The epistatic interaction between dopamine D3 receptor and dysbindin-1 modulates higher-order cognitive functions in mice and humans.

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Running title: D3 by Dysbindin-1 epistasis

Abstract

Dopamine D2 and D3 receptors are implicated in schizophrenia and its pharmacological treatments. These receptors go through intracellular trafficking processes that might be modulated by dysbindin-1 (Dys). Indeed, Dys variations alters cognitive responses to antipsychotic drugs through D2-mediated mechanisms. However, how Dys might selectively interfere with the D3 subtype is unknown. Here, we revealed an interaction between functional genetic variations altering D3 and Dys. In particular, in both patients with schizophrenia and genetically modified mice, the concomitant D3 and Dys reduced expression was associated with better executive and working memory functions. This D3/Dys interaction produced a D2/D3 unbalance in favor of increased D2 levels in the prefrontal cortex (PFC), but not in the striatum. No epistatic effects were evident in clinical PANSS scores while only marginal effects on sensorimotor gating, locomotor functions and social behavior were observed in mice. This D3/Dys genetic interaction point to a D2/D3 unbalance in PFC as a possible target for patients' stratification and pro-cognitive treatments in schizophrenia.

Key words: dopamine D3 receptor, dysbindin-1, schizophrenia, cognition, personalized medicine, genetics.

Introduction

Dopaminergic receptors have important implications in several psychiatric and neurodevelopmental disorders[1]. For schizophrenia, in particular, converging physiological, anatomical, genetics, and pharmacological evidence indicate a major implication of the D2-like receptors[2-5]. In contrast to D1-like receptors (D1 and D5), members of the D2 receptors family (D2, D3, D4) are quickly internalized after agonist stimulation and eventually degraded through the lysosomal intracellular pathway[6, 7]. Intracellular trafficking processes might be altered in schizophrenia[8-10], and are strongly implicated in antipsychotic drugs mode of action[11-15].

The dysbindin-1 protein (Dys), encoded by the dystrobrevin-binding protein 1 gene (DTNBP1), is part of the BLOC-1 (Biogenesis of Lysosome-related Organelles Complex 1) and is implicated in intracellular trafficking processes[16, 17]. In particular, Dys genetic disruption alters intracellular trafficking of D2-like receptors, but not D1, resulting in increased D2 receptors on the neuronal surface[16, 18]. In agreement, in both mice and humans, Dys genetic variations affect cognitive and schizophrenia-relevant behavioral phenotypes trough dopamine/D2-like mechanisms[19-21]. Furthermore, in both mice and humans, Dys genetic variations alters cognitive responses to antipsychotic drugs through D2-mediated mechanisms[18]. However, how Dys-dependent modulation of D2-like receptors intracellular trafficking might selectively interact with D3 signaling is still unknown.

Dopamine D2 and D3 receptors show high structural homology[22], and current available pharmacological tools as well as antipsychotic drugs have high affinity for both these receptors[5, 23-25]. Thus, the unique contribution of each of these receptors in physiological and behavioral functions cannot be fully distinguished. This would be important to address, as recent electrophysiological and morphological analyses identified distinct neuronal

populations expressing either D2 or D3 receptors[26]. Furthermore, D2 and D3 receptors are suggested to differently control mood and cognitive processes[24, 25], and might have different implications in psychiatric disorders and their pharmacological treatments[27, 28].

Here, we adopted a genetic approach to assess the selective contribution of D3 hypofunction in the context of Dys-dependent alterations of D2-like intracellular trafficking. First, we discovered an epistatic functional interaction between D3 and Dys in patients with schizophrenia, enrolled in the NIH Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE). Subsequently, by generating a mouse line with concomitant selective hypofunction of both D3 and Dys genes (i.e. double heterozygous $Dys^{+/-}$*$D3^{+/-}$ mice), we confirmed the functional interaction between D3 and Dys in schizophrenia-relevant phenotypes, as well as in neuronal excitability and extracellular dopamine levels. Our data support the hypothesis that D3 might represent a pharmacological target for pro-cognitive drug treatments in schizophrenia as well as a genetic tool in schizophrenia patients' stratification.

Materials and methods

Human subjects

Patients were enrolled in the CATIE study through the NIMH Center for Collaborative Research and Genomics Resource[29, 30]. Analysis was carried out on samples from 662 schizophrenic patients clinically assessed at baseline and with 18 months follow-up for which cognitive and genetic data were available. Demographic and clinical details included age, sex, age of illness onset, illness duration, medical history (including alcohol and drug use), admission and medication history. We selected from the CATIE study the cognitive performance in the Wisconsin Card Sorting Test (WCST), a widely-used measurement to assess executive function deficits in patients with schizophrenia associated with prefrontal cortex function[31-33], and a composite measure of working memory (WM) based on Letter-Number Span Test and on a computerized test of visuospatial working memory[30]. Subjects were genotyped for 492,900 single nucleotide polymorphisms (SNPs) using the Affymetrix 500K two chip genotyping platform plus a custom 164K fill-in chip following multiple quality control steps for both subjects and SNPs [34]. SNPSs of interest were extracted using the software PLINK for Windows (http://pngu.mgh.harvard.edu/purcell/plink/) and Hardy-Weinberg Equilibrium test (HWE) was performed for each of them.

Mice

We generated a novel mouse line first by breeding $D3^{-/-}$[35] with $Dys^{-/-}$[21] mice to obtain double D3 and Dys heterozygous ($D3^{+/-}$*$Dys^{+/-}$). Both lines had a C57BL/6J genetic background, which is commonly used to facilitate inter-laboratory comparisons. In keeping with the idea that heterozygous mice might better mimic human functional genetic variations than full

knockouts[18, 36, 37] and to avoid uncontrollable gene-environment interactions coming from a possible alteration in maternal behavior, we followed a breeding scheme consisted in mating one male $D3^{+/-}$*$Dys^{+/-}$ with two C57BL6/J female mice. This approach allowed us to evaluate in the generated littermates the life-long effects of genetic variation resulting in normal levels of both D3 and Dys ($D3^{+/+}$*$Dys^{+/+}$), a selective D3 hypofunction ($D3^{+/-}$*$Dys^{+/+}$ single heterozygous mice), Dys hypofunction ($D3^{+/+*}Dys^{+/-}$ single heterozygous mice), and the combination of both decreased D3 and Dys levels in the same individual ($D3^{+/-}$*$Dys^{+/-}$ mice). Only male littermates, 3-6 months old were tested. Animals were group-housed (2–4 mice per cage), with free access to food and water, in an air-conditioned room (22±2°C), with a 12 h light–dark cycle. Genotypes were identified by PCR analysis of tail DNA as previously described[21, 38]. The experimenter handled animals on alternate days during the week preceding the first behavioral test. All experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of both the Catania University and the Istituto Italiano di Tecnologia (IIT).

RNA isolation and real-time-PCR

Total RNA was extracted from isolated brain areas by using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instruction. Total RNA was dissolved in 30 μl of RNase-free water and optical density at 260 and 280 nm was assessed to evaluate RNA concentrations and purity. Total RNA (2 μg) was converted in first-strand cDNA in a 20 μl reaction volume with 200 U of SuperScript III, 50 ng random hexamers, 0.5 mM dNTP mix, 0.01 mM dithiothreitol, 20 mM Tris–HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$ (Invitrogen Life Technologies). The reactions were carried out at 50 °C for 50 min and stopped by heating at 85 °C for 5 min. Aliquots of 100 ng cDNA were amplified in parallel

reactions, using specific primer pairs for D3 (Fw: 5'-GGGGTGACTGTCCTGGTCTA-3'; Rv: 5'-AAGCCAGGTCTGATGCTGAT-3'; product length 110 bp; acc. num.NM007877.2) and Dys (Fw; 5'-TGAAGGAGCGGCAGAAGTT-3'; Rv: 5'-GTCCACATTCACTTCCATG-3'; product length 134 bp; acc. num.NM025772.4) genes. GAPDH was used as reference housekeeping gene (Fw: 5'-CAACTCACTCAAGATTGTCAGCAA-3'; Rv: 5'-GGCATGGACTGTGGTCATGA-3'; product length 118 bp; acc. num.NM001289726.1). Each PCR reaction (20 µl final volume) contained 0.4 µM primers, 1.6 mM Mg^{2+}, and 1X Light Cycler-Fast Start DNA Master SYBR Green I (Roche, Basel, Switzerland). Amplifications were carried out in a Light Cycler 1.5 instrument (Roche). Fold change quantification was obtained by the ΔΔCt comparative method.

Slices surface biotinylation

Mice were anaesthetized with isofluorane and then decapitated. The brain was sectioned in cold carboxygenated Hanks' Balanced Salt Solution (HBSS, Invitrogen Life Technologies) enriched with 4 mM $MgCl_2$, 0.7 mM $CaCl_2$ and 10 mM D-glucose, equilibrated with 95% O_2 and 5% CO_2, pH 7.4.) on a vibratome at a 300 µm thickness. mPFC was dissected from coronal slices. Before starting the surface biotinylation reaction the tissues were washed twice for 5 minutes in ice-cold HBSS buffer, to ensure a gradual cooling of the cells. The filters holding the tissues were transferred to a well containing an excess of biotinylation reagent solution (100 µM NHS-LC-biotin, Pierce, Rockford, IL, USA) in HBSS. After 45 min of incubation, the tissues were transferred to another well and washed twice with the HBSS buffer containing 200 mM Lysine (Sigma, Saint Louis, MO, USA), to block all reactive NHS-LC-biotin in excess. The tissues were washed twice with ice-cold HBSS and immediately placed on ice to mechanically disrupt the tissue in 500 µl lysis buffer (1% Triton X-100, PBS,

and a cocktail of protease inhibitors, Sigma). To discard extra debris, homogenates were centrifuged for 5 min at 4°C at 13,000 r.p.m. and supernatants were collected. To precipitate the biotinylated proteins from the homogenates, 50 µl of immobilized Streptavidin beads (Pierce) were added to the samples and the mixture was rotating for three hours at 4°C. The precipitates were collected by a brief centrifugation, mixed with 50 µl of SDS-PAGE loading buffer, boiled for 5 minutes and stored at -80°C until use. Equal amounts of proteins (100 µg) were loaded onto 10% SDS/PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Western analyses were performed using antibodies against D2-like (sc-5303), Synaptophysin (sc-365488) and Transferrin Receptor (sc-21011), purchased from Santa Cruz Biotechnology (Heidelberg, Germany), and the anti-actin antibody from Sigma. Western blot analyses of brain samples were performed as previously described [8]. Quantifications were performed using NIH ImageJ (version 1.42q, Bethesda, MD, USA) software. All the data was obtained blind to the treatment and the genotype of the animals.

Behavioral tasks

Discrete paired-trial variable delay T-maze task. In this test[21, 39], mice were exposed to a sequence of randomly chosen forced runs, each followed by a choice run such that the mice were required to integrate information from the forced run with the learned rule (non-match to sample). The T-maze apparatus was built out of transparent plexiglass (0.5 cm thick; dimensions of the alleys: 40x10.2x17.5 cm; light levels were 20±2 lux in the main alley and 10±2 lux in side alleys). A recessed cup at the end of each side alley concealed the food reinforcement from view. All visual cues that could be used by animals to guide their choices were carefully removed and behavioral studies were carried out in a room without visual landmarks or windows. After a week of single housing, body weight and 24-hour food intake

were monitored for 3 consecutive days. Then, animals were force to a food restriction regimen throughout the experiments to maintain 90% body weight. During the first week of food restriction, each animal was also trained to the food reinforcement (14 mg, 5TUL, TestDiet, Richmond, IN, USA) for 3 consecutive days, in the home cage. Thereafter, animals were trained to the T-maze apparatus and allowed to retrieve the food reinforcement for 10 minutes/day, for 2 consecutive days, with both arms open. The day after, the animals were exposed to 10 forced-alternation runs: in each run the animals were placed into the T-maze with one goal arm closed off; the animals were allowed for up to 2 minutes to locate and eat the food reinforcement in the open arm; after consuming the food pellet an inter-trial period of at least 20 minutes was given to mice in the home cage, and then they were placed back into the maze for another forced run, where the food reinforcement was located in the opposite arm. The next day, mice were tested as follows (discrete trial delayed alternation test): a randomly chosen forced run, a 4-second delay interval in the home cage, a choice run (access to both arms); the food reinforcement was located in the opposite arm entered in the previous forced trial. After an inter-trial period of 20 minutes, the animal was placed back in the maze for another forced run-choice run paired trial. The test included ten paired runs per day and was repeated every day, until the mouse successfully performed 8 correct choices out of 10 daily trials (80% choice accuracy), for 3 consecutive days. Animals that did not reach this criterion within 20 days were eliminated. A different pseudo-randomly chosen pattern of forced runs (e.g., R-R-L-R-L-L-R-L-R-L) was used every day, but on a given day the same pattern was used for all animals. Once the mouse performed consistently at the 4-second intra-trial delay, testing at three additional intra-trial delays (30, 60 and 240 seconds presented in a random fashion, with a 20-second inter-trial delay) was carried out. Mice were given 4 trials of each delay on 4 consecutive days of testing, for a total of 16 trials for each

delay. After each trial, the apparatus was cleaned with water and ethanol 10%, with special attention to the choice point of the T-maze.

Acoustic startle response and prepulse inhibition (PPI). Two hours before the test, animals were acclimatized to the testing room. Acoustic startle response and PPI were measured using a SR-Lab System apparatus (San Diego Instruments, San Diego, CA, USA). The procedure was performed as previously described[8, 37]. PPI test session began by placing the animal in the plexiglas holding cylinder for a 5-min acclimation period with a 65 dB background noise. Animals were then exposed to a series of trial types, which were presented in pseudorandom order. The inter trial interval (ITI) was 5-60 seconds. One trial type measured the response to no stimulus (baseline movement), and another one measured the startle stimulus alone (acoustic amplitude), which was a 40 ms 120 dB sound burst. Other four trial types were acoustic prepulse plus acoustic startle stimulus trials. Prepulse tones lasted 20 ms at 70, 75, 80 and 85 dB, presented 100 ms before the startle stimulus. PPI was calculated by using the following formula: 100 × [pulse-only units − (prepulse + pulse units)]/(pulse-only units).

Open field test. Animals were tested in an evenly illuminated (9±1 lux) square open field, 40 x 40 x 40 cm, divided into sixteen quadrants by lines on the floor (Ugo Basile, Gemonio, Italy) over a 30 minute-period. Locomotor activity and rearing behavior were assessed during the first exposure to the empty open field arena. The apparatus was cleaned with a 10% ethanol solution in between each test to prevent olfactory cues. Locomotor activity was quantified by counting the number of crossings (number of squares entered) with all four paws. The

behavior of animals was recorded by using a video camera (Sony Videocam PJ330E) and analyzed by one observer who was unaware of the genotype.

Habituation/dishabituation social interaction test. Animals were tested as previously described[40] in 2150E Tecniplast cages (35.5 × 23.5 × 19 cm) slightly illuminated (5±1 lux) and video-recorded using a video camera (Sony Videocam PJ330E). Mice were individually housed in a clean testing cage for 1 h of habituation in the testing room. The test began when a stimulus male mouse was introduced into the testing cage for a 1-min trial. At the end of this first trial, the stimulus mouse was removed from the testing cage and placed into the home cage. The subject mouse was left in the testing cage over a 3-min inter-trial interval (ITI). In trial 2, the same stimulus mouse was re-introduced for a 1-min trial. The same procedure was repeated for three additional trials. In a fifth dishabituation trial, a different stimulus mouse was introduced to the cage of the subject mouse for a 1-min session, to control for the habituation of the subject mouse to social investigation of the previous stimulus mouse. Social interactions were scored from the videotapes for the cumulative duration of the following behavioral responses performed by the subject mouse: ano-genital sniffing (direct contact with the ano-genital area), body sniffing (sniffing or snout contact with the flank area), nose-to-nose sniffing (sniffing or snout contact with the head/neck/mouth area). One observer uninformed of the genotype conducted scoring.

Electrophysiology

Slice preparation. Medial prefrontal cortex (mPFC) slices were prepared from postnatal day (PND) 13 to PND 22. After decapitation, brains were removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) with the following composition in mM: 124 NaCl; 2.5 KCl; 1.25

NaH_2PO_4; 1.2 $MgSO_4$; 2 $CaCl_2$; 26 $NaHCO_3$; 10 D-glucose at pH 7.3. ACSF was oxygenated and pH buffered by constant bubbling with a gas mixture of O_2 (95%) and CO_2 (5%). Brains were glued onto a Leica VT1200 vibratome specimen holder and cut in 300 µm thickness coronal sections in a modified ACSF with the following composition in mM: 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 4.0 $MgCl_2$ and 250 sucrose, equilibrated with O_2 (95%) and CO_2 (5%). Slices were then incubated at 30–32°C for 30 min in regular ACSF and subsequently stored at room temperature in the same buffer up to 2.5 h prior to experiments.

Whole-cell patch-clamp recordings. Slices were transferred to a recording chamber, kept at 30-32°C, and were perfused with oxygenated regular ACSF at 1 ml/min. Neurons in the mPFC were visualized using two water-immersion objectives (HCX/APO L 10X/0.30 and 40X/0.80) with infrared differential interference contrast (DMLFS microscope, Leica, Wetzlar, Germany) connected with an infrared-sensitive camera. Patch pipettes were filled with an intracellular solution containing (in mM): 115 K-gluconate, 20 KCl, 2 EGTA, 10 HEPES, 2 Mg-ATP, and 0.3 Na_2-GTP, pH 7.25 adjusted with KOH. Tip resistance range of pipettes filled with such solution was 3-5 MΩ. Neuronal firing was evoked in a current-clamp configuration using an EPC7 amplifier (HEKA Electronik, Germany). Data were acquired at 2 kHz, filtered at 10 kHz using a 3-pole Bessel filter and digitized using CED 1401 Plus and Signal 1.9 software (Cambridge Electronic Design, UK). For each neuron, resting potential was set at -65 mV and depolarizing steps were injected from 0 to 200 pA, with 50 pA interval. Data were analyzed in pClamp 10 (Molecular devices, Sunnyvale, CA, USA).

In vivo microdialysis

Concentric dialysis probe, with a dialysis portion of 2.0 mm, were prepared as previously described[8, 41]. Mice were anesthetized with isoflurane and then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) for the probe implantation. The probe was implanted into the mPFC, according to the Paxinos and Franklin mouse brain atlas (AP: +1.9; ML: ±0.1; DV: -3.0 from Bregma). Microdialysis sessions started 24 hours after the surgical procedures. Probes were perfused with Ringer's solution (composition mM: 147 NaCl, 2.2 $CaCl_2$ and 4.0 KCl) at a constant flow rate of 1 µl/min. Collection of basal dialysate samples (20 µl) started 30 minutes after implantation. Dialysate samples were injected into a HPLC equipped with a reverse phase column (C8 3.5 µm, Waters, Mildford, MA, USA) and DA was quantified by a coulometric detector (ESA, Coulochem II, Bedford, MA). At the end of the experiment, mice were anesthetized with isoflurane and euthanized. Brains were removed and serial coronal sections of mPFC were cut with a vibratome to confirm the correct location of the probs. All measurements were performed blind to the genotype.

Statistical analysis

Data were analyzed using RStudio(v1.1.447; Boston, MA) or Graphpad Prism 7 (GraphPad Software, La Jolla, CA, USA) and subjected to one- or two-way analysis of variance (ANOVA) and two-way ANOVA with repeated measures when appropriate. For all data analysis, differences among individual means were assessed using Fisher Least Significant Difference (LSD) and Newman–Keuls *post hoc* tests as well as the Holm-Sidak method "multcomp" package (Bretz, Hothorn,Westfall, 2010). P values <0.05 were considered significant. The estimate of dispersion was shown as the standard error of the mean (s.e.m.), and variances were found to be similar between groups. All data were presented as mean ± s.e.m.

Results

Epistatic interaction between D3 and Dys functional genetic variations in cognitive functions in patients with schizophrenia.

We first questioned whether an epistatic interaction would be detectable in humans, in clinical behavioral readouts. Therefore, we analyzed data from 662 patients with schizophrenia gathered from the CATIE database, a clinical trial which monitored these patients from time 0 up to 18 months following assignment to an antipsychotic drug treatment[29]. In particular, we investigated the interaction between the D3 receptor Ser/Gly functional genetic variation, where the Ser genotype have been associated with decreased dopamine affinity[42], and the Dys rs1047631 functional genetic variation, where TT carries have decreased Dys expression[18].

We tested the effect of the interaction between these genotypes on cognitive functions known to be altered in schizophrenia (i.e. executive functions and working memory) and on clinical symptom rating scales (i.e. PANSS score), both at baseline and at the end of the study. No D3-by-Dys interaction was evident at baseline (Fig. 1A, 1C and Supplementary Table 1). In contrast, we observed a significant D3-by-Dys interaction in cognitive performances at month 18 (Fig. 1B, 1D and Supplementary Table 1). In particular, in both the WCST and WM performance, patients carrying the genetic variations increasing dysbindin-1 expression (C-carriers) and increasing D3 affinity to dopamine (Gly/Gly) had lower scores and did not show any cognitive improvements compared to baseline. No genetic interaction was evident in PANSS scores (Fig. 1E-J and Supplementary Table 2). No significant differences in age, sex and years of education were found between genotypes (Supplementary Table 3).

These results suggest an interaction between functional variations altering D3 and Dys expression in schizophrenia core cognitive deficits.

The epistatic interaction between D3 and Dys functional genetic variations has different molecular outcomes in the cortex compared to the striatum.

To selectively address the D3-Dys genetic interaction, we generated a new mouse line with concomitant hypofunction of both D3 and Dys genes (i.e. double heterozygous $D3^{+/-}$*$Dys^{+/-}$). This approach would circumvent possible confounding factors linked with human studies such as genetic heterogeneity, environment, pathological state and pharmacological treatments. In particular, reduced levels of both D3 and Dys in $D3^{+/-}$*$Dys^{+/-}$ mice should mimic the human condition of carriers of both the D3 Ser/Ser and Dys TT functional polymorphisms. We focused on the medial PFC (mPFC) and the striatum as main areas involved in the dopamine hypothesis of schizophrenia[3, 43].

We found increased levels of D3 mRNA expression in $Dys^{+/-}$ mice in both the mPFC (Fig. 2A) and striatum (Fig. 2B). These increased D3 levels were rescued in $D3^{+/-}$*$Dys^{+/-}$ double mutant mice down to wild-type levels (Fig. 2A-B). In contrast, Dys mRNA expression was decreased in both $D3^{+/+}Dys^{+/-}$ and $D3^{+/-}$*$Dys^{+/-}$ mice in the mPFC (Fig. 2C). However, in the striatum Dys expression was increased in $D3^{+/-}$ mice, and rescued in $D3^{+/-}$*$Dys^{+/-}$ double mutant mice (Fig. 2D). Alterations in Dys expression can change D2 recycling[16, 21]. Thus, we analyzed total and surface protein levels of D2-like receptors. Total level of D2-like receptors expression in both the mPFC and striatum were unchanged by either Dys or D3 genotypes *per se* or by their interaction (Fig. 2E-F), consistent with previous findings[8, 16]. Single D3 hypofunction did not altered D2-like receptor cell surface expression, while single Dys

hypofunction increased D2-like receptor expression on the cellular surface in both the mPFC and striatum (Fig. 2G-H), consistent with previous studies [8]. However, in the mPFC of $D3^{+/-}$*$Dys^{+/-}$ an even larger increment in cellular surface D2-like receptors was detected (Fig. 2G). In contrast, cellular surface D2-like receptor in the striatum were rescued in the $D3^{+/-}$*$Dys^{+/-}$ double heterozygous mice down to wild-type level (Fig. 2H). Overall, these results confirmed in mice a genetic interaction between functional variations in D3 and Dys. Moreover, these data indicate that the D3/Dys interaction might act in different manner in the PFC compared to the striatum.

Epistatic interaction between D3 and Dys in striatal-related locomotor activity, startle and PPI responses.

Based on the apparent dichotomy in the D3-by-Dys interaction at the molecular level, we assessed whether this could reflect distinct behavioral phenotypes related to striatal and/or PFC functioning. Striatal dopamine/D2-D3 pathways are implicated in the mediation of locomotor activity[21, 44, 45], and an altered stimulation of D2-like receptors in striatal regions is related to schizophrenia positive symptoms[43, 46].

Consistent with previous reports[21, 35], both Dys ($D3^{+/+}$*$Dys^{+/-}$) and D3 ($D3^{+/-}$*$Dys^{+/+}$) single heterozygous mice were more active than their wild-type littermates ($D3^{+/+}$*$Dys^{+/+}$) when tested in an open field arena. In contrast, $D3^{+/-}$*$Dys^{+/-}$ double heterozygous mice showed a wild-type-like behavior (Fig. 3A). Analysis of rearing behavior revealed no differences between the genotypes (Fig. 3B). Thus, concomitant reduction of D3 and Dys gene expression abolished the hyperactive phenotype produced by either genetic variation.

D2-like receptors within the striatum are also crucial for the expression of startle and

prepulse inhibition (PPI) of an acoustic startle in mice and humans[47-49]. Moreover, decreased PPI is found in patients with schizophrenia[50] as well as in mouse models relevant to schizophrenia[8, 51, 52]. In Dys single heterozygous ($D3^{+/+}$*$Dys^{+/-}$), startle reactivity was increased (Fig. 3C), consistent with previous findings[21]. Conversely, D3 single heterozygous mice ($D3^{+/-}$*$Dys^{+/+}$), in agreement with other studies[53], showed a lower reactivity to the startle stimulus, as compared to wild-type (Figure 3C). In contrast, the double heterozygous $D3^{+/-}$*$Dys^{+/-}$ showed a restored wild-type-like reactivity to the startle stimulus (Fig. 3C). The levels of basal activity in the apparatus, when no stimulus was presented, did not differ between genotypes (Figure 3C). Similarly to locomotor activity, these results show that concomitant partial disruption of D3 and Dys genes rescues the alterations in startle responses driven by each single mutation.

Consistent with evidence that startle and PPI are distinct behavioral responses[54], we found a distinct impact of D3/Dys genotypes in PPI measures compared to startle reactivity. In fact, in contrast to Dys single heterozygous ($D3^{+/+}$*$Dys^{+/-}$), but similarly to D3 single heterozygous ($D3^{+/-}$*$Dys^{+/+}$), $D3^{+/-}$*$Dys^{+/-}$ double heterozygous exhibited a PPI response higher than both WT and $D3^{+/+}$*$Dys^{+/-}$ (Fig. 3D). Overall, these results show that, consistent with D2 and D3 levels in the striatum (Fig. 1), concomitant D3 and Dys hypofunction rescued to WT levels locomotor and startle alterations caused by the single disruption of the D3 or Dys genes.

D3 hypo-function improves PFC-dependent working memory without changing social behavior.

We next moved to explore behavioral tasks more related to cortical activity. In particular, we used a well-validated working memory discrete paired-trial variable-delay non-match to

place T-maze task[21, 39] which relies on mPFC functioning[4, 39], and is sensitive to dopaminergic modulation[21, 39].

All genotypes displayed a delay-dependent performance, progressively increasing the number of errors with longer delays (Fig. 4A). As previously shown[19, 21], Dys single heterozygous ($D3^{+/+}$*$Dys^{+/-}$) exhibited a worse performance compared to WT ($D3^{+/+}$*$Dys^{+/+}$), at both the 4 and 30s intra-trial intervals (Fig. 4A). Conversely, the hypofunction of the D3 receptor gene rescued the Dys-dependent deficits, and improved the working memory performance of $D3^{+/-}$*$Dys^{+/-}$ double heterozygous over the level of wild-type mice (Fig. 4A). All genotypes required the same amount of time to learn the basic version of the task (Fig. 4B). Moreover, all genotypes equally learned to run quickly through the maze to retrieve the reward (Fig. 4C). Thus, the concomitant D3/Dys hypo-function not only rescued the working memory deficits related to Dys reduction, but improved working memory abilities in this mPFC-dependent task. Noteworthy, the concomitant D3/Dys hypo-function was not able to rescue Dys-dependent social behavioral deficits. Indeed, both $D3^{+/+}$*$Dys^{+/-}$ and $D3^{+/-}$*$Dys^{+/-}$ revealed social interaction deficits in an habituation/dishabituation social interaction test (Fig. 4D), while the single partial deletion of D3 did not affect social behaviors (Fig. 4D). Thus, we unraveled a major role of D3 hypo-function in PFC-dependent cognitive abilities with no effects on sociability/social novelty measures.

D3 hypo-functioning rescues Dys-dependent physiological alterations in the mPFC.

Human and mouse data both pointed to a D3-by-Dys genetic interaction in PFC-dependent cognitive functions. Thus, we investigated the physiological role of D3/Dys interaction in the mPFC.

Whole-cell recordings were performed on layer V in mPFC slices, because D3 are mainly

expressed in this cortical layer[26, 55]. The firing frequencies increased in parallel with the injected current for all genotypes ($D3^{+/+}$*$Dys^{+/+}$, $D3^{+/+}$*$Dys^{+/-}$, $D3^{+/-}$*$Dys^{+/+}$, $D3^{+/-}$*$Dys^{+/-}$). However, the increase in the current injection from 50 to 200 pA induced less spikes in $D3^{+/+}$*$Dys^{+/-}$ pyramidal neurons compared to wild-type mice (Fig. 5A-C). This difference was particularly marked at 1-s and 150 pA depolarization step (Figure 5C). This phenotype was partially ameliorated in the double $D3^{+/-}$*$Dys^{+/-}$ mice as no statistical difference compared to wild-type mice was evident in the spike frequency of neurons (Fig. 5A-C). This data indicate that D3 hypo-function ameliorated the disrupted excitability of layer V pyramidal neurons triggered by Dys reduction. Next, to investigate if these electrophysiological changes could be associated with an altered dopaminergic transmission, we performed an *in vivo* microdialysis assessment in the mPFC of freely-moving D3/Dys mutant mice (Fig. 5D-E). Basal extracellular dopamine levels in the mPFC were higher in single heterozygous Dys ($D3^{+/+}$*$Dys^{+/-}$) compared to wild-type ($D3^{+/+}$*$Dys^{+/+}$). In contrast, $D3^{+/-}$*$Dys^{+/-}$ double heterozygous showed restored, WT-like, dopamine levels. Taken together, these electrophysiological and neurochemical data show that D3 hypo-function can ameliorate Dys-dependent neuronal and dopaminergic basal abnormalities in the mPFC.

Discussion

This study shows an epistatic (gene-by-gene) interaction between D3 and Dys (DTNBP1) genes. In particular, Dys-dependent alterations in the intracellular trafficking of D2-like receptors interact with D3 receptors, with prominent effects in higher-order cognitive functions in both humans and mice.

The approach employed, whereby functional genetic variations relatively changed the expression of different genes simultaneously, allows to distinguish, phenotypes regulated by epistasis (gene-by-gene interaction) from phenotypes in which D3 and Dys exert independent or no effects. Moreover, the similar findings in humans and mice strength the conclusion that concomitant reduced D3 and Dys functionality brings to cognitive advantages in patients with schizophrenia. Indeed, cognitive deficits measured by the WCST and working memory tasks are described as core cognitive features of schizophrenia, and are related to dopamine signaling within the PFC[19, 31, 56-59]. Similarly, the working memory task used here for mice rely on medial PFC functioning and dopaminergic modulation[4, 21, 59]. We previously demonstrated that higher-order cognitive functions modulated by Dys depend on D2 receptor signaling within the PFC[18, 19, 21]. However, beside D2, D3 might also have high clinical relevance, because most currently prescribed antipsychotic drugs bind with similar affinity to D2 and D3 receptors[5, 23, 60]. Notably, the effects we revealed were more prominent in cognitive functions relevant to schizophrenia while general clinical assessment such as positive and negative PANSS scores in humans as well as social behavior in mice were not observed any D3-by-Dys interaction. This could be in agreement with studies suggesting that D3 blockade might enhance cognitive functions[25, 61], without inducing the D2-related side effects of antipsychotic drugs[5, 24]. Furthermore, in line with previous findings[28], we also found that D3 genetic hypofunction increased PPI scores, a sensorimotor gating ability which is usually impaired in

patients with schizophrenia[49]. Thus, in a clinical perspective, our current findings suggest that the cognitive beneficial effects of D3 blockade should be considered along with epistatic interaction with the Dys gene.

The molecular data we generated reinforce the meaning of the D3/Dys genetic interaction. In particular, altered D3 expression by either D3 or Dys single genetic variations was restored to wild-type-level in both the mPFC and striatum of $D3^{+/-}$*$Dys^{+/-}$ double mutant mice. This is consistent with a recent *in vitro* study, showing that Dys might also influence the expression of D3 receptors[62]. In contrast, the D3/Dys genetic interaction rescued Dys expression to wild-type-level in the striatum, but not in the PFC. Supporting this area-specific effect, we found that D2 receptors trafficking was rescued in $D3^{+/-}$*$Dys^{+/-}$ double mutant mice to wild-type-level in the striatum, but not in the PFC (Fig. 1). Indeed, Dys expression levels are strictly linked to D2-like receptor trafficking[16, 18, 21]. This D3/Dys epistatic normalization of striatal D2-like receptors signaling was further corroborated by the normalized locomotor activity and startle reactivity (Fig. 3). In contrast with striatum, in the PFC, the D3-Dys interaction produced a D2/D3 unbalance in favor of increased D2 neuronal surface levels (Fig. 2), with normalized basal extracellular dopamine levels (Fig. 5E). A potentiation of D2 signaling in PFC in the context of normalized dopamine levels improves higher-order cognitive functions[18]. Thus, these findings are in line with the improved working memory performances driven by D3 hypo-functioning in the context of reduced Dys.

The differential effect of the D3/Dys interaction on the D2/D3 relative balance in the striatum versus the PFC suggests a peculiar region-specific effect which will require further investigations. However, in contrast to striatum, which contains only two principle classes of medium spiny neuron co-expressing D2 and D3, in the PFC D3 receptors are expressed in a distinct subclass of L5 pyramidal cells compared to D1 and D2 expressing cells[26]. Moreover,

while mPFC L5 D2-expressing neurons principally project subcortically[63, 64], L5 D3 positive pyramidal neurons are a cortically projecting neuronal subtype[26]. Finally, D2 expressing neurons are relatively more present in layers 2/3 while D3 positive neurons in layer 5[26]. Thus, the reduced excitability of layer 5 pyramidal neurons which we found in Dys hypo-functioning mice (Fig. 5) might be related to their increased D3 basal signaling (Fig. 2). In agreement, the D3 hypo-function in $Dys^{+/-}$ mice ameliorated their altered excitability. Postmortem studies revealed a 2-fold increase of D3 receptors in brains of long-term hospitalized drug-free patients with schizophrenia[65] while patients with early psychosis display augmented levels of D3 receptors mRNA in T lymphocytes[66]. Thus, based on the present data, it is tempting to suggest that schizophrenia-related phenotypes might be associated with a genetic background relatively increasing D3 function. Therefore, a selective blockade of D3 receptors might shift the D2/D3 balance in favor of increased D2, ultimately improving cognitive performances.

In conclusion, the present study support the D3 receptors as a valid target for improving psychiatric-related higher-order cognitive deficits. Furthermore, these new epistatic interaction might provide additional tools towards a better stratification of patients with schizophrenia which will be required if a more personalized therapeutic approach will be applied.

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Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. **Genetic variations concomitantly and relatively increasing both D3 affinity and Dys expression are associated with neurocognitive disadvantages in patients with schizophrenia.** Wisconsin Card Sorting Test (WCST) performance across genotypes (**A**) at month 0 and (**B**) at month 18. Two-way ANOVA analysis of performance revealed a significant effect of genotype on WCST score after 18 months of treatment with antipsychotic drugs. [$F_{(2,387)}$=3.14, p=0.04]. Post hoc: *P<0.05, ***P<0.001 versus Gly/Gly & C-carriers subjects. Working Memory composite score (WM) across genotypes (**C**) at month 0 and (**D**) at month 18. Two-way ANOVA analysis of performance showed a tendency in genotype effect on WM score after 18 months of treatment with antipsychotic drugs [$F_{(2,408)}$=2.52, p=0.08]. Post hoc: *P<0.05, **P<0.01 versus Gly/Gly & C-carriers subjects. There was no significant genotype effect on PANSS scores both at beginning and at the end of the study (positive scale, **E**-**F**; negative scale. **G**-**H**; total score, **I**-**J**). Values represent mean±s.e.m.

Figure 2. The D3/Dys epistatic interaction normalized single genes molecular changes in the striatum while generating a D2/D3 unbalance in the prefrontal cortex (PFC). Abundance of (**A**, **B**) D3 and (**C**, **D**) Dys mRNA in the mPFC and striatum of $D3^{+/+}$*$Dys^{+/+}$, $D3^{+/-}$ *$Dys^{+/+}$, $D3^{+/+}$*$Dys^{+/-}$ and $D3^{+/-}$*$Dys^{+/-}$ littermates measured by quantitative RT-PCR. Mean fold changes are expressed relative to transcript levels of controls ($D3^{+/+}$*$Dys^{+/+}$). One-way analyses of variance (ANOVAs) revealed a genotype effect for D3 expression in the mPFC [$F_{(3,20)}$=16.8; P<0.001] and striatum [$F_{(3,18)}$=20.76; P<0.001] and a genotype effect for Dys in the mPFC [$F_{(3,36)}$=6.95; P<0.001] and in the striatum [$F_{(3,36)}$=25.02; P<0.001). Western blot and densitometric analysis of (**E**, **F**) total and (**G**, **H**) surface D2-like receptors (52kDa) in

the mPFC and striatum of $D3^{+/+}$*$Dys^{+/+}$, $D3^{+/-}$*$Dys^{+/+}$, $D3^{+/+}$*$Dys^{+/-}$ and $D3^{+/-}$*$Dys^{+/-}$ littermates. The results presented are normalized to Transferrin receptor proteins (95kDa) and on $D3^{+/+}$*$Dys^{+/+}$ control group average. Synaptophysin (39kDa) has been used as cytosolic control. One-way ANOVAs revealed no genotype effect for the total levels of D2 receptors in both the mPFC [$F_{(3,\ 21)}$=0.0753, P=0.972] and striatum [$F_{(3,\ 25)}$=1.963, P=0.145]. One-way ANOVAs revealed a genotype effect for surface D2 receptors expression in both the mPFC [$F_{(3,\ 23)}$=5.382, P=0.0059] and striatum [$F_{(3,\ 25)}$=4.4296, P=0.0125]. Post hoc: ***P<0.001, **P<0.01, *P<0.05 vs $D3^{+/+}$*$Dys^{+/+}$. ††P<0.01, †P<0.05 vs $D3^{+/+}Dys^{+/-}$. Each histogram depict the mean (s.e.m.) from 5/8 different samples.

Figure 3. **The partial deletion of the D3 gene rescues the increased hyperlocomotion and acoustic startle response of Dys mutant mice**. $D3^{+/+}$*$Dys^{+/+}$ (*n*=14), $D3^{+/-}$*$Dys^{+/+}$ (*n*=10), $D3^{+/+}$*$Dys^{+/-}$ (*n*=15) and $D3^{+/-}$*$Dys^{+/-}$ (*n*=13) were tested in an open field arena for 30 minutes. Repeated measures ANOVAs revealed a genotype [$F_{(3,\ 48)}$=3.374; P=0.0258] and time effects [$F_{(5,\ 240)}$=6.026; P<0.001] on locomotor activity (crossings, **A**) and but not genotype effects [$F_{(3,\ 48)}$=1.742; P=0.171] on rearing behavior (**B**). (**C**) Animal movement displayed by $D3^{+/+}$*$Dys^{+/+}$ (*n*=24), $D3^{+/-}$*$Dys^{+/+}$ (*n*=23), $D3^{+/+}$*$Dys^{+/-}$ (*n*=17) and $D3^{+/-}$*$Dys^{+/-}$ (*n*=13) littermates during no stimulus trials or following the presentation of a 120-dB stimulus (Startle). Two-way ANOVAs revealed a stimulus x genotype interaction [$F_{(3,\ 146)}$=11.02, P<0.001], a stimulus effect [$F_{(1,\ 146)}$ =197.2, P<0.001] and a genotype effect [$F_{(3,\ 146)}$=10.73; P<0.001). (**D**) Percent PPI of the acoustic startle response displayed by the same mice after the presentation of 70, 75, 80 and 85 prepulse sound stimuli. Repeated measures ANOVAs revealed a stimulus x genotype interaction [$F_{(9,\ 219)}$=2.034, P=0.0369], a stimulus effect [$F_{(3,}$

$_{219)}$=93.52, P<0.001] and a genotype effect [$F_{(3, 73)}$=2.893; P=0.0410]. Post hoc: ***P<0.001, **P<0.01, *P<0.05 vs $D3^{+/+}$*$Dys^{+/+}$ and †††P<0.001, ††P<0.01, †P<0.05 *vs* $D3^{+/+}$*$Dys^{+/-}$. Values represent mean ± s.e.m.

Figure 4. The partial deletion of the D3 gene rescues the working memory deficits of Dys mutant mice in a PFC-dependent working memory task, but not the social interaction deficit. (**A**) Percentage of correct choices displayed by $D3^{+/+}$*$Dys^{+/+}$ (*n*=12), $D3^{+/-}$*$Dys^{+/+}$ (*n*=9), $D3^{+/+}$*$Dys^{+/-}$ (*n*=10) and $D3^{+/-}$*$Dys^{+/-}$(*n*=9) mice during the discrete paired-trial variable-delay T-maze test with different intra-trial delays randomly presented (4, 30, 60 and 240 s) and an inter-trial delay of 20 s. The 50% value corresponds to chance levels of correct choices. Repeated measures ANOVAs revealed a genotype [$F_{(3, 36)}$=8.351, P=0.0002] and a delay effect [$F_{(3, 108)}$ = 50.35, P < 0.0001]. (**B**) Days needed to reach criterion and (**C**) latency to retrieve the hidden food pellet during the discrete paired-trial T-maze task. There was no genotype difference in the acquisition of the task [One-way ANOVAs, genotype effect: $F_{(3, 36)}$=1.152; P=0.3413] and in retrieving the food [Two-way ANOVAs, genotype effect $F_{(3, 36)}$=2.542; P=0.0716 , and day effect: $F_{(1, 36)}$=143.3; P<0.0001]. (**D**) show the quantity of time spent by $D3^{+/+}$*$Dys^{+/+}$ (*n*=15), $D3^{+/-}$*$Dys^{+/+}$ (*n*=16), $D3^{+/+}$*$Dys^{+/-}$ (*n*=11) and $D3^{+/-}$*$Dys^{+/-}$ (*n*=9) in investigating the same unfamiliar male mouse during each of four successive 1-min trials. A fifth 'dishabituation' trial depicts the social investigation activity of the subject mice to the presentation of a new unfamiliar male. Repeated measures ANOVAs revealed a genotype effect [$F_{(3, 47)}$=3.793; P=0.0162], and a trial effect [$F_{(4, 188)}$ = 25.71; P<0.0001]. Post hoc: ***P<0.001, **P<0.01, *P<0.05 vs $D3^{+/+}$*$Dys^{+/+}$ and ††P<0.01, †P<0.05 vs $D3^{+/+}Dys^{+/-}$. Values represent mean ± s.e.m.

Figure 5. **The partial deletion of D3 gene rescues the decreased excitability of pyramidal neurons as well as the increased DA levels in mPFC of heterozygous Dys mutant mice.** Representative traces **(A)** of neuronal firing recorded in mice with different genotype. Pyramidal neurons of mPFC layer V were selected. Spikes were evoked in current-clamp configuration during depolarizing steps from 0 to 200 pA with 50 pA interval. Traces obtained during 1s depolarizing step at 150 pA are shown. Summary of spike frequency (**B)** obtained in pyramidal neurons at different interval of depolarizing steps from mice with different genotype: $D3^{+/+}$*$Dys^{+/+}$ (*n*=10); $D3^{+/-}$*$Dys^{+/+}$ (*n*=10); $D3^{+/+}$*$Dys^{+/-}$ (*n*=13); $D3^{+/-}$*$Dys^{+/-}$ (*n*=11). Bar diagram showing spike frequency (**C)** observed in different genotype mice at 150 pA depolarizing step to highlight differences. Repeated measures ANOVAs revealed an intensity x genotype interaction [$F_{(9, 120)}$=3.254, P=0.0014], an intensity effect [$F_{(3, 120)}$=397.7, P<0.0001] as well as a genotype effect [$F_{(3, 40)}$=3.833, P=0.0167]. (**D**) Localization of probe dialyzing portion within the mPFC, number represent the antero-posterior position of the slice in mm, relative to bregma. (**E**) Basal extracellular DA levels in the mPFC of $D3^{+/+}$*$Dys^{+/+}$ (*n*=8); $D3^{+/-}$*$Dys^{+/+}$ (*n*=4); $D3^{+/+}$*$Dys^{+/-}$ (*n*=7); $D3^{+/-}$*$Dys^{+/-}$ (*n*=6). One-way ANOVAs revealed a genotype effect [$F_{(3, 21)}$=3.997, P=0.0213]. Post hoc: *P<0.05 vs $D3^{+/+}Dys^{+/+}$ and †P<0.05 vs $D3^{+/+}Dys^{+/-}$. Values represent mean ± s.e.m.

Figure 1

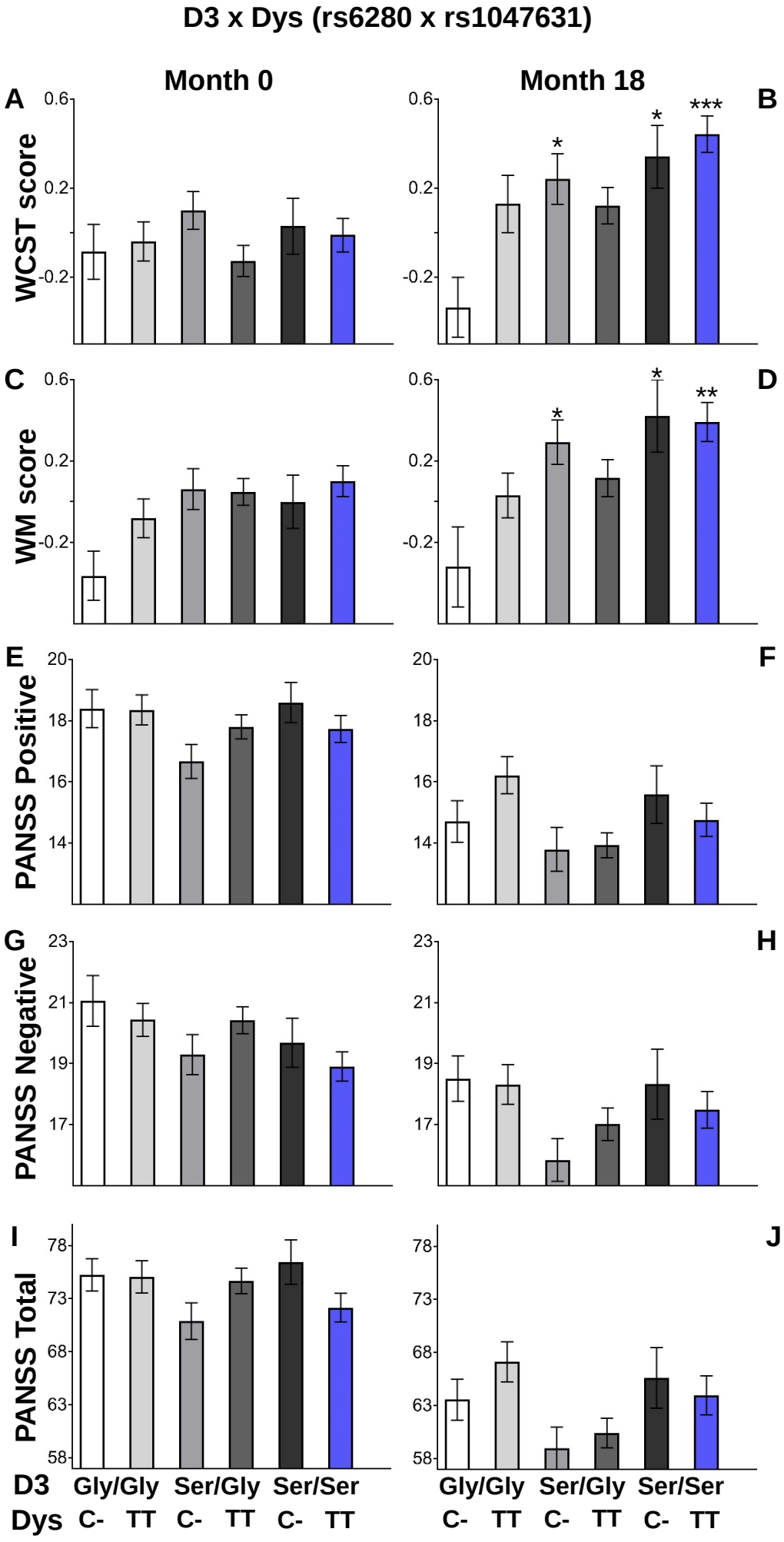

D3 x Dys (rs6280 x rs1047631)
Month 0
Month 18
A
B
C
D
E
F
G
H
I
J
WCST score
WM score
PANSS Positive
PANSS Negative
PANSS Total
0.6
0.2
-0.2
20
18
16
14
23
21
19
17
78
73
68
63
58
*
**
***
D3
Dys
Gly/Gly
C-
TT
Ser/Gly
Ser/Ser

Fig 2

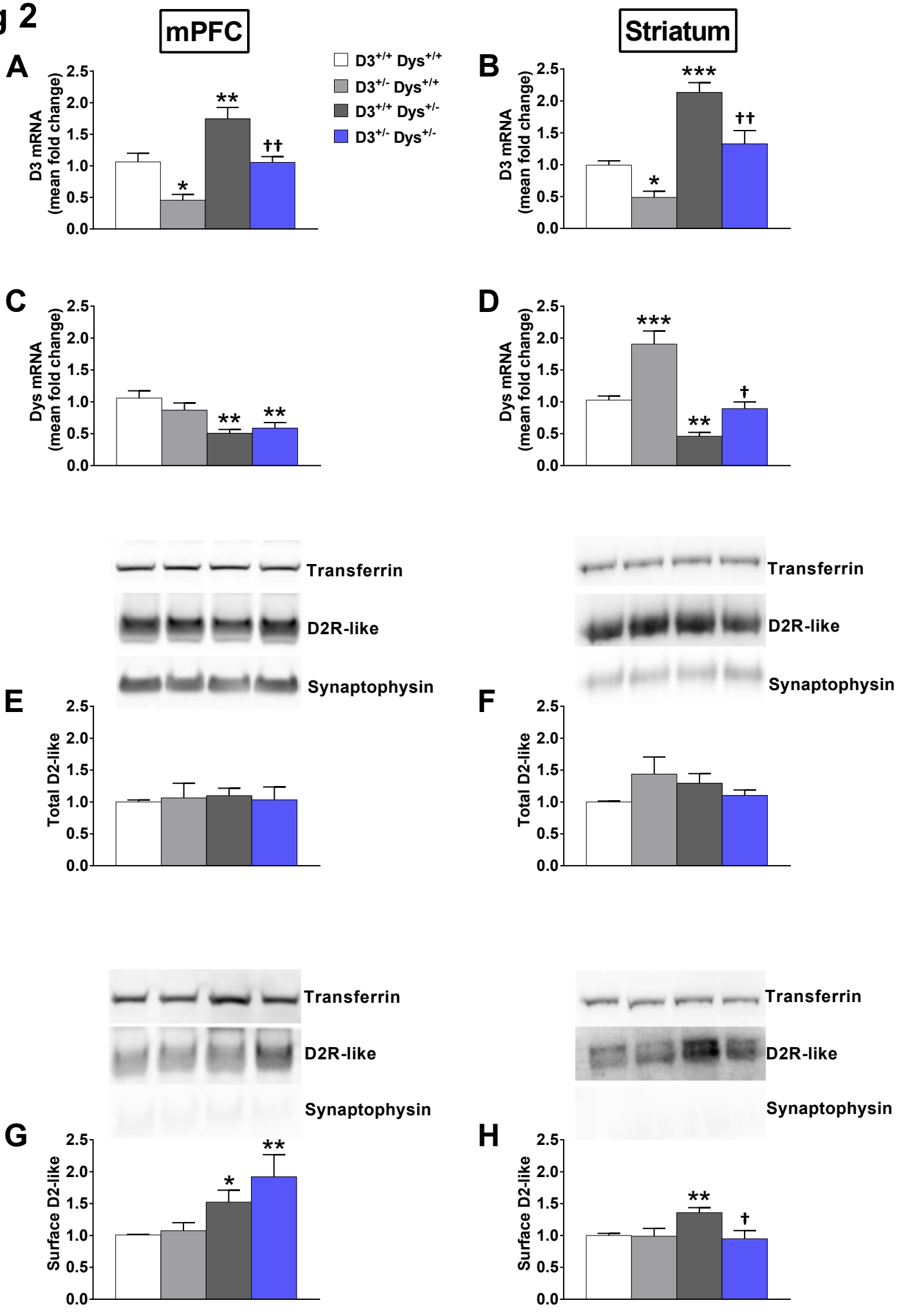

mPFC
Striatum
A
B
C
D
E
F
G
H
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
D3 mRNA (mean fold change)
Dys mRNA (mean fold change)
Total D2-like
Surface D2-like
Transferrin
D2R-like
Synaptophysin
0.0
0.5
1.0
1.5
2.0
2.5
*
**
***
†
††

Fig 3

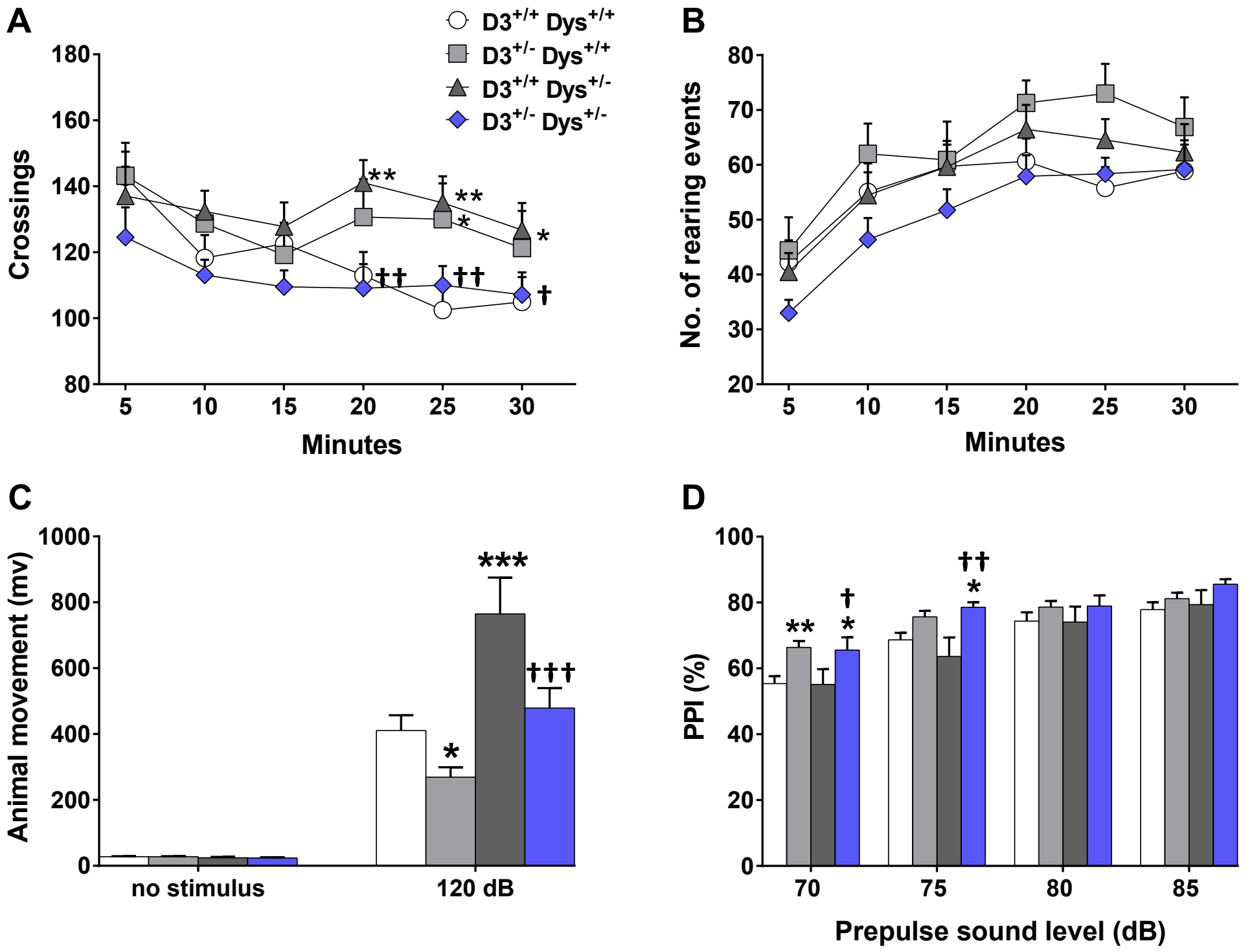

A
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
Crossings
Minutes
B
No. of rearing events
Minutes
C
Animal movement (mv)
no stimulus
120 dB
D
PPI (%)
Prepulse sound level (dB)

Fig 4

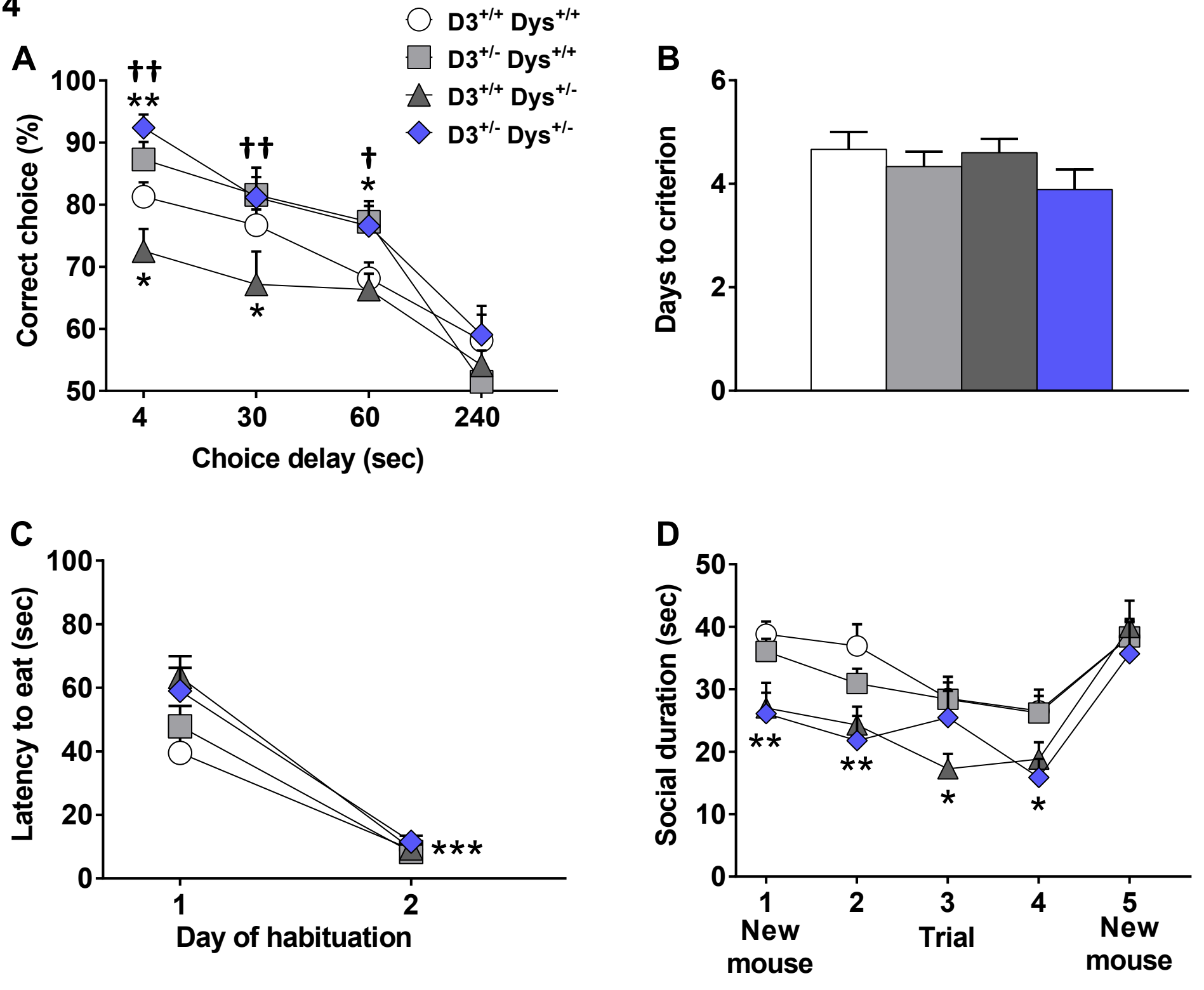
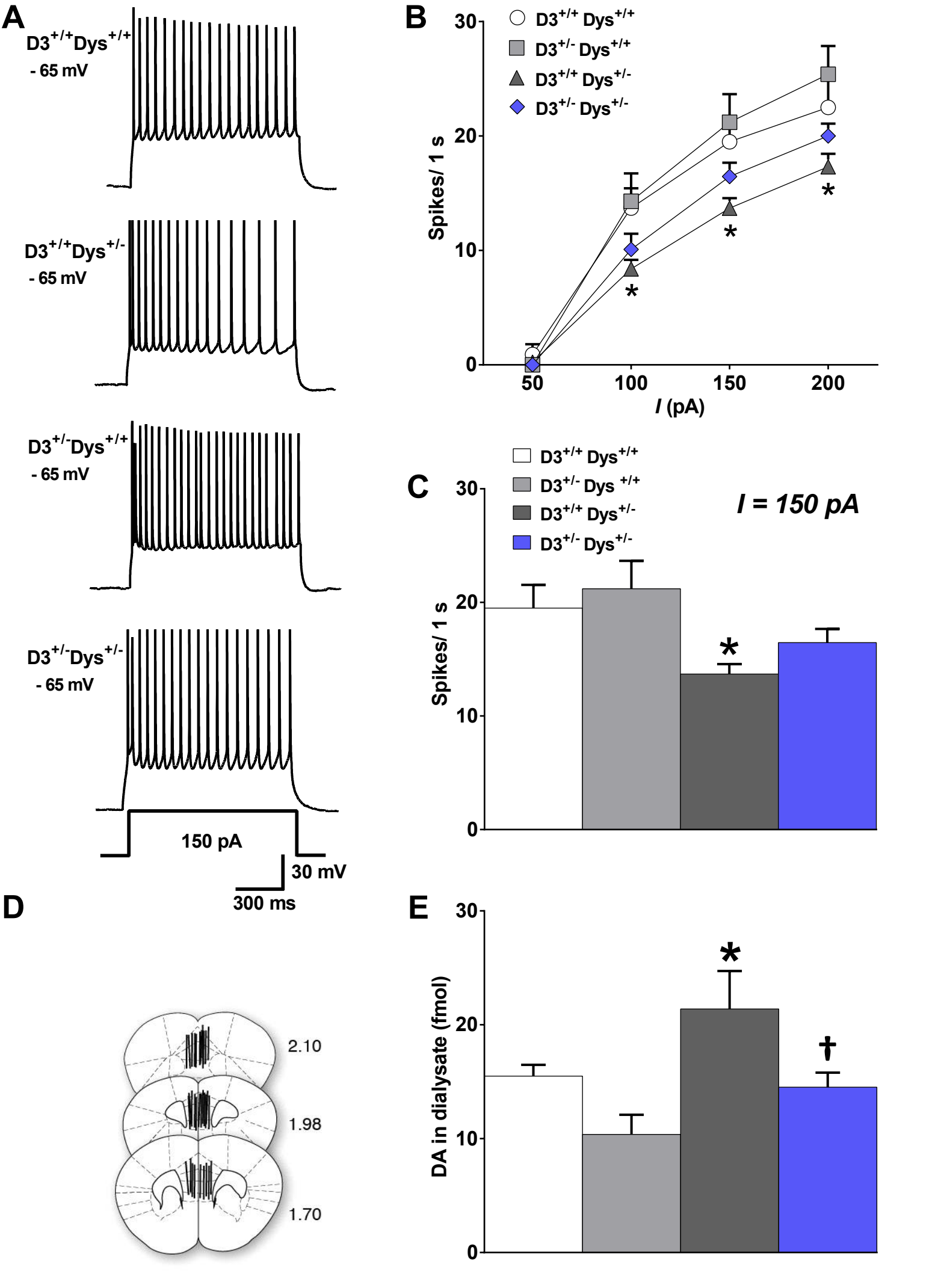

A
B
C
D
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
Correct choice (%)
Choice delay (sec)
4
30
60
240
Days to criterion
Latency to eat (sec)
Day of habituation
Social duration (sec)
New mouse
Trial
New mouse

Fig 5


A
D3+/+Dys+/+
- 65 mV
D3+/+Dys+/-
- 65 mV
D3+/-Dys+/+
- 65 mV
D3+/-Dys+/-
- 65 mV
150 pA
30 mV
300 ms
B
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
Spikes/ 1 s
I (pA)
C
D3+/+ Dys+/+
D3+/- Dys +/+
D3+/+ Dys+/-
D3+/- Dys+/-
I = 150 pA
Spikes/ 1 s
D
2.10
1.98
1.70
E
DA in dialysate (fmol)