Wisden W, Seeburg PH (1992). The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12:4151-4172.

11. Fritschy JM, Mohler H (1995) GABAA-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol 359:154-194.

12. Klausberger T (2009) GABAergic interneurons targeting dendrites of pyramidal cells in the CA1 area of the hippocampus. Eur J Neurosci 30:947-957.

13. Olsen RW, Sieghart W (2009) GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology 56:141-148.

14. Böhme I, Rabe H, Lüddens H (2004). Four amino acids in the αsubunits determine the γ-aminobutyric acid sensitivities of GABAA receptor subtypes. J Biol Chem 279: 35193–35200.

15. Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662-3667.

16. Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors. Nat Rev Neurosci 6: 215–229.

17. Zheleznova NN, Sedelnikova A, Weiss DS (2009) Function and modulation of δ-containing GABAA receptors. Psychoneuroendocrinology 34: S67–S73.

18. Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW (2002)

Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. J Neurosci 22:5572-5580.

19. Martin LJ, Zurek AA, MacDonald JF, Roder JC, Jackson MF, Orser BA (2010) Alpha5GABAA receptor activity sets the threshold for long-term potentiation and constrains hippocampus-dependent memory. J Neurosci 30:5269-82.

20. Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H, Rudolph U (2002) Trace fear conditioning involves hippocampal alpha5 GABAA receptors. Proc Natl Acad Sci U S A 99:8980-8985.

21. Yee BK, Hauser J, Dolgov VV, Keist R, Mohler H, Rudolph U, Feldon J (2004) GABA receptors containing the alpha5 subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. Eur J Neurosci 20:1928-1936.

22. Atack JR (2010) Preclinical and clinical pharmacology of the GABAA receptor alpha5 subtype-selective inverse agonist alpha5IA. Pharmacol Ther 125:11-26.

23. Mohler H (2012) Cognitive enhancement by pharmacological and behavioral interventions: the murine Down syndrome model. Biochem Pharmacol 84:994-999.

24. Atack JR, Bayley PJ, Seabrook GR, Wafford KA, McKernan RM, Dawson GR (2006) L-655,708 enhances cognition in rats but is not proconvulsant at a dose selective for alpha5-containing GABAA receptors. Neuropharmacology 51:1023-1029.

25. Ballard TM, Knoflach F, Prinssen E, Borroni E, Vivian JA, Basile J, Gasser R, Moreau JL, Wettstein JG, Buettelmann B, Knust H, Thomas AW, Trube G, Hernandez MC (2009) RO4938581, a novel cognitive enhancer acting at GABAA a5 subunit-containing receptors. Psychopharmacology 202:207-223.

26. Nutt DJ, Besson M, Wilson SJ, Dawson GR, Lingford-Hughes AR (2007) Blockade of alcohol's amnestic activity in humans by an alpha5 subtype benzodiazepine receptor inverse agonist.Neuropharmacology 53:810-20.

27. Bittles AH, Bower C, Hussain R, Glasson EJ (2007) The four ages of Down syndrome. Eur J Public Health 17:221-225.

28. Lott IT, Dierssen M (2010) Cognitive deficits and associated neurological complications in individuals with Down's syndrome. Lancet Neurol 9:623-33.

29. Sturgeon X, Gardiner KJ (2011) Transcript catalogs of human chromosome 21 and orthologous chimpanzee and mouse regions: Mamm Genome 22:261-71.

30. Duchon A, Raveau M, Chevalier C, Nalesso V, Sharp AJ, Herault Y (2011) Identification of the translocation breakpoints in the Ts65Dn and Ts1Cje mouse lines: relevance for modeling Down syndrome. Mamm Genome 22:674–684.

31. Gupta M, Dhanasekaran AR, Gardiner KJ (2016) Mouse models of Down syndrome: gene content and consequences. Mamm Genome 27:538-555.

32. Bartesaghi R, Guidi S, Ciani E (2011) Is it possible to improve neurodevelopmental abnormalities in Down syndrome? Rev Neurosci 22:419-55.

33. Haydar TF, Reeves RH (2012) Trisomy 21 and early brain development. Trends Neurosci 35:81-91.

34. Rueda N, Florez J, Martinez-Cue C (2012) Mouse models of Down syndrome as a tool to unravel the causes of mental disabilities. Neural Plast 2012:584071.

35. Siarey RJ, Carlson EJ, Epstein CJ, Balbo A, Rapoport SI, Galdzicki Z (1999) Increased synaptic depression in the Ts65Dn mouse, a model for mental retardation in Down syndrome. Neuropharmacology 38: 1917-1920.

36. Kleschevnikov AM, Belichenko PV, Villar AJ, Epstein CJ, Malenka RC, Mobley WC (2004) Hippocampal long-term potentiation suppressed by increased inhibition in the Ts65Dn mouse, a genetic model of Down syndrome. J Neurosci 24:8153-60.

37. Costa AC, Grybko MJ (2005) Deficits in hippocampal CA1 LTP induced by TBS but not HFS in the Ts65Dn mouse: a model of Down syndrome. Neurosci Lett 382:317-22.

38. Mojabi FS, Fahimi A, Moghadam S, Moghadam S, McNerneny MW, Ponnusamy R, Kleschevnikov A, Mobley WC, Salehi A (2016) GABAergic hyperinnervation of dentate granule cells in the Ts65Dn mouse model of Down syndrome: Exploring the role of App. Hippocampus 26:1641-1654.

39. Deidda G, Bozarth IF, Cancedda L (2014) Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives. Front Cell Neurosci 8:119.

40. Martínez-Cué C, Delatour B, Potier MC (2014) Treating enhanced GABAergic inhibition in Down syndrome: use of GABA α5-selective inverse agonists. Neurosci Biobehav Rev,46:218-27.

41. Fernandez F, Morishita W, Zuniga E, Nguyen J, Blank M, Malenka RC, Garner CC (2007) Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome. Nat Neurosci 10:411-413.

42. Rueda N, Florez J, Martinez-Cue C (2008) Chronic pentylenetetrazole but not donepezil treatment rescues spatial cognition in Ts65Dn mice, a model for Down syndrome. Neurosci Lett 433:22-27.

43. Colas D, Chuluun B, Warrier D, Blank M, Wetmore DZ, Buckmaster P, Garner CC, Heller HC (2013) Short-term treatment with the GABAA receptor antagonist pentylenetetrazole produces a sustained pro-cognitive benefit in a mouse model of Down's syndrome. Br J Pharmacol 169:963-973.

44. Braudeau J, Delatour B, Duchon A, Pereira PL, Dauphinot L, de Chaumont F, Olivo-Marin JC, Dodd RH, Herault Y, Potier MC (2011) Specific targeting of the GABA-A receptor alpha5 subtype by a selective inverse agonist restores cognitive deficits in Down syndrome mice. J Psychopharmacol 25:1030-1042.

45. Martínez-Cué C, Martínez P, Rueda N, Vidal R, García S, Vidal V, Corrales A, Montero JA, Pazos A, Flórez J, Gasser R, Thomas AW, Honer M, Knoflach F, Trejo JL, Wettstein JG, Hernandez MC (2013) Reducing GABAA α5 receptor mediated inhibition rescues functional and neuromorphological deficits in a mouse model of Down syndrome. J Neurosci 33:3953-3966.

46. Liu F, Liang Z, Wegiel J, Hwang YW, Iqbal K, Grundke-Iqbal I, Ramakrishna N, Gong CX (2008) Overexpression of Dyrk1A contributes to neurofibrillary degeneration in Down syndrome: Faseb j 22:3224-33.

47. Bowes C, Li T, Frankel WN, Danciger M, Coffin JM, et al. (1993) Localization of a retroviral element within the rd gene coding for the beta subunit of cGMP phosphodiesterase. Proc Natl Acad Sci USA 90:2955–2959.

48. Rodgers FC, Zrnowska ED, Laha KT, Engin E, Zeller A, Keist R, Rudolph U, Pearce RA (2015) Etomidate impairs long-term potentiation in vitro by targeting a5-subunit containing GABAA receptors in nonpyramidal cells. J Neurosci 35:9707-9716.

49. Sur C, Fresu L, Howell O, McKernan RM, Atack JR (1999) Autoradiographic localization of alpha5 subunit-containing GABAA receptors in rat brain. Brain Res 822:265-70.

50. Steele RJ, Morris RG (1999) Delay-dependent impairment of a matching-to place task with chronic intrahippocampal infusion of the NMDA-antagonist DAP5.Hippocampus 9:118–136.

51. Chen G, Chen KS, Knox J, Inglis J, Bernard A, et al. (2000) A learning deficit related to age and beta-amyloid plaques in a mouse model of Alzheimer's disease. Nature 408:975–979.

52. Savonenko A, Xu GM, Melnikova T, Morton JL, Gonzales V, et al. (2005) Episodic-like memory deficits in the APPswe/PS1dE9 mouse model of Alzheimer's disease: relationships to beta-amyloid deposition and neurotransmitterabnormalities. Neurobiol Dis 18: 602–617.

53. Saab BJ, Saab AMP, Roder JC (2011) Statistical and theoretical considerations for the platform re-location water maze. J Neurosci Methods 198:44–52.

54. Corrales A, Martínez P, García S, Vidal V, García E, Flórez J, Sanchez-Barceló EJ, Martínez-Cué C, Rueda N (2013) Long-term oral administration of melatonin improves spatial learning and memory and protects against cholinergic degeneration in middle-aged Ts65Dn mice, a model of Down syndrome. J Pineal Res 54:346-58.

55. Salehi A, Faizi M, Colas D, Valletta J, Laguna J, et al. (2009) Restoration of norepinephrine-modulated contextual memory in a mouse model of Down syndrome. Sci Trans Med 18: 7ra17.

56. LLorens-Martín MV, Torres-Alemán I, Trejo JL (2006) Pronounced individual variation in the response to the stimulatory action of exercise on immature hippocampal neurons. Hippocampus 16:480–490.

57. Savanthrapadian S, Meyer T, Elgueta C, Booker SA, Vida I, Bartos M (2014) Synaptic properties of SOM- and CCK-expressing cells in Dentate gyrus interneuron network. The Journal of Neuroscience 34: 8197-8209.

58. Han Z-S, Buhl EH, Lörinczi Z, Somogyi P (1993) A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. European Journal of Neuroscience 5: 395-410.

59. Scharfman HE, Myers CE (2012) Hilar mossy cells in the dentate gyrus a historical perspective. Frontiers in neural circuits 6: 106.

60. Förster E, Zhao S, Frotscher M (2006) Laminating the hippocampus. Nature Neuroscience reviews 7: 259-267.

61. Overstreet LS, Hentges ST, Bumaschny VF, de Souza FS, Smart JL, Santangelo AM, Low MJ, Westbrook GL, Rubinstein M (2004) A transgenic marker for newly born granule cells in dentate gyrus. J Neurosci 24:3251–3259.

62. von Bohlen O, Halbach (2007) Immunohistochemical markers for staging neurogénesis in adult hippocampus. Cell Tissue Res 329: 409-240.

63. Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Sander AB, von der Beherens W, Kempermann G (2003) Transient calretinina expression defines early potmitotic step of neuronal differentiation in the adult hippocampal neurogénesis of mice. Molecular and Cellular Neuroscience 24: 603-613.

64. Glykys J, Mody I (2006) Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABAA receptor α5 subunit-deficient mice. J Neurophysiol 95: 2796-2807.

65. Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL (2006) Synaptic and nonsynaptic localization of GABAA receptors containing the alpha5 subunit in the rat brain. J Comp Neurol 499:458-470.

66. Moore CS (2006) Postnatal lethality and cardiac anomalies in the Ts65Dn Down syndrome mouse model. Mamm Genome 17:1005-1012.

67. Roper RJ, St John HK, Philip J, Lawler A, Reeves RH (2006) Perinatal loss of Ts65Dn Down syndrome mice. Genetics 172: 437-443.

68. Campeau S, Davis M (1995) Involvement of subcortical and cortical afferents to the lateral nucleus of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. J Neurosci 15:2312-27.

69. Goosens KA, Maren S (2001) Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. Learn Mem 8:148-55.

70. Braudeau J, Dauphinot L, Duchon A, Loistron A, Dodd RH, Herault Y, Delatour B, Potier MC (2011) Chronic Treatment with a Promnesiant GABA-A alpha5-Selective Inverse Agonist Increases Immediate Early Genes Expression during Memory Processing in Mice and Rectifies Their Expression Levels in a Down Syndrome Mouse Model. Adv Pharmacol Sci 2011:153218.

71. Kleschevnikov AM, Belichenko PV, Gall J, George L, Nosheny R, Maloney MT, Salehi A, Mobley WC (2012) Increased efficiency of the GABAA and GABAB receptor-mediated neurotransmission in the Ts65Dn mouse model of Down syndrome. Neurobiol Dis 45: 683-691.

72. Hanson JE, Blank M, Valenzuela RA, Garner CC, Madison DV (2007) The functional nature of synaptic circuitry is altered in area CA3 of the hippocampus in a mouse model of Down's syndrome. J Physiol 579: 53–67.

73. Stagni F, Magistretti J, Guidi S, Ciani E, Mangano C, Calzà L et al. (2013) Pharmacotherapy with fluoxetine restores functional connectivity from the dentate gyrus to field CA3 in the Ts65Dn mouse model of down syndrome. PLoS One 8:e61689.

74. Chakrabarti L, Best TK, Cramer NP, Carney RSE, Isaac JTR, Galdzicki Z, et al. (2010) Olig1 and Olig2 triplication causes developmental brain defects in Down syndrome. Nat Neurosci 13: 927–934.

75. Best TK, Cramer NP, Chakrabarti L, Haydar TF, Galdzicki Z (2012) Dysfunctional hippocampal inhibition in the Ts65Dn mouse model of Down syndrome. Exp Neurol 233: 749–757.

76. Szemes M, Davies RL, Garden CLP, Usowicz MM (2013) Weaker control of the electrical properties of cerebellar granule cells by tonically active GABAA receptors in the Ts65Dn mouse model of Down's syndrome. Mol Brain 6:33.

77. Kurt MA, Davies DC, Kidd M, Dierssen M, Florez J (2000) Synaptic deficit in the temporal cortex of partial trisomy 16 (Ts65Dn) mice. Brain Res 858: 191–197.

78. Kurt AM, Kafa IM, Dierssen M, Davies CD (2004) Deficits of neuronal density in CA1 and synaptic density in the dentate gyrus, CA3 and CA1, in a mouse model of Down syndrome. Brain Res 1022: 101–109.

79. Belichenko PV, Kleschevnikov AM, Masliah E, Wu C, TakimotoKimura R, Salehi A, et al. (2009) Excitatory-inhibitory relationship in the fascia dentata in the Ts65Dn mouse model of Down syndrome. J Comp Neurol 512: 453–466.

80. García-Cerro S, Martínez P, Vidal V, Corrales A, Flórez J, Vidal R, Rueda N, Arbonés ML, Martínez-Cué C (2014) Overexpression of Dyrk1A is implicated in several cognitive, electrophysiological and neuromorphological alterations found in a mouse model of Down syndrome. PLoS One 9:e106572.

81. Deidda G, Parrini M, Naskar S, Bozarth IF, Contestabile A, Cancedda L (2015) Reversing excitatory GABAAR signaling restores synaptic plasticity and memory in a mouse model of Down syndrome. Nat Med 21: 318–326.

82. Belichenko PV, Kleschevnikov AM, Salehi A, Epstein CJ, Mobley WC (2007) Synaptic and cognitive abnormalities in mouse models of Down syndrome: exploring genotype-phenotype relationships. J Comp Neurol 504: 329–345.

83. Popov VI, Kleschevkikov AM, Klimenko OA, Stewart MG, Belichenko PV (2011) Three-dimensional ultrastructure in the dentate gyrus and hippocampal area CA3 in the Ts65Dn mouse model of Down syndrome. *The Journal of Comparative Neurology* 519:1338–1354.

84. Souchet B, Guedj F, Sahún I, Duchon A, Daubigney F, Badel A, Yanagawa Y, Barallobre MJ, Dierssen M, Yu E, Herault Y, Arbones M, Janel N, Créau N, Delabar JM (2014) Excitation/inhibition balance and learning are modified by Dyrk1a gene dosage. Neurobiol Dis 69:65-75.

85. Voronov SV, Frere SG, Giovedi S, Pollina EA, Borel C, Zhang H, Schmidt C, Akeson EC, Wenk MR, Cimasoni L, Arancio O, Davisson MT, Antonarakis SE, Gardiner K, De Camilli P, Di Paolo G (2008) Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome. Proc Natl Acad Sci U S A 105: 9415-9420.

86. Martenson JS, Tomita SS (2015) Synaptic localization of neurotransmitter receptors: comparing mechanisms for AMPA and GABAA receptors. Curr Opin Pharmacol 0: 102-198.

87. Cellot G, Cherubini E (2013) Functional role of ambient GABA in refining neuronal circuits early in postnatal development. Frontiers in Neural Circuits 7: 1-9.

88. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-593.

89. Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47:803-815.

90. Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H, Luscher B (2007) GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. J Neurosci 27:3845-3854.

91. Bortone D, Polleux F (2009) KCC2 expression promotes the termination of cortical interneuron migration in a voltage-sensitive calcium-dependent manner. Neuron 62:53-71.

92. Deprez F, Vogt F, Floriou-Servou A, Lafourcade C, Rudolph U, Tyagarajan SK, Fritschy JM (2016) Partial inactivation of GABAA receptors containing the α5 subunit affects the development of adult-born dentate gyrus granule cells. Eur J Neurosci 44:2258-2271.

93. Song J, Zhong C, Bonaguidi MA, Sun GJ, Hsu D, Gu Y, et al. (2012) Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. Nature 489: 150–154.

94. Pontes A, Zhang Y, Hu W (2013) Novel functions of GABA signaling in adult neurogenesis. Front Biol 8, 496–507.

95. Pallotto M, Deprez F (2014) Regulation of adult neurogenesis by GABAergic transmission: signaling beyond GABAA-receptors. Front Cell Neurosci 8:166.

96. Aimone JB, Wiles J, Gage FH (2006) Potential role for adult neurogenesis in the encoding of time in new memories. Nat Neurosci 9:723-7.

97. Ambrogini P, Lattanzi D, Ciuffoli S, Agostini D, Bertini L, Stocchi V, Santi S, Cuppini R (2004) Morpho-functional characterization of neuronal cells at different stages of maturation in granule cell layer of adult rat dentate gyrus. Brain Res 1017:21-31.

98. Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory?: Nat Rev Neurosci 11:339-50.

99. Garthe A, Kempermann G (2013) An old test for new neurons: refining the Morris water maze to study the functional relevance of adult hippocampal neurogenesis. Front Neurosci 7: 63.

100. Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

101. Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372-376.

102. Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-84.

103. Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153-1161.

104. Siarey RJ, Villar AJ, Epstein CJ, Galdzicki Z (2005) Abnormal synaptic plasticity in the Ts1Cje segmental trisomy 16 mouse model of Down syndrome. Neuropharmacology 49: 122–128.

105. Olson LE, Roper RJ, Sengstaken CL, Peterson EA, Aquino V, Galdzicki Z et al. (2007) Trisomy for the Down syndrome 'critical region' is necessary but not sufficient for brain phenotypes of trisomic mice. Hum Mol Genet 16: 774–782.

106. Yu T, Li Z, Jia Z, Clapcote SJ, Liu C, Li S, et al. (2010) A mouse model of Down syndrome trisomic for all human chromosome 21 syntenic regions. Hum Mol Genet 19: 2780–2791.

107. Yu T, Liu C, Belichenko P, Clapcote SJ, Li S, Pao A, et al (2010) Effects of individual segmental trisomies of human chromosome 21 syntenic regions on hippocampal long-term potentiation and cognitive behaviors in mice. Brain Res 1366: 162–171.

108. Belichenko PV, Kleschevnikov AM, Becker A, Wagner GE, Lysenko LV, Yu EY, et al (2015) Down syndrome cognitive phenotypes modeled in mice trisomic for all HSA 21 homologues. PLoS One 10:e0134861.

109. Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87: 1215–1284.

110. Wang DD, Kriegstein AR (2009) Defining the role of GABA in cortical development. J Physiol 587: 1873–1879.

111. Kilb W, Kirischuk S, Luhmann HJ (2013) Role of tonic GABAergic currents during pre- and early postnatal rodent development. Front Neural Circuits 7:139.

112. Luhmann HJ, Fukuda A, Kilb W (2015) Control of cortical neuronal migration by glutamate and GABA. Front Cell Neurosci 9:4.

113. Dawson GR, Maubach KA, Collinson N, Cobain M, Everitt BJ, MacLeod AM, Choudhury HI, McDonald LM, Pillai G, Rycroft W, Smith AJ, Sternfeld F, Tattersall FD, Wafford KA, Reynolds DS, Seabrook GR, Atack JR (2006) An inverse agonist selective for a5 subunit-containing GABAA receptors enhances cognition. The Journal of pharmacology and experimental therapeutics 316:1335-1345.

FIGURE CAPTIONS

Fig 1. Crossings performed to obtain the six genotypes of mice characterized in this study (a), schematic representation showing where in the hippocampus different experiments were performed (b), and percentage of animals born with each genotype (c). Dotted bars represent the expected probability of birth (Mendelian distribution) in each experimental group. ΦΦ: p<0.01 TS vs. CO χ^2 'karyotype'. DG: dentate gyrus, GCL: granular cell layer, IML: inner molecular layer, ML: molecular layer, SGZ: subgranular zone. Quantification of VGAT+ boutons was performed in the IML, of Ki67+ cells in the SGZ and of DCX/CLR (+ and/or –cells) and of DAPI+ cells in the GCL of the DG. LTP induction and recordings were performed in the CA3/CA1 hippocampal regions.

Fig 2. The mean ± S.E.M. of the latency to reach the platform (a), the percentage of time spent in the periphery of the maze (b) during the acquisition sessions of the Morris water maze, the number of freezing episodes (c) and the time spent freezing (d) displayed by TS and CO mice with different gene dosage of the *Gabra5* gene during the cued- and context- conditioning test sessions in the fear conditioning tests. *: p<0.05, **: p<0.01, ***: p<0.01 TS vs. CO, #: p<0.05; ##: p<0.01, ###: p<0.001 *Gabra5* +/+ vs. *Gabra5* +/- or vs. *Gabra5* -/-. Bonferroni tests following significant ANOVAs. Φ: p<0.05 TS vs. CO, ANOVA 'karyotype'.

Fig 3. Representative *in vitro* autoradiographical images of coronal brain sections in which the anterior hippocampus is observed from CO and TS mice with different gene dosages of the *Gabra5* gene (a). The mean ± S.E.M. of the density of the α5 $GABA_A$ receptors as measured by *in vitro* [^{3}H]-RO0154513 autoradiography in the anterior CA1, CA3, and DG; the mean of these three anterior hippocampal areas (anterior Hc) and the posterior CA1 area (b) and the mean of the specific radioligand binding found in all these hippocampal areas (c). *: p<0.05, **: p<0.01 TS +/- vs. CO +/-; #: p<0.05, ##: p<0.01, ###: p<0.001 *Gabra5* +/+ vs. +/- or +/+ vs. -/-; δ: p<0.05, δδ: p<0.01, δδδ: p<0.001 *Gabra5* +/- vs. -/-. Bonferroni tests following significant ANOVAs.

Fig 4. Representative images of the area occupied by the VGAT+ boutons in the DG IML in the 6 groups of mice (a) and the mean ± S.E.M of the area occupied by the VGAT+ boutons in this area of the hippocampus (b) in TS and CO mice with different gene dosages of the *Gabra5* gene. *: p<0.05 TS vs. CO; #: p<0.05; ##: p<0.01 *Gabra5* +/+ vs. +/- or vs. -/-. Bonferroni tests following significant ANOVAs.

Fig 5. Representative images of the Ki67+ staining in the SGZ of the hippocampus in the 6 groups of mice (a) and the mean ± S.E.M. of the density of the Ki67+ cells in the SGZ of the hippocampus in TS and CO mice with different *Gabra5* gene dosages (b). ***: p<0.001 TS +/+/+ vs. CO +/+/+. Bonferroni tests following significant ANOVAs. ΦΦΦ: p<0.05 TS vs. CO ANOVA 'karyotype'. Arrowheads in (a) signal single Ki67+ cells.

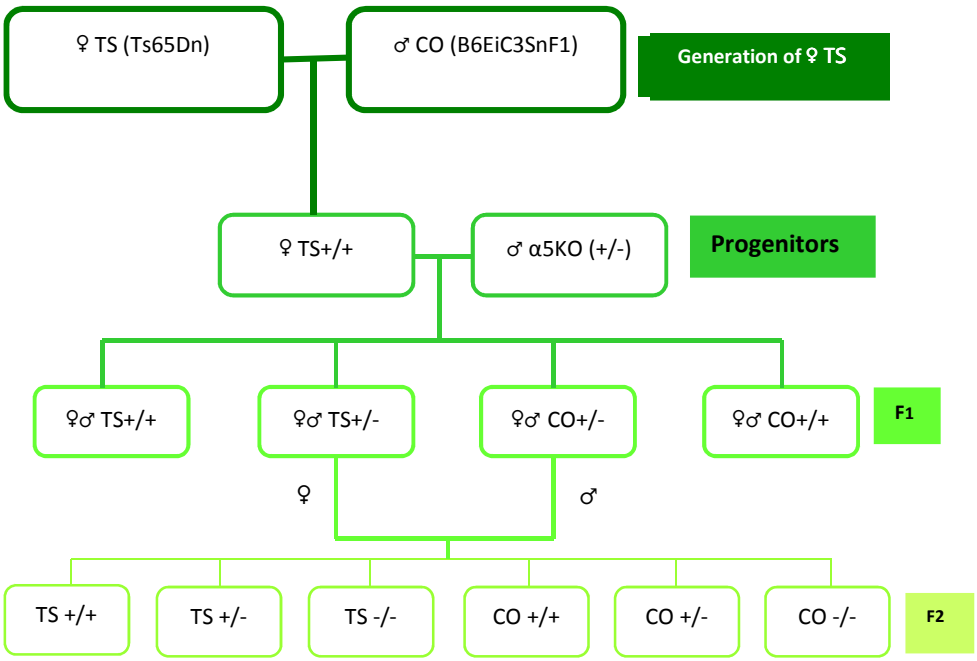
Fig 6. Representative images of the number of DCX+/CLR- and DCX+/CLR+ positive cells in the DG in the 6 groups of mice (a) and the mean ± S.E.M of the density of the DCX+/CLR- cells and DCX+/CLR+ cells found in the hippocampus in the 6 groups of animals (b). *: $p<0.05$; **: $p<0.01$ TS +/+/+ vs. CO +/+/+, #: $p<0.05$ *Gabra5* +/+ vs. +/- or -/-. Bonferroni tests following significant ANOVAs. ΦΦ: $p<0.01$ TS vs. CO ANOVA 'karyotype'.

Fig 7. Representative images of DAPI+ cells in the GCL in the 6 groups of mice (a) and the mean ± S.E.M of the density of the DAPI+ cells in the GCL of the hippocampus in TS and CO mice with different dosages of the *Gabra5* gene (b). ***: $p<0.01$ TS +/+/+ vs. CO +/+/+; #: $p<0.05$ *Gabra5* +/+ vs. +/- or vs. -/-. Bonferroni tests following significant ANOVAs. ΦΦΦ: $p<0.001$ TS vs. CO ANOVA 'karyotype'.

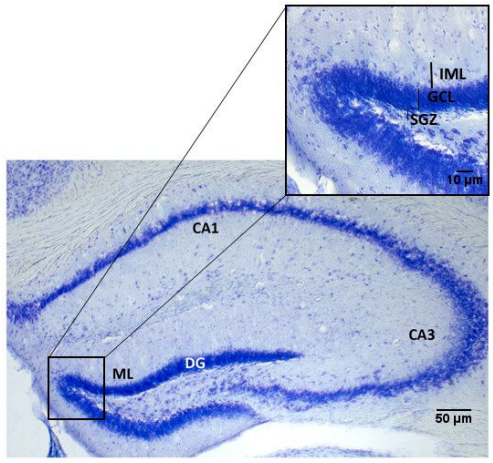
Fig 8. Time courses of the initial slope of the field excitatory postsynaptic potentials (fEPSPs) recorded from the apical dendritic layer of the CA1 region in hippocampal slices after stimulating the Schaffer collateral commissural pathway at 30 s intervals. Following 20 min of stable baseline recording, a theta burst stimulus induced robust LTP in the hippocampal slices of the vehicle treated CO, but not TS, mice. Reduction in the gene dosage of *Gabra5* resulted in an enhanced LTP in slices from TS, but not from CO, mice. Data are presented as the means ± S.E.M.

Figure 1

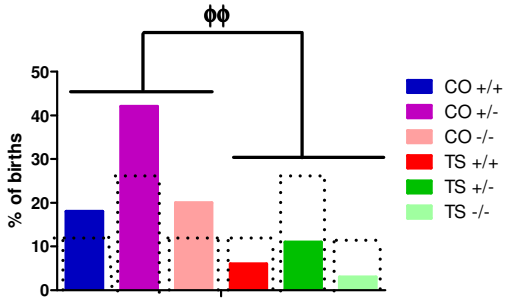
a


♀ TS (Ts65Dn)
♂ CO (B6EiC3SnF1)
Generation of ♀ TS
♀ TS+/+
♂ α5KO (+/-)
Progenitors
♀♂ TS+/+
♀♂ TS+/-
♀♂ CO+/-
♀♂ CO+/+
F1
♀
♂
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
F2

b


IML
GCL
SGZ
10 µm
CA1
CA3
DG
ML
50 µm

c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% of births
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CO +/+
CO +/-
CO -/-
TS +/+
TS +/-
TS -/-

Figure 2

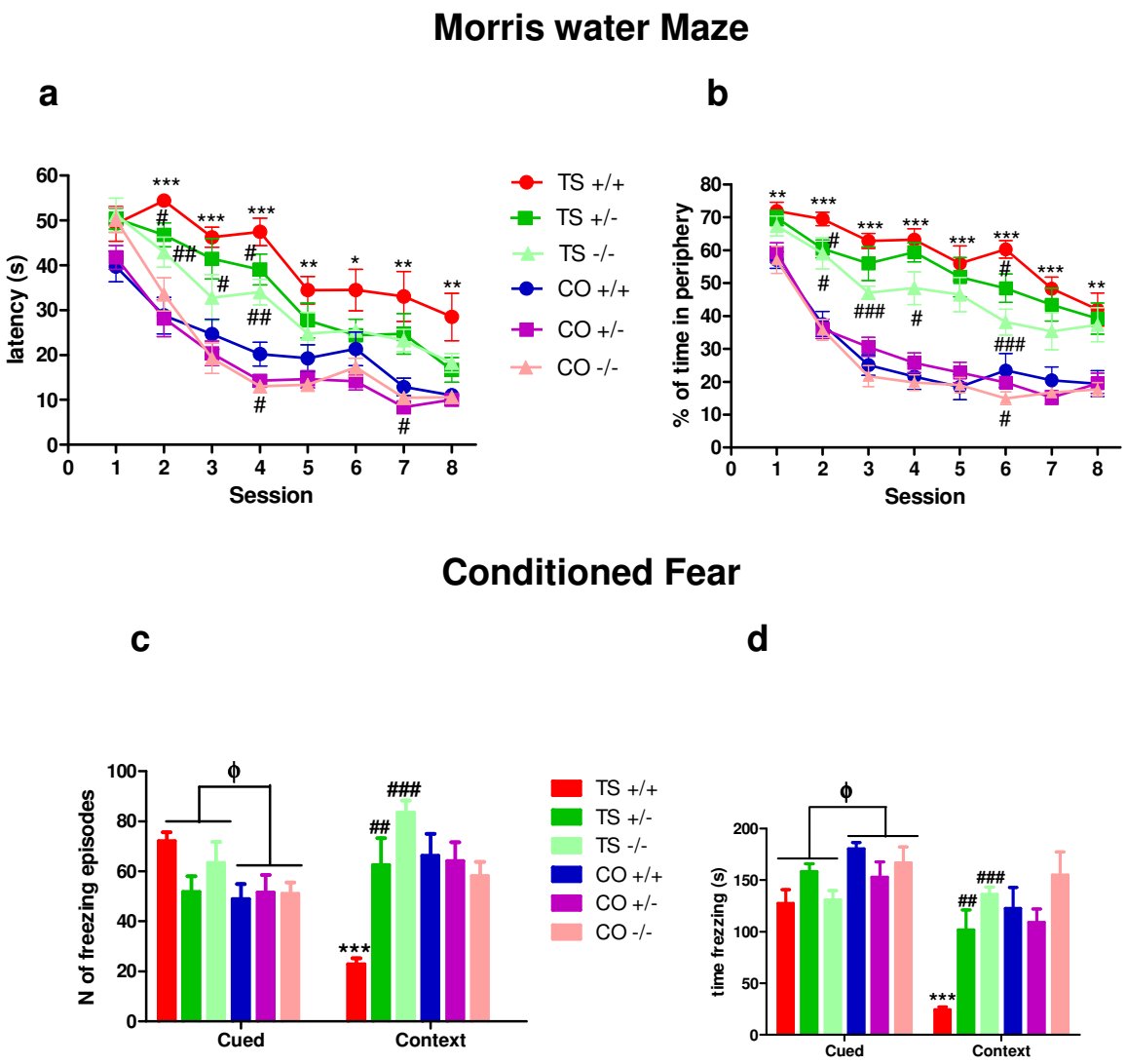
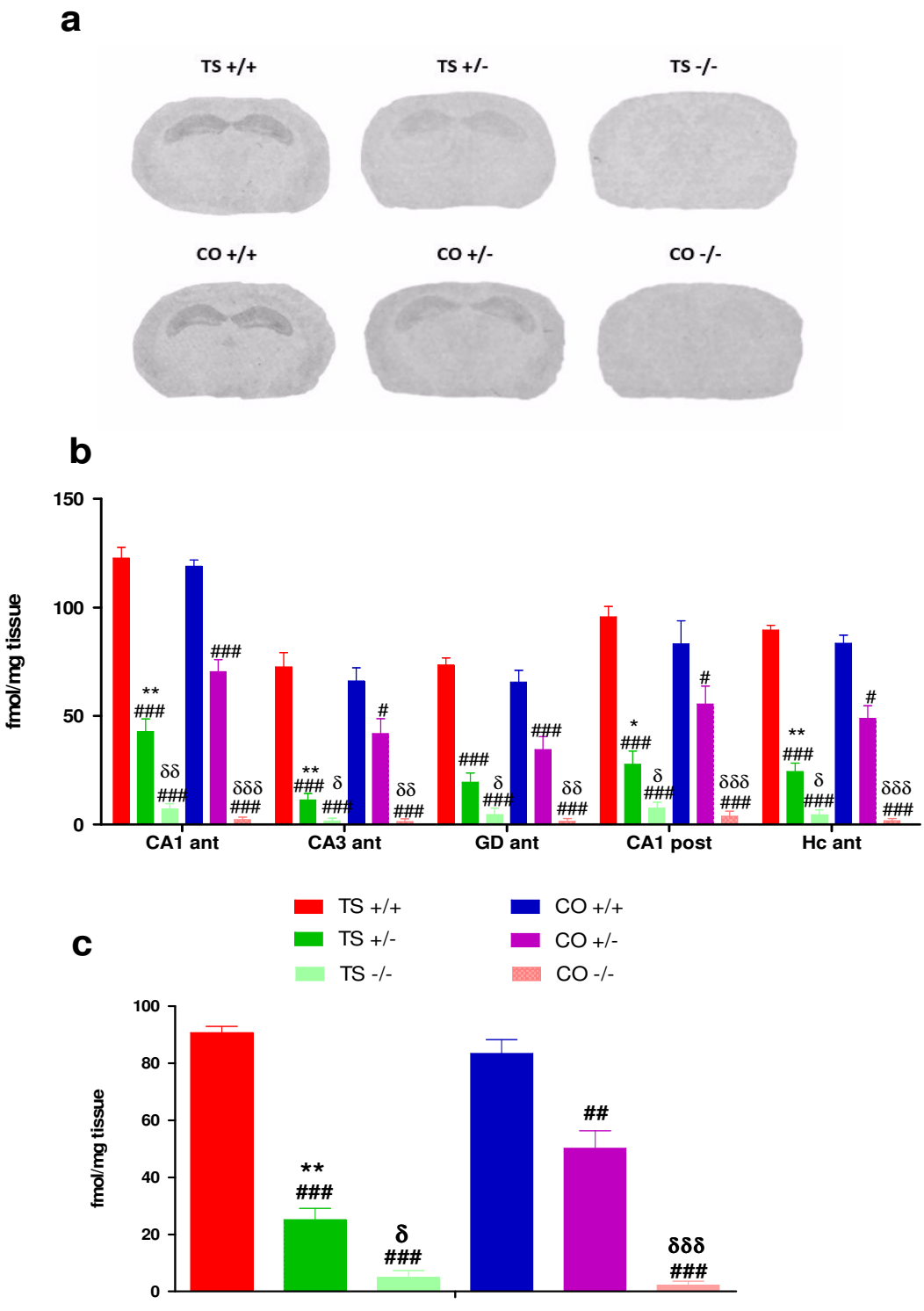

Morris water Maze
a
b
latency (s)
Session
% of time in periphery
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
Conditioned Fear
c
d
N of freezing episodes
time frezzing (s)
Cued
Context
ϕ

Figure 3


a
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
b
fmol/mg tissue
150
100
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CA1 ant
CA3 ant
GD ant
CA1 post
Hc ant
TS +/+
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CO +/+
CO +/-
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fmol/mg tissue
100
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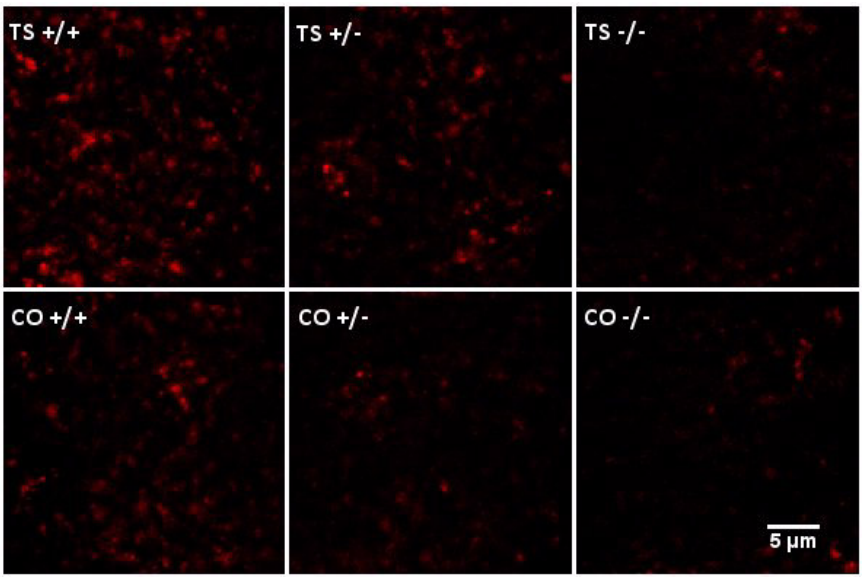
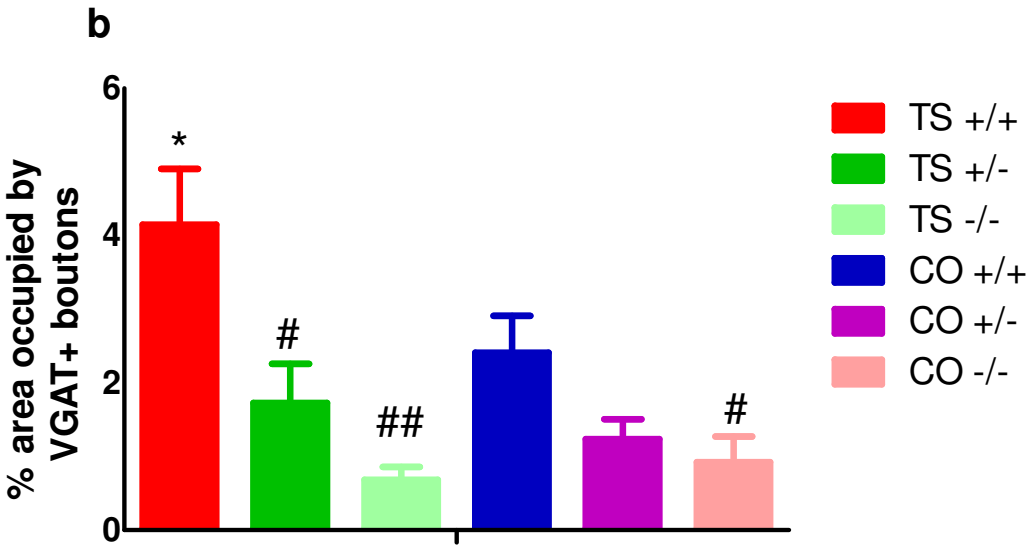
Figure 4

a


TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
5 µm

b


% area occupied by VGAT+ boutons
6
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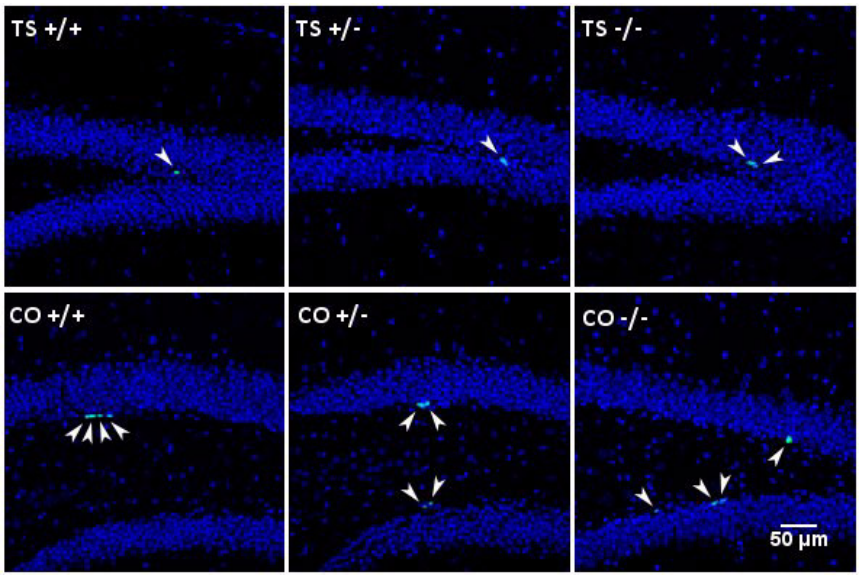
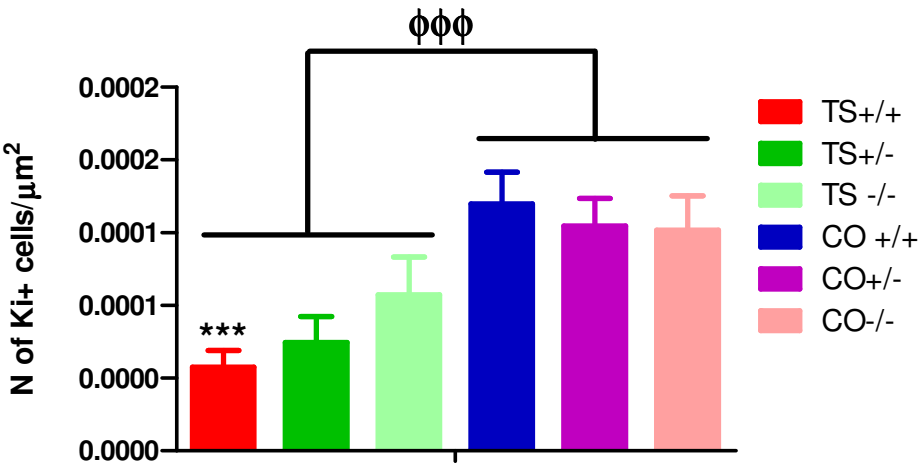
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-

Figure 5

a


TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
50 µm

b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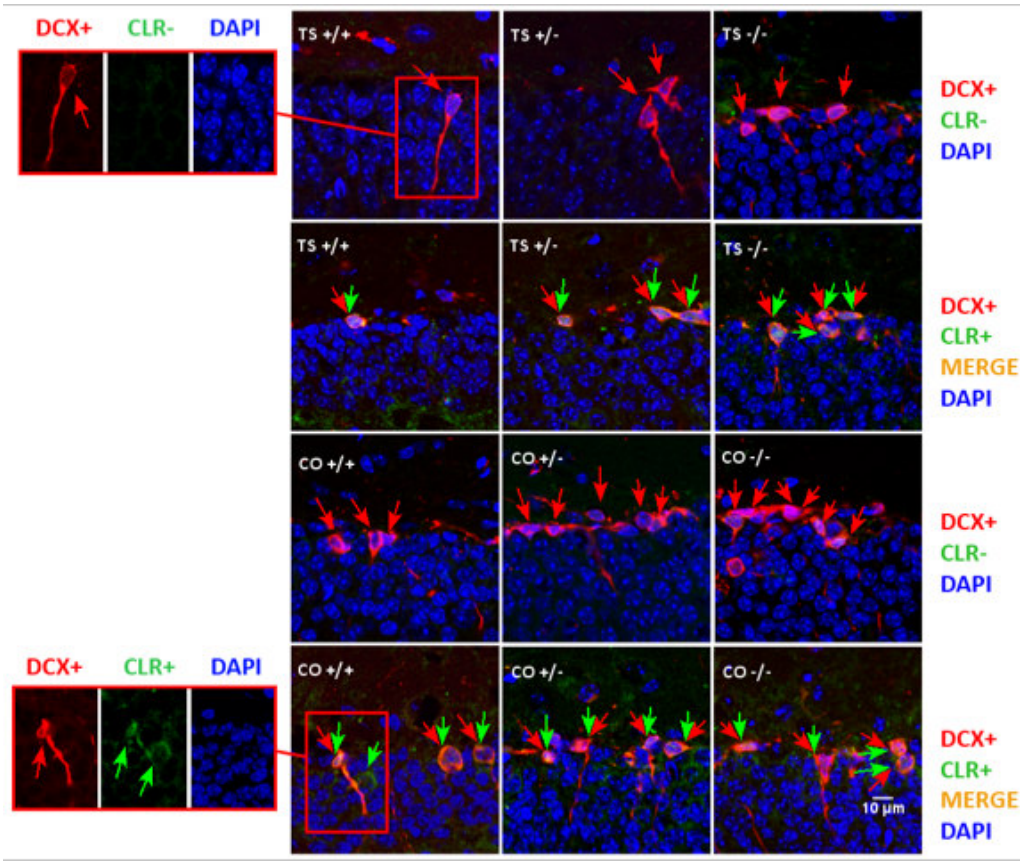
0.0000
0.0000
N of Ki+ cells/µm2
***
TS+/+
TS+/-
TS -/-
CO +/+
CO+/-
CO-/-

Figure 6

a


DCX+
CLR-
DAPI
TS +/+
TS +/-
TS -/-
DCX+
CLR-
DAPI
TS +/+
TS +/-
TS -/-
DCX+
CLR+
MERGE
DAPI
CO +/+
CO +/-
CO -/-
DCX+
CLR-
DAPI
DCX+
CLR+
DAPI
CO +/+
CO +/-
CO -/-
10 µm
DCX+
CLR+
MERGE
DAPI

b

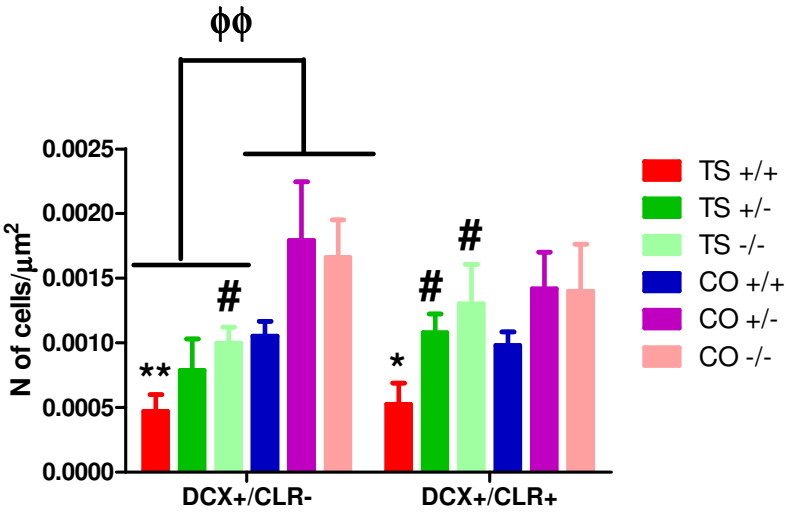
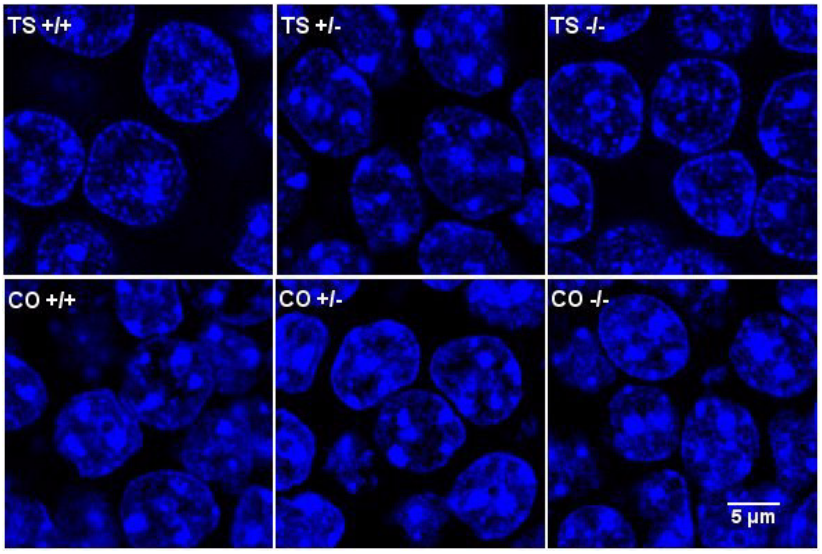

ϕϕ
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
N of cells/µm2
**
#
*
#
#
DCX+/CLR-
DCX+/CLR+
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-

Figure 7

a


TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-
5 µm

b

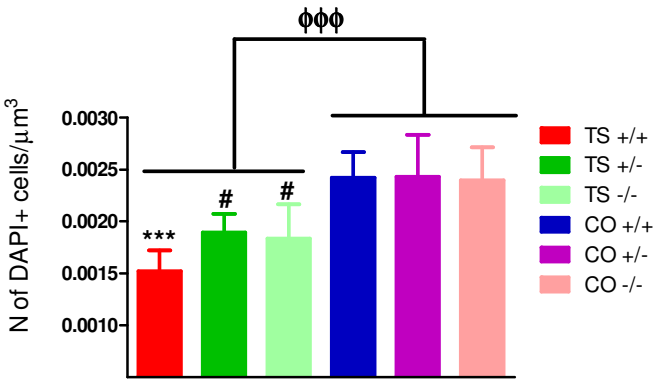
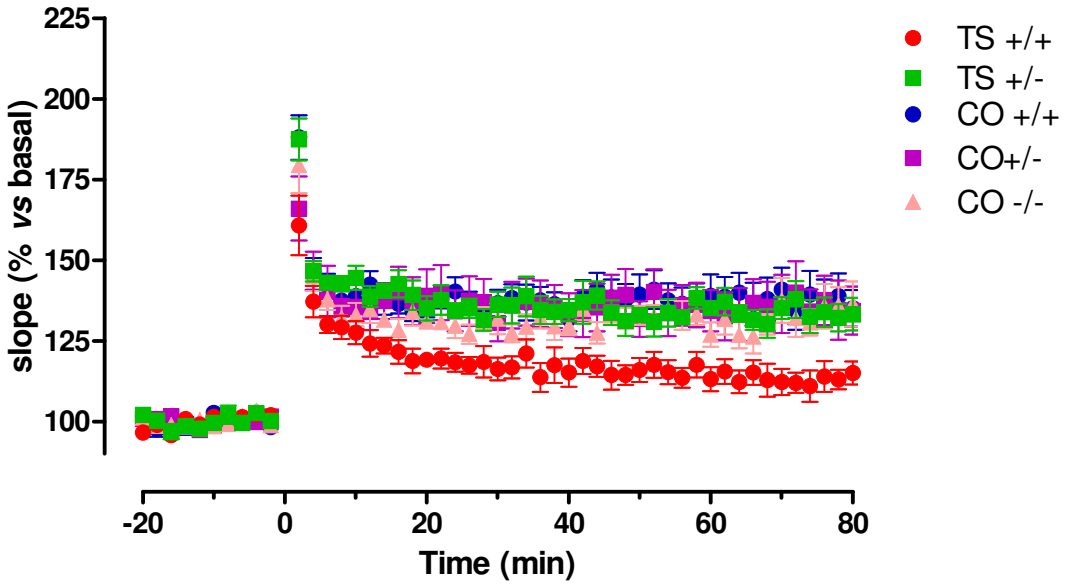

ϕϕϕ
N of DAPI+ cells/µm3
0.0030
0.0025
0.0020
0.0015
0.0010
***
#
#
TS +/+
TS +/-
TS -/-
CO +/+
CO +/-
CO -/-

Figure 8


225
200
175
150
125
100
slope (% vs basal)
-20
0
20
40
60
80
Time (min)
TS +/+
TS +/-
CO +/+
CO+/-
CO -/-