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

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Abstract
The dopamine D2 and D3 receptors are implicated in schizophrenia and its pharmacological treatments. These receptors undergo intracellular trafficking processes that are modulated by dysbindin-1 (Dys). Indeed, Dys variants alter cognitive responses to antipsychotic drugs through D2-mediated mechanisms. However, the mechanism by which Dys might selectively interfere with the D3 receptor subtype is unknown. Here, we revealed an interaction between functional genetic variants altering Dys and D3. Specifically, both in patients with schizophrenia and in genetically modified mice, concomitant reduction in D3 and Dys functionality was associated with improved executive and working memory abilities. This D3/Dys interaction produced a D2/D3 imbalance favoring increased D2 signaling in the prefrontal cortex (PFC) but not in the striatum. No epistatic effects on the clinical positive and negative syndrome scale (PANSS) scores were evident, while only marginal effects on sensorimotor gating, locomotor functions, and social behavior were observed in mice. This genetic interaction between D3 and Dys suggests the D2/D3 imbalance in the PFC as a target for patient stratification and procognitive treatments in schizophrenia.

Introduction
Dopaminergic receptors have important implications in several psychiatric and neurodevelopmental disorders [1]. Particularly for schizophrenia, converging physiological, anatomical, genetic, and pharmacological evidence strongly imply the importance of D2-like receptors [2–5]. In contrast to D1-like receptors (D1 and D5), members of the D2 receptor family (D2, D3, and D4) are quickly internalized after agonist stimulation and eventually degraded through the intracellular lysosomal pathway [6, 7]. Intracellular trafficking processes might be altered in schizophrenia [8–10] and are implicated in antipsychotic drug modes of action [11–15].

The dysbindin-1 (Dys) protein, encoded by the dystrofribin-binding protein 1 gene (DTNBP1), is part of the biogenesis of lysosome-related organelles complex 1 and is implicated in intracellular trafficking processes [16, 17]. In particular, genetic disruption of Dys alters the intracellular trafficking of D2-like but not D1 receptors, resulting in increased expression of D2 receptors on the neuronal surface [16, 18]. Consistent with this observation,
in both mice and humans, genetic variations in Dys affect cognition- and schizophrenia-relevant behavioral phenotypes through dopamine/D2-like mechanisms [19–22]. Furthermore, in both mice and humans, genetic variations in Dys alter cognitive responses to antipsychotic drugs through D2-mediated mechanisms [18]. However, the mechanism by which Dys-dependent modulation of D2-like receptor intracellular trafficking might selectively interact with D3 signaling is unknown.

Dopamine D2 and D3 receptors show high structural homology [23], and currently available pharmacological tools, as well as antipsychotic drugs have high affinity for both of these receptors [5, 24–26]. Thus, the unique contribution of each of these receptors to physiological and behavioral functions cannot be fully distinguished. This limitation is important to address, as recent electrophysiological and morphological analyses have identified distinct neuronal populations expressing either D2 or D3 receptors [27]. Furthermore, D2 and D3 receptors are suggested to differentially control mood and cognitive processes [25, 26] and might be implicated differently in psychiatric disorders and their pharmacological treatments [28, 29].

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) study. Subsequently, by establishing a mouse line with concomitant selective hypofunction of both the D3 and Dys genes (i.e., double heterozygous D3+/− × Dys+/− mice), we confirmed functional interaction between D3 and Dys in schizophrenia-relevant phenotypes, as well as in neuronal excitability, extracellular dopamine levels, and responses to antipsychotic drugs. Our data support the hypothesis that D3 might be a pharmacological target for procognitive drug treatments, as well as a genetic tool for patient stratification toward more personalized treatments in schizophrenia.

Materials and methods

Human subjects

Patients were enrolled in the CATIE study through the NIMH Center for Collaborative Research and Genomics Resource [30, 31]. Analysis was carried out on samples from 662 patients with schizophrenia clinically assessed at baseline and with an 18-month follow-up for which cognitive and genetic data were available. Demographic and clinical details included age, sex, age of illness onset, illness duration, and medical (including alcohol and drug use), admission and medication histories. From the CATIE study, we selected the cognitive performance on the Wisconsin Card Sorting Test (WCST), a measurement widely used to assess executive function deficits associated with prefrontal cortex (PFC) function in patients with schizophrenia [32–34], and a composite measure of working memory (WM) based on the letter-number span test and a computerized test of visuospatial WM [31]. For details, see the Supplementary Information.

Mice

We established a novel mouse line first by breeding D3−/− [35] mice with Dys−/− [22] mice to obtain double D3 and Dys heterozygous (D3+/− × Dys+/−) mice. Both lines were on a C57BL/6J genetic background, which is commonly used to facilitate interlaboratory comparisons. Consistent with the idea that heterozygous mice might mimic human functional genetic variations better than full knockout mice [18, 36, 37] and to avoid uncontrollable gene–environment interactions stemming from possible alterations in maternal behavior, we followed a breeding scheme consisting of mating one male D3+/− × Dys+/− mouse with two C57BL6/J female mice. This approach allowed us to evaluate, in the generated littermates, the lifelong effects of genetic variations resulting in normal levels of both D3 and Dys (D3+/+ × Dys+/+), selective D3 hypofunction (D3+/− × Dys+/+ single heterozygous mice), Dys hypofunction (D3+/+ × Dys−/− single heterozygous mice), and decreased levels of both D3 and Dys in the same individual (D3+/− × Dys−/− mice). Only 3- to 6-month-old male littermates were tested to directly compare the results with our relevant previous study [18]. For details, see the Supplementary Information.

Drugs and treatments

Risperidone (Sigma, Dorset, UK), clozapine and b-lonanserin (Sigma–Aldrich, St. Louis, MO, United States) were dissolved in 20 μl of acetic acid and further brought up to volume with physiological saline (0.9% NaCl); the pH was adjusted to 6 with 0.1 M NaOH. All drug solutions were prepared daily and administered intraperitoneally (i.p.) in an injection volume of 10 ml/kg. For details, see the Supplementary Information.

Behavioral tasks

Temporal order recognition (TOR) test

This test was carried out as previously described [8, 38].

Discrete paired-trial variable-delay T-maze task

In this test [22, 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 (nonmatch to sample).

**Acoustic startle response and prepulse inhibition (PPI) test**

Before 2 h of the test, animals were acclimatized to the testing room. The acoustic startle response and PPI were measured using an SR-Lab System apparatus (San Diego Instruments, San Diego, CA, USA). The procedure was performed as previously described [8, 37].

**Open field test**

Animals were tested in an evenly illuminated (9 ± 1 lux) square open field, 40 × 40 × 40 cm, divided into 16 quadrants by lines on the floor (Ugo Basile, Gemonio, Italy) over a 30-min period. Locomotor activity and rearing behavior were assessed during the first exposure to the empty open field arena.

**Habituation/dishabituation social interaction test**

Animals were tested as previously described [40] in slightly illuminated (5 ± 1 lux) 2150E Tecniplast cages (35.5 × 23.5 × 19 cm), and the test was video recorded using a video camera (Sony Videocam PJ330E).

For detailed information on the behavioral testing, see the Supplementary Information.

**RNA isolation and real-time PCR**

Total RNA was extracted from isolated brain areas [medial prefrontal cortex (mPFC) and striatum]. For details, see the Supplementary Information.

**Slices surface biotinylation**

Mice were anesthetized with isoflurane and were then decapitated. The brain was sectioned in cold carboxygenated Hanks’ balanced salt solution (HBSS, Invitrogen Life Technologies) enriched with 4 mM MgCl₂, 0.7 mM CaCl₂, and 10 mM d-glucose and equilibrated with 95% O₂ and 5% CO₂; pH 7.4.) on a vibratome at a thickness of 300 μm. The mPFC was dissected from coronal slices. For details, see the Supplementary Information.

**Electrophysiology**

**Slice preparation**

mPFC slices were prepared from mice of postnatal day (PND) 13 to PND 22.

Whole-cell patch-clamp recordings

Slices were transferred to a recording chamber, maintained at 30–32 °C, and perfused with oxygenated regular artificial cerebrospinal fluid (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. For details, see the Supplementary Information.

**In vivo microdialysis**

A concentric dialysis probe with a dialyzing portion of 2.0 mm was prepared as previously described [8, 41]. Mice were anesthetized with isoflurane and were then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) for 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 the bregma). For details, see the Supplementary Information.

**Statistical analysis**

Data were analyzed using RStudio (v1.1.447, Boston, MA) or GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). For details, see the Supplementary Information.

**Results**

Epistatic interaction between D3 and Dys functional genetic variants affects cognitive functions in patients with schizophrenia

We first investigated whether an epistatic interaction would be detectable in humans in clinical behavioral readouts. We analyzed data from 662 patients with schizophrenia extracted from the CATIE database, a data repository for a clinical trial that monitored these patients following assignment to an antipsychotic drug treatment [30]. Specifically, we investigated the interaction between the D3 receptor Ser/Gly rs6280 and the Dys rs1047631 functional genetic variants at the first (Month 0), after 6 months, and at last assessment (Month 18) of this clinical trial. Considering the drop out of patients between the different time points of assessments and some missing genetic data [18, 31, 42], we performed longitudinal analyses including those patients for which all cognitive and genetic data were available in all three assessment time points considered. Several consistent lines of evidence reported that the D3 haplotype rs6280 with the Ser allele is associated with a lower affinity for...
dopamine than the Gly allele [43] and that Dys rs1047631 TT carriers have decreased Dys expression [18] (Supplementary Fig. 1).

We examined the effect of the interaction between these genotypes on cognitive functions known to be altered in schizophrenia (i.e., executive functions and WM) and for which we could have equivalent tasks in mouse models [8, 18, 20, 44, 45]. Moreover, we assessed possible genotype-dependent effects on clinical symptom rating scales (i.e., the positive and negative syndrome scale (PANSS) score), both at baseline and at the end of the study.

A genotype-by-time of assessment effect was evident for the WCST score (Fig. 1a, and Supplementary Tables 1). Specifically, patients carrying genetic variants increasing Dys expression (C-carriers) and increasing D3 affinity for dopamine (Gly/Gly) had lower WCST scores than patients without these variants after 18th months follow-up and did show a cognitive deterioration with time (Fig. 1a, and Supplementary Tables 1). In contrast, there was a significant improvement in WCST score in TT-Ser/Gly, C-carriers Ser/Ser, and TT-Ser/Ser patients (Fig. 1a, and Supplementary Tables 1). For the WM scores we observed a main effect of genotype and time, but no genotype-by-time interaction (Fig. 1b, and Supplementary Tables 1). Specifically, patients carrying genetic variants increasing Dys expression (C-carriers) and increasing D3 affinity for dopamine (Gly/Gly) had lower WM scores than all other patients without these variants (Fig. 1b, and Supplementary Tables 1).

No genotype-by-time interaction was evident in the positive and total PANSS scores (Fig. 1c, e, and Supplementary Tables 1). For the PANSS negative scores, a significant genotype-by-time interaction showed an improvement with time in all genotypes, but no differences between genotypes within each single time of assessment (Fig. 1d, and Supplementary Tables 1). No significant differences in age, sex, or years of education were found between genotypes (Supplementary Tables 2). These results suggest an interaction between functional variants altering D3 and Dys expression that affects core cognitive deficits in schizophrenia.

**Epistatic interaction between D3 and Dys functional genetic variants affects cognitive functions in genetically modified mice with or without treatment with antipsychotics**

To selectively address the D3–Dys genetic interaction, we established a new mouse line with concomitant hypofunction of both the D3 and Dys genes (i.e., double heterozygous D3+/− × Dys+/− mice). This approach circumvented possible confounding factors linked with human studies, such as genetic heterogeneity, environmental effects, and pathological state. Specifically, reduced levels of both D3 and Dys in D3+/− × Dys+/− mice should approximate the human genetic condition of carriers of both the D3 Ser/Ser and Dys TT functional polymorphisms.

The cognitive deficits we found in the WCST and WM tasks in human patients (Fig. 1) are usually linked to dysfunctional dopaminergic signaling within the PFC [19, 31, 46–49]. Moreover, all patients with schizophrenia were under treatments with antipsychotic drugs. Thus, we first tested wild-type (D3+/− × Dys+/−), single (D3+/− × Dys+/+ and D3+/− × Dys+/−), and double mutant (D3+/− × Dys+/−) littermates in a TOR test that is sensitive to dopaminergic alterations within the mPFC [8], following no manipulations, or chronic treatments with vehicle or different antipsychotic drugs (Fig. 2a). In particular, we treated mice with risperidone (as one of the most commonly used antipsychotic [18]), clozapine (as the antipsychotic with a more different pharmacological profile and possibly higher therapeutic efficacy [5]), and blonanserin (for its antagonistic activity on D3 [50]).

Chronic treatment with all drugs rescued the TOR memory impairment seen in drug-naïve D3+/− × Dys+/− mice (Fig. 2b, c). However, only risperidone and blonanserin improved the TOR performance of all mutant mice compared to vehicle- and drug-treated D3+/− × Dys+/− mice (Fig. 2b, c). Whereas no difference in the total amount of object exploration during the test phase was found between genotypes (Fig. 2d, e), D3+/− × Dys+/− naïve mice showed a decrease of the total exploration of the objects only during the sample phase 1 (Supplementary Fig. 2a–d). Considering that risperidone was the only drug in common between the human (Fig. 1) and mouse data (Fig. 2), the cognitive performance of risperidone-treated mice was remarkably similar to that shown by patients with schizophrenia when stratified by D3 and Dys functional genetic variants (Fig. 1b, d vs Fig. 2c).

**D3 hypofunction improves PFC-dependent working memory**

We next aimed to further explore the consequences of the revealed D3-by-Dys genetic interaction in a more selective and demanding WM-discrete paired-trial variable-delay T-maze task [22, 39], which also relies on mPFC functioning [4, 39] and is sensitive to dopaminergic modulation [22, 39].

All genotypes displayed delay-dependent performance, with a progressive increase in the number of errors with longer delays (Fig. 3a). As previously shown [20, 22], Dys single heterozygous (D3+/− × Dys+/−) mice performed worse than wild-type (D3+/+ × Dys+/+) mice at both the 4 and 30 s intra-trial intervals (Fig. 3a). Conversely, hypofunction of the D3 receptor gene rescued the Dys-dependent deficits and improved the WM performance of D3+/− × Dys+/− double heterozygous mice over that of wild-type mice (Fig. 3a). mice of all genotypes required the same amount of time to learn the
basic version of the task (Fig. 3b). Moreover, mice of all genotypes learned equally to run quickly through the maze to retrieve the reward (Fig. 3c). Thus, concomitant D3/Dys hypofunction not only rescued the WM deficits related to Dys hypofunction but also improved working memory abilities on this mPFC-dependent task.

Marginal effects of D3–Dys genetic interactions in social behavior, locomotor activity, startle and PPI responses

Because D3-by-Dys effects in humans were most evident in cognitive abilities rather than other behavioral alterations (i.e., PANSS scores, Fig. 1), we next tested D3–Dys mutant mice in other behavioral processes that might be relevant for schizophrenia-like behavioral alterations.

Concomitant D3/Dys hypofunction did not rescue Dys-dependent social behavioral deficits. Indeed, both D3\(^{+/+}\) × Dys\(^{+/−}\) and D3\(^{+/−}\) × Dys\(^{+/−}\) mice exhibited social interaction deficits in a habituation/dishabituation social interaction test (Fig. 3d), while single partial deletion of D3 did not affect social behaviors (Fig. 3d). Thus, D3 hypofunction has negligible effect on sociability/social novelty measures, which might be related to negative symptoms of schizophrenia.

Consistent with previous reports [22, 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 area. In contrast, D3−/− × Dys−/− double heterozygous mice showed wild-type-like behavior (Fig. 3e). Analysis of rearing behavior revealed no differences among the genotypes (Fig. 3f). Thus, the concomitant reduction in D3 and Dys gene expression abolished the hyperactive phenotype produced by either genetic variant.

Startle and PPI responses to an acoustic startle stimulus can be measured in mice and humans [51–53], and decreased PPI is found in patients with schizophrenia [54], as well as in mouse models relevant to schizophrenia [8, 55, 56]. In Dys single heterozygous (D3+/− × Dys+/+) mice, startle reactivity was increased (Fig. 3g), consistent with previous findings [22]. Conversely, D3 single heterozygous mice (D3+/− × Dys−/+), in agreement with findings from other studies [57], were less reactive to the startle stimulus than wild-type mice (Fig. 3g). In contrast, wild-type-like reactivity to the startle stimulus was restored in double heterozygous (D3+/− × Dys−/+) mice. The levels of basal activity in the apparatus in the absence of a stimulus did not differ among genotypes (Fig. 3g). Similar to the locomotor activity results,
these results show that concomitant partial disruption of the D3 and Dys genes rescues the alterations in startle responses driven by each single mutation.

Consistent with evidence that startle and PPI responses are distinct behavioral responses [58], we found a distinct impact of D3/Dys genotypes in PPI measures compared to that in startle reactivity. In fact, in contrast to Dys single heterozygous (D3+/− × Dys+/−) mice but similar to D3 single heterozygous (D3+/− × Dys+/+) mice, D3+/− × Dys+/− double heterozygous mice exhibited a PPI response higher than that in both wild-type and D3+/+ × Dys+/− mice (Fig. 3h). Overall, these results show that concomitant D3 and Dys hypofunction returned the locomotor and startle alterations caused by single disruption of the D3 or Dys gene to wild-type levels.

**Epistatic interaction between D3 and Dys functional genetic variants produces different molecular outcomes in the cortex and the striatum**

Prompted by the behavioral effects driven by D3-by-Dys genetic interaction, we sought to identify if this would be
We focused on the mPFC and striatum as the main areas involved in the dopamine hypothesis of schizophrenia [3, 59]. We found increased levels of D3 mRNA expression in Dys$^{+/−}$ mice in both the mPFC (Fig. 4a) and striatum (Fig. 4b). These increased D3 levels were reversed to wild-type levels in D3$^{+/+} \times$ Dys$^{+/−}$ double mutant mice (Fig. 4a, b). In contrast, Dys mRNA expression was decreased in the mPFC of both D3$^{+/+} \times$ Dys$^{+/−}$ and D3$^{+/−} \times$ Dys$^{+/−}$ mice (Fig. 4c). However, Dys expression in the striatum was increased in D3$^{+/−}$ mice, but this increase was reversed in D3$^{+/−} \times$ Dys$^{+/−}$ double mutant mice (Fig. 4d). Alterations in Dys expression can change D2 recycling [16, 22]. Thus, we analyzed the total and surface protein levels of D2-like receptors. The total levels of D2-like receptor expression in both the mPFC and striatum were unchanged by alterations in either the Dys or D3 genotype individually or interactively (Fig. 4e, f), consistent with previous findings [8, 16]. Single mutant-induced D3 hypofunction did not alter D2-like receptor cell surface expression, while single mutant-induced Dys hypofunction increased D2-like receptor expression on the cell surface in both the mPFC and striatum (Fig. 4g, h), consistent with findings from previous studies [8]. However, in the mPFC of D3$^{+/+} \times$ Dys$^{+/−}$ mice, an even larger increase in cell surface D2-like receptor expression was detected (Fig. 4g). In contrast, cell surface D2-like receptor expression in the striatum was returned to the wild-type level in D3$^{+/+} \times$ Dys$^{+/−}$ double heterozygous mice (Fig. 4h). Overall, these results confirmed a genetic interaction between D3 and Dys functional variants in mice. Moreover, these data indicate that the D3/Dys interaction might act differently in the PFC and the striatum.

**D3 hypofunction rescues Dys-dependent physiological alterations in the mPFC**

Both the human and mouse data suggested a D3-by-Dys genetic interaction in PFC-dependent cognitive functions. Thus, we investigated more in depth the physiological role of the D3/Dys interaction in the mPFC.

Whole-cell recordings were performed on layer V in mPFC slices because D3 is mainly expressed in this cortical layer [27, 46]. The firing frequencies increased in parallel with the injected current for all genotypes (D3$^{+/+} \times$ Dys$^{+/+}$, D3$^{+/+} \times$ Dys$^{+/−}$, D3$^{+/−} \times$ Dys$^{+/+}$, and D3$^{+/−} \times$ Dys$^{+/−}$). However, increasing the current injection from 50 to 200 pA induced fewer spikes in the pyramidal neurons of D3$^{+/+} \times$ Dys$^{+/−}$ mice than in those of wild-type mice (Fig. 5a, b). This difference was particularly marked at the 1 s and 150 pA depolarization steps (Fig. 5b inset). This phenotype was partially ameliorated in double mutant (D3$^{+/−} \times$ Dys$^{+/−}$) mice, as the spike frequency of neurons was not statistically different from that in wild-type mice (Fig. 5a, b). These data indicate that D3 hypofunction ameliorated the disrupted excitability of layer V pyramidal neurons triggered by Dys reduction.

To investigate whether these electrophysiological changes could be associated with altered dopaminergic transmission, we performed an in vivo microdialysis assessment in the mPFC of freely moving mice with D3 and/or Dys mutation (Fig. 5c). The basal extracellular dopamine levels in the mPFC were higher in single heterozygous Dys (D3$^{+/+} \times$ Dys$^{+/−}$) mice than in wild-type (D3$^{+/+} \times$ Dys$^{+/+}$) mice. In contrast, D3$^{+/−} \times$ Dys$^{+/−}$ double heterozygous mice exhibited restored, wild-type-like dopamine levels (Fig. 5c). Risperidone treatment restored the basal...
dopamine to wild-type levels in Dys^{+/−} mice but did not affect D3^{+/−} × Dys^{+/−} or wild-type mice (D3^{+/+} × Dys^{+/+}; Fig. 5d).

Notably, the infusion of the D2-preferring agonist quinpirole into the mPFC by reverse dialysis in freely moving mice, revealed that the functionality of D2-like receptors was disrupted in single D3 heterozygous Dys (D3^{+/−} × Dys^{+/−}) mice, but restored in double heterozygous (D3^{+/−} × Dys^{+/−}; Fig. 5e). Furthermore, following risperidone treatment, quinpirole had again no effects on dopamine levels in single D3 heterozygous (D3^{+/−} × Dys^{+/−}; Fig. 5f), but increased dopamine levels in D3^{+/−} × Dys^{+/−} double heterozygous mice (Fig. 5f, g). These results are similar to the quinpirole-induced increase in mPFC dopamine levels found in risperidone-treated Dys^{+/−} mice with lentiviral-mediated D2 silencing [18], they might be related to the unselective nature of risperidone and/or quinpirole towards D2 and D3, and further support the D2/D3 imbalance in D3^{+/−} × Dys^{+/−} double heterozygous mice.

Taken together, these electrophysiological and biochemical data show that D3 hypofunction can ameliorate Dys-dependent neuronal and dopaminergic basal abnormalities in the mPFC. Moreover, combined with the biochemical data obtained (Figs. 4 and 5), these data
Fig. 4 D3/Dys epistatic interaction normalizes single-gene molecular changes in the striatum while generating a D2/D3 imbalance in the medial prefrontal cortex (mPFC). a, b Abundance of D3 in the mPFC [D3/i+ × Dys/i+ (n = 5), D3/i− × Dys/i+ (n = 6), D3/i+ × Dys/i− (n = 6), D3/i− × Dys/i− (n = 6)] and striatum [D3/i+ × Dys/i+ (n = 5), D3/i− × Dys/i− (n = 6), D3/i+ × Dys/i− (n = 6), D3/i− × Dys/i+ (n = 6)] measured by quantitative RT-PCR. c, d Abundance of Dys in the mPFC [D3/i+ × Dys/i+ (n = 11), D3/i− × Dys/i+ (n = 10), D3/i+ × Dys/i− (n = 10), D3/i− × Dys/i+ (n = 9)] and striatum [D3/i+ × Dys/i+ (n = 10), D3/i− × Dys/i− (n = 11), D3/i+ × Dys/i− (n = 10)] measured by quantitative RT-PCR. Mean fold changes are expressed relative to transcript levels in control mice (D3/i+ × Dys/i+). One-way ANOVAs revealed a genotype effect for D3 expression in the mPFC [F(3, 29) = 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, 29) = 6.95; P < 0.001] and striatum [F(3, 30) = 25.02; P < 0.001]. e, f Western blot and densitometric analysis of total expression of D2-like receptors (52 kDa) in the mPFC [D3/i+ × Dys/i+ (n = 8), D3/i− × Dys/i+ (n = 5), D3/i− × Dys/i− (n = 6)] and striatum [D3/i+ × Dys/i+ (n = 9), D3/i− × Dys/i− (n = 6), D3/i+ × Dys/i− (n = 7), D3/i− × Dys/i− (n = 7)]. g, h Western blot and densitometric analysis of surface expression of D2-like receptors (52 kDa) in the mPFC [D3/i+ × Dys/i+ (n = 9), D3/i− × Dys/i+ (n = 6), D3/i− × Dys/i− (n = 6)] and striatum [D3/i+ × Dys/i+ (n = 9), D3/i− × Dys/i+ (n = 6), D3/i− × Dys/i− (n = 7), D3/i− × Dys/i− (n = 7)]. The results presented are normalized to transferrin receptor protein (95 kDa) levels and to the D3/i+ × Dys/i+ control group average. Synaptophysin (39 kDa) was used as the cytosolic control. One-way ANOVAs revealed no genotype effect for the total level of D2 receptor expression in either the mPFC [F(3, 21) = 0.0753, P = 0.972] or striatum [F(3, 25) = 1.963, P = 0.145]. One-way ANOVAs revealed a genotype effect for surface D2 receptor expression in both the mPFC [F(3, 29) = 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/i+ × Dys/i+ mice. † †P < 0.01, † P < 0.05 vs D3/i+ × Dys/i− mice. Each histogram shows the mean ± s.e.m.

Discussion

This study reveals an epistatic (gene-by-gene) interaction between D3 and Dys (DTNPBP1) genes. In particular, Dys-dependent alterations in the intracellular trafficking of D2-like receptors interact with D3 receptors, exerting prominent effects on higher-order cognitive functions in both humans and mice.

The approach employed, wherein functional genetic variants change the relative expression of different genes simultaneously, allows us to distinguish phenotypes regulated by epistasis (gene-by-gene interaction) from phenotypes for which D3 and Dys exert independent or no effects. Moreover, the similar findings in humans and mice strengthen the conclusion that a concomitant reduction in D3 and Dys functionality yields cognitive advantages in patients with schizophrenia. Indeed, the cognitive deficits measured by the WCST and WM tasks are described as core cognitive features of schizophrenia and are related to dopamine signaling within the PFC [20, 32, 44, 45, 47, 48]. Similarly, both the TOR and the WM task used here in mice relies on D2/D3 signaling and dopaminergic modulation [4, 8, 22, 45]. We previously demonstrated that higher-order cognitive functions modulated by Dys depend on D2 receptor signaling within the PFC [18, 20, 22]. However, in addition to D2, D3 might be highly clinically relevant, because most currently prescribed antipsychotic drugs bind with similar affinity to D2 and D3 receptors [5, 24, 49]. Notably, the effects that we found were more prominent in cognitive functions relevant to schizophrenia while no D3-by-Dys interaction was observed for general clinical assessments, such as positive and negative PANSS scores in humans and social behavior in mice. This finding could agree with those from studies suggesting that D3 blockade enhances cognitive functions [26, 60] without inducing the D2-related side effects of antipsychotic drugs [5, 25]. Furthermore, consistent with previous findings [29], D3 genetic hypofunction increased the scores for the PPI, a sensorimotor gating ability that is usually impaired in patients with schizophrenia [53]. Thus, from a clinical perspective, our current findings suggest that the beneficial cognitive effects of D3 blockade should be considered in combination with epistatic interactions with the Dys gene.

Our molecular data reinforce the meaning of the D3/Dys genetic interaction. Specifically, D3 expression altered by single genetic variants of either D3 or Dys was restored to the wild-type level in both the mPFC and striatum of D3+i × Dys+i double mutant mice. This pattern is consistent with that found in a recent in vitro study, showing that Dys might also influence the expression of D3 receptors [61]. In contrast, D3/Dys genetic interaction rescued Dys expression to the wild-type level in the striatum but not in the PFC. In support of this area-specific effect, we found that D2 receptor trafficking was rescued in D3+i × Dys+i double mutant mice to the wild-type-level in the striatum but not in the PFC. Indeed, Dys expression levels are strictly linked to D2-like receptor trafficking [16, 18, 22]. This D3/Dys epistatic normalization of striatal D2-like receptor signaling was further corroborated by the normalized locomotor activity and startle reactivity. Unlike in the striatum, in the PFC, D3–Dys interaction produced a D2/D3 imbalance favoring increased D2 neuronal surface levels, with normalized basal extracellular dopamine levels. Potentiation of D2 signaling in the PFC in the context of normalized dopamine levels improves higher-order cognitive functions [18]. Thus, these findings are consistent with the improved WM performance driven by D3 hypo-functioning in the context of reduced Dys expression.
cognitive performance driven by D3 hypofunction in the context of reduced Dys expression.

The differential effect of D3/Dys interaction on the relative D2/D3 balance in the striatum vs that in the PFC suggests a distinctive region-specific effect requiring further investigation. However, in contrast to the striatum, which contains only two principal classes of medium spiny neurons coexpressing D2 and D3, the PFC features D3 receptor expression in a subclass of L5 pyramidal cells distinct from D1- and D2-expressing cells [27]. Moreover, while L5 D2-expressing neurons in the mPFC principally project subcortically [62, 63], L5 D3-positive pyramidal neurons are a cortically projecting neuronal subtype [27]. Finally, D2-expressing neurons are relatively more abundant in layers 2 and 3, while D3-positive neurons are relatively more abundant in layer 5 [27]. Thus, the reduced excitability of
Fig. 5 Partial deletion of the D3 gene reverses the decreased excitability of pyramidal neurons, which we found in mice with D3 hypofunction. Representative traces of neuronal firing recorded in mice with different genotypes. 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 intervals. Traces obtained during the 1-s depolarizing step at 150 pA are shown. Summary of spike frequencies (b) obtained for different intervals of depolarizing steps in pyramidal neurons of mice with different genotypes: D3+/− × Dys+/− (n = 10); D3+/− × Dys+/+ (n = 5); D3+/− × Dys−/− (n = 7); and D3−/− × Dys+/− (n = 6) mice. Bar diagram showing the spike frequency (b) observed in mice with different genotypes, with a 150 pA depolarizing step to highlight differences. Repeated measures 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+/−. Extracellular dopamine levels in the mPFC of D3+/− × Dys+/− (n = 8), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 7), and D3−/− × Dys+/− (n = 6) mice. One-way ANOVAs revealed a genotype effect [F(3, 40) = 3.833, P = 0.0167]. Localization of the dialyzing portion of the probe within the mPFC. The number represents the antero-posterior position of the slice (in mm), relative to the bregma and basal extracellular DA levels in the mPFC of D3+/− × Dys+/− (n = 8), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 7), and D3−/− × Dys+/− (n = 6) mice. Post hoc: *P < 0.05 vs D3+/− × Dys+/− and †P < 0.05 vs D3−/− × Dys+/−. Extracellular dopamine levels in the mPFC of D3+/− × Dys+/− (n = 7), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 5), D3−/− × Dys+/− (n = 9) or vehicle (V) [D3+/− × Dys+/+ (n = 11), D3+/− × Dys−/− (n = 12), D3−/− × Dys+/− (n = 10)]. Two-way ANOVAs revealed a genotype effect [F(1, 13) = 7.004, P = 0.0005]. Post hoc: ***P < 0.001 vs vehicle-vehicle-treated mice. f Quinpirole-induced dopamine release in the mPFC of D3+/− × Dys+/− (n = 10), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 9), D3+/− × Dys+/− (n = 12) following chronic treatment (14 days) with risperidone (R) [D3+/− × Dys+/− (n = 7), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 5), D3−/− × Dys+/− (n = 9)] or vehicle (V) [D3+/− × Dys+/− (n = 11), D3+/− × Dys−/− (n = 12), D3−/− × Dys+/− (n = 10)]. Two-way ANOVAs revealed a genotype effect [F(3, 53) = 11.842, P < 0.0001]. Post hoc: *P < 0.05 vs D3+/− × Dys+/− vehicle-treated mice. g Quinpirole-induced dopamine release in the mPFC of D3+/− × Dys+/− (n = 10), D3+/− × Dys+/+ (n = 5), D3+/− × Dys−/− (n = 9), D3−/− × Dys+/− (n = 12) following chronic treatment (14 days) with risperidone or vehicle. Two-way ANOVAs revealed a treatment effect [F(1, 19) = 8.95, P = 0.0075]. Post hoc: at 40-min ***P < 0.001 and 60-min **P < 0.01 vs D3+/− × Dys+/− vehicle-treated mice. Values are the means ± s.e.m. V vehicle, R risperidone, B baseline, Q quinpirole, W washout.

In conclusion, the present study supports D3 receptors as a valid target for improving psychiatric-related higher-order cognitive deficits. Furthermore, these new epistatic interactions might provide additional tools for improved stratification of patients with schizophrenia, which will be required for the application of a more personalized therapeutical approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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