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Pharmacological and genetic modulation of Dopamine D3 receptor: Schizophrenia relevant phenotypes

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The experiments for this PhD thesis were carried out in the laboratories directed by **Prof. Filippo Drago** at the Department of Biomedical and Biotechnological Sciences (BIOMETEC), Section of Pharmacology, University of Catania.

During the 3rd year of my PhD program, I spent nine months working as a visiting PhD student in laboratory directed by **Prof. Rafael Maldonado**, at the Department of Experimental and Health Sciences, Parc Recerca Biomedica de Barcelona (PRBB), University Pompeu Fabra in Barcelona.

During this period, I worked on the project entitled: "Neurobiological mechanisms involved in food addiction"

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List of abbreviation

7-OH-DPAT 7-hydroxy-N,N-di-*n*-propyl-aminotetraline

AC Adenilate Ciclase

ADHD Attention Deficit Hyperactivity Disorder

BLOC-1 Biogenesis of Lysosomes -Related Organelles Complex 1

cAMP 3',5'-cyclic Adenosine Monophosphate

CNS Central Nervous System

D1R Dopamine D1 receptor

D2R Dopamine D2 receptor

D3R Dopamine D3 receptor

D4R Dopamine D4 receptor

D5R Dopamine D5 receptor

$D3R^{-/-}$ Dopamine D3 receptor deficient mice

DA Dopamine

DISC-1 Disrupted in Schizophrenia 1

DR Discrimination Ratio

DSM-5 Diagnostic and Statistical Manual of Mental Disorders

DTNBP-1 Dystrobrevin Bindin Protein-1

Dys Dysbindin

GIRK G protein-coupled inwardly-rectifying potassium channel

GLAST Glutamate-Aspartate Transporter

GLUT Glutamate

GPCR G-Protein Coupled Receptor

I.p. Intra-Peritoneal

KO Knocked Out

MAPK Mitogen-Activated Protein Kinase

mPFC Medial Prefrontal Cortex

NAc Nucleus Accumbens

NMDA N-methyl-D-aspartate

PCP Phencyclidine

PET Positron Emission Tomography

PKA Protein Kinase A

PPI Pre-Pulse Inhibition

Sdy Sandy Mice

SNP Single Nucleotide Polymorphism

TOR Temporal Order Recognition

VTA Ventral Tegmental Area

WCST Wisconsin Card Sorting Test

WM Working Memory

WT Wild Type mice

Preface

Schizophrenia is a chronic and severe psychiatry disorder affecting 1% of the worldwide population, characterized by a heterogeneous genetic and neurobiological background that influence early brain development. The characteristic symptoms of the disease can be divided in three categories: positive, negative and cognitive, the latter expressed as a varied set of cognitive dysfunctions. The etiopathogenesis of schizophrenia is not fully understood, due to the complexity of the disease and to the large number of molecular targets involved. Yet, the current understanding of schizophrenia is represented by the "dopamine hypothesis" stating that the disease is caused by an imbalance in the dopaminergic transmission in both cortical and subcortical brain areas. The first line treatment for schizophrenic patients is represented by the first- and second-generation antipsychotic drugs, that act mainly as antagonist or partial agonist to dopamine D2-like receptors, that include D2R, D3R, and D4R. Among dopamine receptors, theD3R create interest because of its limited expression in limbic brain areas involved in cognition and emotional processes. To date, several preclinical studies show the involvement of D3R in the regulation of the activity of DA neurons in the mesolimbic, mesocortical and nigrostriatal dopaminergic pathways. Further, evidence show the role of D3R in physiological mechanisms underlying mPFC-dependent cognitive functions, suggesting that blocking D3R may impact cognitive impairment. Consequently, D3R could be considered as a new pharmacological target for schizophrenia. However, to date no available antipsychotic show a higher selectivity for the D3R over D2R.

Based on data present in literature, the aims of this thesis are 1) to study the role of D3R as a therapeutic target for pro-cognitive treatment and 2) investigate the role of D3R as a genetic tool for patient stratification.

Chapter I

General introduction

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) define psychiatric disorders as "complex syndromes characterized by significative disturbance in an individual's cognition, emotion regulation or behavior that reflects a dysfunction in the psychological, biological, or developmental processes underlying mental functioning" [1]

The polygenetic architecture of all the major psychiatric disorders points out a crucial role of genetic factors in the etiopathology of disorders as anxiety disorders, autism, attention deficit hyperactivity disorder (ADHD) and schizophrenia.

Schizophrenia is a severe and chronic mental illness with a prevalence of 0.8%-1%. Recent evidence show that the onset of this pathology generally begins in early adolescence and not in early adulthood, as initially thought, with a slow decline in cognitive and social functioning. The life expectancy in schizophrenic patients is reduced by 20 years compared with the general population, with suicide being the main contributor during the early years and cardiovascular disease during the late period [2, 3] The main risk factors for schizophrenia include complications in prenatal and perinatal events (complications of pregnancy, abnormal fetal growth, etc…) [4] and persistent abuse of amphetamine and cocaine, that produce a state almost identical to that of paranoid schizophrenia [5]. The administration of cannabis, instead, is known to exacerbate existing psychosis, particularly if used during early adolescence [6].

The diagnostic criteria for schizophrenia are not easy to classify. Indeed, psychosis is not exclusive for schizophrenia and occurs in different categories of psychiatric disorders. However, is possible to distinguish three core clinical feature for schizophrenia: positive, negative and cognitive symptoms.

Positive symptoms include hallucination (auditory, visual, etc..), delusion, movement disorders, confused thoughts, and disorganized speech. The amotivational syndrome is characterized by negative symptoms that include anhedonia, social withdrawal and affective flattening [7]. Cognitive impairment, instead, is expressed as a variety of cognitive deficits including dysfunctions in working memory, attention, verbal and visual learning with deficits in reasoning, planning and problem-solving[8].

The positive psychotic symptoms tend to improve in response to antipsychotic treatment, however, recovery is achieved in a limited number of patients with schizophrenia. In contrast, negative symptoms and cognitive deficits are less responsive to the drug treatment in the first episode of psychosis [9] but also in chronic schizophrenia[10]. To date, no antipsychotics show robust effects on cognitive deficits or impaired social processing, which are the most clinically relevant dimension of the disease [11]. The reason for the partial effectiveness of current antipsychotic drugs is the pathophysiology of schizophrenia, which is not fully understood due to the complexity of the disease and the involvement of a variety of molecular targets[12].

The ongoing understanding of schizophrenia is constituted by the "dopaminergic hypothesis", which refers to alterations of dopamine neurotransmission [13, 14] complemented by the "Glutamate hypothesis".

The dopamine and dopamine-glutamate hypothesis will be discussed in the next section.

Dopaminergic system in the CNS

Dopamine (DA) is a catecholamine neurotransmitter involved in multiple functions in the central nervous system (CNS) including locomotion, cognition, reward and emotional behavior [15, 16].

DA exerts its functions through four dopaminergic pathways: the *nigrostriatal pathway*, which consists of neurons whose cell bodies originate in the substantia nigra pars compacta and terminate in the dorsal striatum. This pathway is involved in motor planning and its degeneration is one of the main pathological features of Parkinson's disease [17].

The dopaminergic neurons in the *mesocortical pathways* arise from the ventral tegmental area (VTA) and project to the prefrontal cortex (PFC) and are thought to be involved in cognitive control, emotional and motivation response. Abnormal functioning of this pathway is thought to be associated with the onset of the negative symptoms of schizophrenia [18].

The *mesolimbic pathway* also arises from the VTA but innervates the ventral striatum, which includes the nucleus accumbens and the olfactory tubercle. This system, also named as "reward pathway", plays a key role in reward, craving, and aversion [19, 20]. Moreover, its deregulation is thought to induce the onset of the positive symptoms of schizophrenia [21].

Lastly, the *tuberoinfundibulnar pathway* refers to a group of DA neurons in the arcuate nucleus of the hypothalamus that project to the mediane eminence, which in turn controls prolactin secretion from the anterior pituitary gland [22-24].

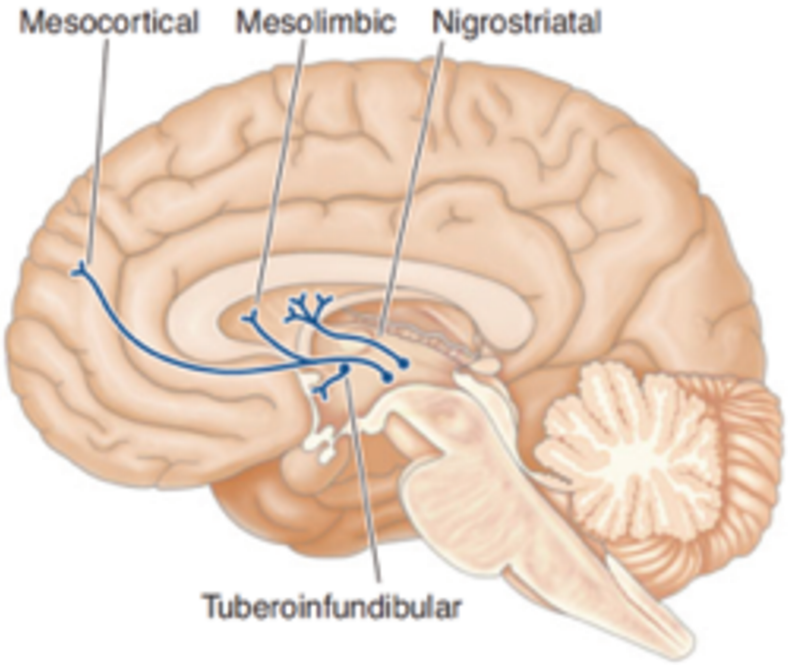

Figure 1. DAergic pathways in the central nervous system [25].

The activity of dopamine is mediated by five receptors, belonging to the superfamily of seven transmembrane domain G-protein coupled receptors (GPCR), divided into two subfamilies: D_1-like and D_2-like receptors.

The D_1-like subtype, which includes dopamine D1R and D5R receptors, is coupled to $G\alpha s$ protein and its activation stimulates adenilate cyclase (AC) to produce adenosine 3′,5′-cyclic monophosphate (cAMP) activating cAMP-dependent intracellular pathways, including protein kinase A (PKA) and mitogen activating protein kinase (MAPK) signals [26].

On the other hand**,** dopamine D_2, D_3, and D_4 receptors belong to the D_2-like subfamily, which is coupled to $G\alpha i$ proteins, through which induce the inhibition of the AC and cAMP-generating system [27, 28].

D1R is the most abundant dopamine receptor in the CNS, with a greater expression compared to other subtypes [29].

The localization of D1R comprises brain areas known to be under the control of dopamine such as striatum, nucleus accumbens and olfactory tubercles, lower levels of D1R were found also in the limbic system, hypothalamus and thalamus.

D5R, on the contrary, has a significantly low expression level compared to D1R, though it is endowed with a 10-fold higher affinity for dopamine than D1R. D5R expression is limited to the hippocampus and thalamus, in particular to the lateral mamillary nucleus and the parafascicular nucleus of the thalamus [30, 31].

D2R is the second dopamine receptor in terms of levels of expression in the brain. D2R mRNA was found especially in the striatum, nucleus accumbens and olfactory tubercles but it is also found in the substatia nigra pars compacta and VTA, in which the D2Rs are expressed by dopaminergic neurons.

In contrast to D1R that are mainly post-synaptic, D2Rs are localized on both pre-synaptic dopaminergic neurons and neurons targeted by dopaminergic projections[32, 33].

Interestingly, D3Rs are mainly confined to the limbic system, including nucleus accumbens, olfactory tubercles and island of Calleja. Moreover, D3R mRNA has been found in the medial prefrontal cortex (mPFC), nucleus accumbens and ventral pallidum but also in the ventral striatum, thalamus and orbitofrontal cortex [34, 35]

D4Rs, instead, has low mRNA levels in basal ganglia in contrast to higher expression levels in frontal cortex, medulla, amygdala and hypothalamus.

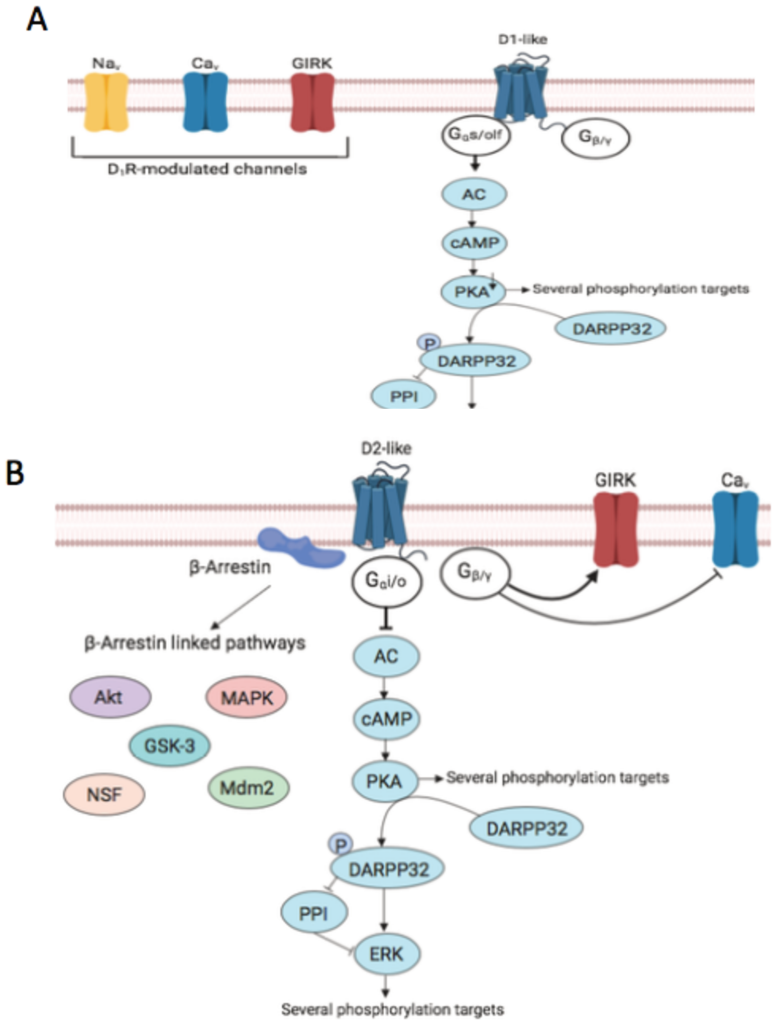

Figure 2. DAergic receptors intracellular signaling. Intracellular signaling pathways activated by D1-(**A**) and D2-(**B**) like receptors. **AC**, adenylate cyclase; **cAMP**, 3'-5'-cyclic adenosine monophosphate; **DARPP32**, cyclic AMP-regulated phosphoprotein 32kDa; **PPI**, protein-phosphatase 1; **ERK**, extracellular signal-regulated kinase protein kinase; **PLC**, phospholipase C; **DAG**, diacylglycerol; **PKC**, protein kinase C; **IP$_3$**, inositol triphosphate, **GIRK**, G-protein gated inwardly rectifying K^+ channels; **Ca$_v$**, voltage-activated Ca^+ channels; **Na**, voltage-activated Na^+channels, **Akt**, thymoma viral proto-oncogene; **GSK-3**, Glycogen Synthase Kinase-3; **MAPK**, mitogen-activated protein kinase; **Mdm2**, mouse double minute 2 homolog; **c-Src**, proto-oncogene non-receptor tyrosine kinase, **NSF**, N-ethylmaleimide-sensitive factor

Dopaminergic hypothesis of schizophrenia

The DA hypothesis of schizophrenia represents one of the most enduring pathogenetic hypothesis in psychiatry, being postulated upon the fortunate discovery of antipsychotic drugs acting as D2R antagonists.

The first hypothesis, postulated in 1970, stated that schizophrenic symptoms were the result of an excess of dopamine transmission and that blockade of dopamine receptors was the only way to treat the psychosis [36, 37].

In the light of new evidence, the above-mentioned hypothesis was subsequently reformulated. The revised dopaminergic hypothesis, postulated in 1991 [38], suggests an imbalance between hyperactive dopamine transmission in the mesolimbic areas and hypoactive dopamine transmission in the prefrontal cortex [39-43]. Indeed, evidence suggest a prefrontal deficit in schizophrenia and the main role of DA in mediating prefrontal-dependent cognitive processes [38, 44].

Moreover, dopamine dysregulation is also observed in the amygdala and prefrontal cortex, brain regions involved in emotional regulation [45].

Positron Emission Tomography (PET) studies have identified differences in dopamine contents in the prefrontal cortex, cingulate cortex and hippocampus in schizophrenic patients compared to healthy individuals; in particular, schizophrenic patients display a hyperactivation of the dopaminergic transmission in the hippocampus[46].

Besides DAergic dysfunction, alteration in glutamatergic transmission occurs with the DA-GLUT hypothesis, which represents an integration to the above-mentioned pathogenetic theory for schizophrenia.

The DA-GLUT hypothesis suggests an imbalance in the dopaminergic system consisting in (i) a strengthened subcortical release of dopamine, which increase D2 receptor activation in the associative striatum, leading to a disrupted cortical pathway through the Nacc[47], (ii) a reduced D1 receptor activation [48] in the prefrontal cortex and decreased activity of the nucleus caudatus [47], and (iii) modification in prefrontal connectivity involving glutamate transmission at N-methyl-D-aspartate (NMDA) receptors [49].

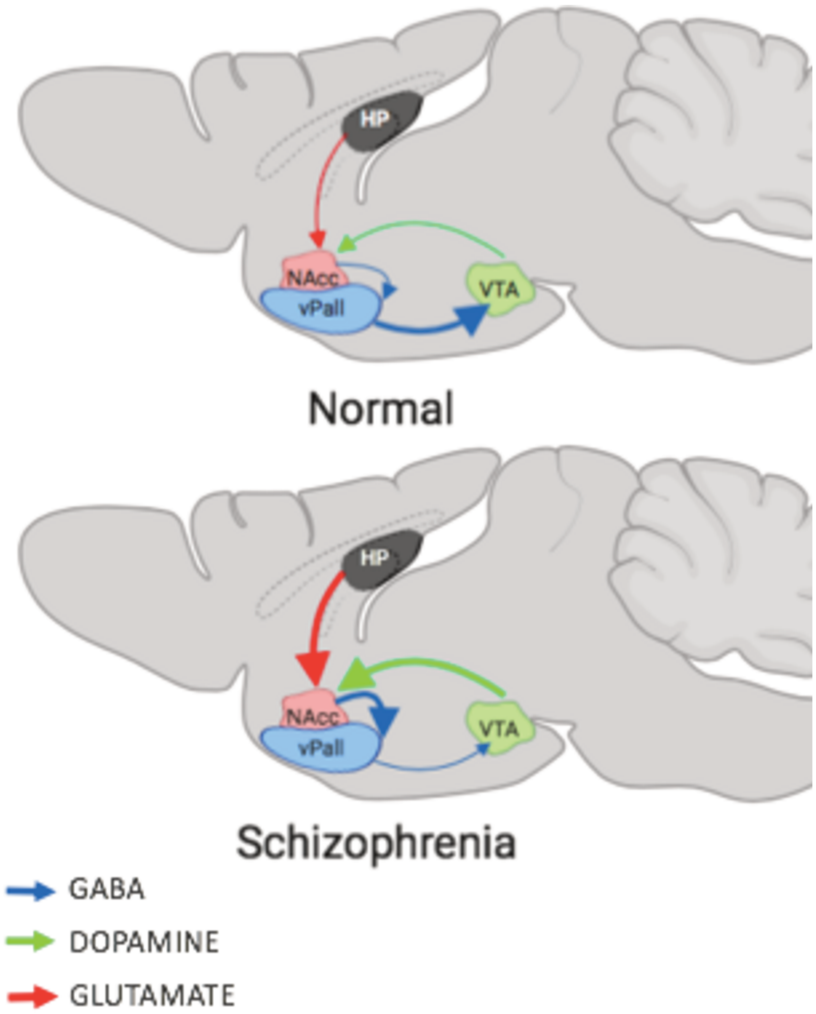

Figure 3. Comparison between the regulation of the dopaminergic system in healthy and pathological condition.
Hippocampal hyperactivity, suggested to be associated with a decrease in GABA transmission (not illustrated), results in an increase of glutamate transmission onto the Nucleus Accumbens (Nacc). The subsequent increase in Nacc GABAergic output inhibits the ventral palladium (vPall) resulting in the disinhibition of the dopaminergic neurons located in the VTA.

Animal models of neuropsychiatry disorders

Recently, nonetheless the development of novel noninvasive technologies to study human brain structure and functions, slow progresses have been made in the understanding of the pathophysiology of neuropsychiatry disorders, such as bipolar disorders, autism, major depression and schizophrenia, as well as in the identification of new molecular targets.

Modelling accurate and predictive animal models for complex psychiatry disorders is fundamental to increase our knowledge of the neurobiological basis of these disorders and for the development of new and more effective therapeutic treatment. The main difficulty in the development of a reliable animal model lies in the impossibility to determine, in mice, the presence of uniquely human symptoms [50], such as hallucinations, memory, thoughts, and delusion, used as criteria to establish psychiatry disorders in patients [51]. On the other hand, symptoms as altered social behavior, working memory and executive functions, even if occur in animal, the correspondence with patients may be only approximated [51].

Although these difficulties, animal models of complex neuropsychiatric disorders represent a rapid and effective pre-clinic tool to investigate the progression of the disease, giving the opportunity to accomplish invasive analysis of structural and molecular changes that may improve the current knowledge of the disorder and test the efficacy of novel molecules which is not possible in patients.

An accurate and useful animal model should fulfill three

sets of validators, first elaborated by Wilner in 1986: face, construct and predictive validity [52].

Face validity refers to the analogy between the symptoms observed in the animal model and the human organism.

Construct validity is defined as the replication of core theoretical neurobiological rationale and neurochemical and brain structural defects;

Predictive validity shows if the animal model has the same response to drug treatment as in patients.

Animal models of schizophrenia

Animal models used to investigate the etiopathology of schizophrenia include not only models of the full disorder but also models of specific symptoms, because of the complexity of this pathology and the heterogeneity of the factors involved in the disorder that cannot be easily reproduced in animals [53].

Some rodents models of schizophrenia may display behavioral alterations similar to positive-like symptoms, reflecting an enhanced mesolimbic dopaminergic activity, while others, such as methylazoxymethanol (MAM) or chronic phencyclidine administration, involve cortical dopaminergic dysfunction or deficit in the sensory motor-gating, which reflect an altered development of frontal cortico-limbic circuits.

The currently used animal models of schizophrenia fit into 3 different categories: neurodevelopment, pharmacologic and genetic models.

- *Neurodevelopment animal models of schizophrenia*

Several evidence demonstrated that exposure to adverse insults or environment in the gestation or perinatal period, such as maternal stress, malnutrition, immune activation or obstetric complication during birth may increase the risk to develop schizophrenia [54]. Manipulation of the environment or drug administration during the perinatal period are usually used to produce irreversible changes in the CNS development. The best characterized model of this category is the one proposed by Lipska and Weinberger [55, 56] involving neonatal lesions of the ventral hippocampus, which produce behavioral impairments in the post pubertal period.

Other neurodevelopmental models involve impairment in neurogenesis during the gestational period, induced by the administration of MAM, post-weaning social isolation and perinatal or maternal immune activation.

The above-mentioned models induce long-term and irreversible behavioral changes that usually occur in the pubertal/post-pubertal period, replicating the time course of the pathology as well.

- *Pharmacologic animal models of schizophrenia*

The most used approach for the development of animal models of schizophrenia involve the use of pharmacologic treatments or drug-induced states. Although the mechanisms that underlie this pathology are not fully understood, the current and most-accepted theory of the etiopathology of the schizophrenia is represented by the DA-Glut hypothesis, suggesting that the dysregulation of the Daergic and glutamatergic system is the cause of the onset of the schizophrenic symptoms. The first pharmacological models were developed with the aim to mimic the DAergic hyperfunction of the mesolimbic pathway. The best-characterized model involves the administration of amphetamine, known to induce, in humans, psychosis with features (hallucination, persecutory delusions..) similar to the positive symptoms of schizophrenia. Thus, studies demonstrated that chronic administration of amphetamine in rodents induced schizophrenia-like symptoms, with behavioral alteration as hyperactivity [57, 58], PPI disruption [59, 60] and deficit in PFC-dependent cognitive task as extra dimensional shift and reversal learning in the attentional set-shifting task [58, 61]. Though, amphetamine administration did not alter social behavior in rodents [62, 63], failing in reproducing the negative symptoms of schizophrenia. The amphetamine-induced

schizophrenia-like symptoms were prevented with the administration of a low dose of haloperidol or clozapine [64].

Recently, many pharmacological evidence suggest that the dysfunction of the glutamatergic system plays a central role in the pathophysiology of the schizophrenia. In particular, the blockade of the NMDA receptor by the administration of non-competitive antagonist, such as ketamine and phencyclidine (PCP), was demonstrated to induce, in healthy patients, schizophrenic-like symptoms as delusions and hallucinations [65, 66].

In addition, acute administration of PCP, in rodents, induce hyperlocomotion [67], social withdrawal [62] and cognition [68] and PPI [69] impairments. Moreover, chronic administration of PCP produces neurochemical changes that resemble those that occur in schizophrenia, as the hyper-responsiveness of the mesolimbic dopamine system [70], reduced DA levels in the PFC [71] and increased PFC glutamate-aspartate transporter (GLAST) levels [72].

Recently, was demonstrated that the administration of MK-801, a NMDA non-competitive antagonist, mimic schizophrenia-like symptoms in rodents, including positive, negative and cognitive symptoms [73-76].

Indeed, a single administration of MK-801 cause behavioral anomalies in mice that resemble schizophrenic-like features. Interestingly, rodents treated acutely with MK-801 show cognitive impairments in the Y-maze test, anxiety in the open field and elevated plus maze, hyperlocomotion and reduced PPI [77, 78].

- *Genetic animal models of schizophrenia*

Schizophrenia is a neuropsychiatric disorder with a heritability estimated around 80%, as demonstrated by family studies [79]; however, no single genetic alteration is sufficient to explain the pathophysiology of this complex and heterogeneous disorder. Genomic studies identified a large array of candidate genes associated with the risk to develop schizophrenia [80-82]; in particular in a study by the International Schizophrenia Consortium was demonstrated the involvement of almost 200 genes in the etiophatology of this disorder , including DRD2 and DRD3 genes [83]. Thus, changes in mRNA and protein seen in schizophrenia were fundamental for the development of genetic animal model.

One of the main gene involved in schizophrenia is DISC-1 (*disrupted in schizophrenia-1*), which encode for the homonym protein [84]. DISC-1 is a synaptic protein that plays a key role in pre and post-natal neuronal development, in synaptogenesis, neuronal migration and synaptic plasticity [85].

Interestingly, transgenic mice with partial DISC-1 function show core features of schizophrenia, both neuroanatomical and behavioral.

Behavioral alterations include PPI disruption, hyperlocomotion and disruption of the social interaction [86-88].

In the context of neuroanatomical variation, DISC-1 mice display enlarged lateral ventricles, reduced brain volume, and reduced parvalbumin immunoreactivity in mPFC and hippocampus [86, 89-91].

Another candidate gene for the development of schizophrenia is DTNBP-1, which encode for dysbindin-1 protein. DTNBP-1 mutations determine reduced expression of dysbindin-1 in dorsolateral PFC and hippocampus, anomalies that occur also in schizophrenic patients [82, 92, 93].

More information about the involvement of DTNBP1 and Dysbindin-1 are discussed in the next chapter.

In addition to DISC-1 and Dysbindin-1, other proteins were identified a susceptibility genes as neuregulin-1 and ErbB4, and Reelin.

Schizophrenia-relevant behavioral tests in rodent models

Rodent behavior is defined as critical experimental protocols for the development and testing of animal models.

The main role of behavioral tests is to provide information about the affected circuitry and pathophysiology of the disease; however, the selection of a specific behavioral task should take in consideration a variety of factors including the validity, reliability, sensitivity, utility and specificity of the tests.

Although it's important to take into account that an animal behavior is relevant to schizophrenia if it depends on brain regions known to be implicated in human schizophrenia, as PFC, cingulate cortex and hippocampus [94].

Interestingly, some schizophrenia-related behavioral dysfunction directly measurable in rodents, are exactly the same in both, animal and patients. Such correspondence of behavior is due to the conservation of neural circuitry underlying behavior across species.

Currently, rodent behaviors are used to model various aspects of schizophrenia, consisting in the assessment of the core symptoms of this pathology, represented by the positive, negative and cognitive symptoms.

- *Mouse behavior relevant for positive symptoms of schizophrenia*

Positive symptoms comprise a number of symptoms that it's impossible to replicate in rodent such as hallucination (auditory, visual or olfactory), delusions (somatic, persecutory or religious), confused thoughts and disorganized speech. The only assessable positive symptoms are hyperactivity and movement disorders.

In this context, schizophrenic patients manifest psychomotor agitation, with hyperactivity and increased stereotypic movements.

Hyperactivity, at baseline or in response to stress due to a novel environment, has been demonstrated in different animal model of schizophrenia [95].

Interestingly psychotomimetic drugs as ketamine, PCP or MK-801 are shown to increase the locomotor activity [96], and this effect is prevented, in rodents, by the administration of antipsychotic drugs [97, 98].

Hyperactivity is usually assed in an open field arena and analyzed by a video tracking software or, if the arena is divided in squares, by counting how many squares each mouse cross. The open field test is not a behavioral paradigm exclusive for schizophrenia, indeed, it is used also to assess anxiety-like behavior in rodents. Moreover, increased locomotor activity occurs in several neuropsychiatric disorders, such as ADHD or bipolar disorder.

- *Mouse behavior relevant for negative symptoms of schizophrenia*

Negative symptoms of schizophrenia encompass blunted affect, poverty of speech, amotivation, anhedonia and asociality or social withdrawal.

Considering that mice are highly social animals, impairments of social interaction are relevant to schizophrenia and considered as one of the hallmarks of the disorder [99].

Moreover, the measure of the social interaction in mice has the exact correlate in humans.

The behavioral test to assess the impairment in social interaction consists in the encounter of a “test mouse” with a “stranger mouse”. However, many variants of this paradigm exist.

The behavior paradigm can be performed in an open field arena, in a three-chamber apparatus, or in the test mouse home cage. Moreover, the “stranger” mouse can be freely moving [100]or trapped in a small wire-cage [101, 102].
Unfortunately, task conducted in a novel environment, can trigger exploratory and anxiety-like behaviors that can affect the results.

-*Mouse behavior relevant for cognitive deficits of schizophrenia*

Cognitive deficits that occur in schizophrenia have recently attracted growing attention due to the number of patients affected [103, 104].
This category of symptoms includes information processing, executive functions, recency discrimination, cognitive flexibility, sensory motor gating, attention, memory and visual processing.
The most studied and reproducible cognitive deficits in animal models of schizophrenia is the impairment in working memory [105, 106].
Working memory is the system that temporarily store and manage information required to fulfill complex cognitive tasks such as learning and reasoning [107].
Many behavioral paradigms were developed in order to investigate the presence of working memory impairments in rodents: the 8-arm radial maze[108], delayed alternation or spontaneous alternation task in T-maze or Y-maze [109] and delayed matching to place task in Morris water maze [110].
However, working memory deficits in schizophrenic patients are usually assessed by the Wisconsin Card Sorting Test (WCST) which is usually linked to dysfunction in dopaminergic signaling within the PFC [111-113]. Analogous behavioral tests in rodents are represented by the temporal order recognition (TOR) test [114, 115], which relies on dopaminergic alteration within the mPFC and the discrete paired

trial variable delay T-maze task [116, 117]. The TOR test, usually performed in an open field arena, consists in two sample phases and a test trial. A delay of 1h occur between the two sample phases, while the test is usually performed 3h after the second sample phase. During the sample phases, the animal is free to explore two identical objects for a fixed amount of time, while in the test trial the same mouse is exposed to a copy of the object from each sample phase. The evaluation of the presence of working memory impairment is given by the Discrimination Ratio (DR).

Unfortunately, the employment of a novel environment, such as the open field apparatus, could trigger anxiety-like behavior, with a subsequent decrease of the exploration time.

On the other hand, a more selective paradigm to assess working memory impairments is the discrete paired-trial variable-delay non match T-maze task, which also relies on dopaminergic modulation within mPFC [116, 117]. In this paradigm mice were exposed to a sequence of randomly chosen forced runs, each followed by a choice run so that the mice were required to integrate information from the forced run with the learned rule (non-match to sample).

Further, several authors suggested that startle response and pre-pulse inhibition (PPI) represent a model to detect differences in the processing of information related to brain structures and systems thought to be fundamental in the developments of schizophrenia such as the mesolimbic system and dopaminergic activity [94].

Moreover, a number of evidence underline that schizophrenic patients show limitation in voluntary control of attention and oversensitivity to sensory stimulation [118]. PPI is among the most used behavioral paradigm in animal models of schizophrenia, that allow to directly measure the sensory motor gating both clinical and pre-clinical studies [118-120].

To measure PPI is first necessary to record the baseline response. To a loud white noise pulse (startle), then the same response is measure in the presence or absence of a smaller, non startling prepulse that precedes the startle pulse by a brief delay of the order of milliseconds.
However, even if the measure of PPI is one of the most used behavior paradigm for the validation of animal models of schizophrenia, PPI deficits occur also in panic disorders [121], obsessive-compulsive disorder [122] and attention deficit disorder [123].
To conclude, no single rodent behavioral task is adequate to to describe the vast array of schizophrenic symptoms and single behavioral task are not exclusive to schizophrenia. Similarly, single symptoms that characterize schizophrenia are not a diagnostic evidence of the presence of the disorder in humans.

Dopamine D3 receptor and its role in schizophrenia

In 1990, Sokoloff and collaborators published a study on the molecular cloning and expression of a full-length cDNA encoding for a novel dopamine receptor subtype [124], extending the dopamine receptor family beyond the existing D_1 and D_2 receptors.

The D_3 receptor was classified as a member of D_2-like subtype because of evidence showing that human D2R and D3R share a homology of the 78% sequence identity in the transmembrane domains, including the binding site [125, 126].

In the same study, Sokoloff also reported that D3R was endowed with a higher affinity for dopamine (70-fold higher) and its agonists compared to D2R [124], suggesting that, in vivo, DA may occupy D3R for extended periods leading to the spontaneous activation of this receptor [127, 128].

D3R is a GPCR associated to $G_{\alpha}i/o$ proteins which regulates two main intracellular pathway. The activation of this receptor inhibit the production of cAMP triggering the consequent reduction of PKA activity [28, 129]. Furthermore, D3R activation regulates other intracellular pathways including the extracellular signaling regulated by kinase 1/2 and Akt cascade through G protein-dependent or independent mechanisms, which may also influence B-arrestins-mediated signaling [130, 131].

D3R, as the most of GPCRs, have been shown to interact with receptors of the same subtype to form homomers (D3R-D3R) and with other dopamine receptor subtype to form heteromeric complexes [132]. Heteromers have been reported with D1R [133, 134] and D2R [135], but also with other GPCRs, such as the Adenosine A2A receptor [136].

As D2Rs, D3Rs regulate the activity of dopamine neurons because of their expression on both pre-and post-sinaptic DAergic neurons [35, 137, 138]. Indeed, *in vitro* evidence show that the stimulation of D3R, expressed in transfected mesencephalic cell line, inhibits dopamine release [139] and synthesis [140] and that agonists, with limited preference for D3R, inhibit dopamine release, synthesis and neuronal electrical activity [141]. Moreover, recent immunocytochemical studies show that D3R is expressed by all mesencephalic dopamine neurons [138], supporting the concept of D3R as auto-receptor.

As mentioned in the previous paragraph, D3R localization is confined to the limbic system, where the largest D3R densities occur in granule cells of the island of Calleja and in the medium-sized spiny neurons of the rostral and ventral shell of the NAcc [137, 142, 143]. Lower levels of D3R mRNA have also been found in the striatum and in specific areas of the PFC .

In this context, recently was demonstrated that, in mouse PFC, D3R characterize a new subclass of layer 5 glutamatergic pyramidal neurons with different elctrophysiological and anatomical features compared to neurons expressing D1R and D2R. In addition, D3R activation, within these neurons, regulates low-voltage activated $Ca_V3.2$ calcium channels, indicating that D3R controls the excitability of a novel PFC neuronal population [144].

The pattern of D3R expression suggest that this dopamine receptor may mediate the effect of dopamine on cognitive and emotional functions and, consequently could be considered to be a target for antipsychotic drug therapy.

Several studies demonstrated the involvement of D3R in the modulation of the mesolimbic dopaminergic pathway. Although few clinical trials with D3R-selective compound have been reported, a

large number of preclinical studies explored the role of D3R in the control of drug seeking behavior and in schizophrenia.[145, 146].

The involvement of D3R on schizophrenia attracted great interest due to its restricted localization in limbic areas, linked to the positive symptoms and cognition or emotional impairment present in schizophrenia [35, 124, 147], and its fundamental role in physiological mechanisms underlying mPFC-dependent cognitive functions [148].

Recent evidence, even if conflicting, suggest that blocking D3R may impact cognitive impairment. Indeed, $D3R^{-/-}$ mice show a better performance in a step-through passive avoidance test compared to WT [149], while no differences were found in mice treated with SB 277011A, a D3R selective antagonist, and WT in the Morris water maze paradigm [150]. Moreover, the over-expression of D3R in striatum doesn't affect cognition, but this condition disrupts motivation, suggesting that variation in D3R expression could be involved in the negative symptoms of schizophrenia [151].

Conversely to human studies, several D3R selective ligands have become available for preclinical studies in order to elucidate the involvement of D3R in schizophrenia.

PET imaging studies with [^{11}C]-(+)-4-propyl-9-hydroxy-naphthoxazine ([^{11}C]-(+)–PHNO)[152], the only available radiotracer for imaging D_3 receptors with a 53-fold D_3/D_2 receptor selectivity in vivo in humans [153], in presence and absence of a D_3 receptor antagonist, SB-277011, confirmed that D3Rs are highly expressed, in vivo, in the ventral pallidum, substantia nigra, thalamus, and habenula; to a limited extent in the ventral striatum; and scarcely in the dorsal caudate and putamen [154].

The development of ([^{11}C]-(+)–PHNO) provided the opportunity to explore, *in vivo,* the role of D3R in both normal and pathological condition.

Interestingly, D3R is thought to be implicated in the pathophysiology of a number of neuropsychiatry disorders usually followed up by cognitive dysfunction, such as schizophrenia [153, 155, 156], drug addiction [157, 158], Parkinson disease [159, 160], dementia [161], mood disorders [161] and autism [162].

However, the association between cognition and D3R in healthy individuals or in the above-mentioned pathological condition was explored directly by only few studies. In particular, while the D3R gene, DRD3, is not directly associated with enhanced cognitive ability or reasoning skills, the Single Nucleotide Polymorphism (SNP) DRD3 Ser/Ser was linked to fewer perseveration errors in the Winsconsin Card sorting test (WCST) [163], while individuals carrying the DRD3 Ser/Gly SNP had more benefit from multimodal cognitive training compared compared to Ser/Ser genotype[164].

Currently, available drug treatments are effective in improving positive symptoms but exhibit limited activity on negative and cognitive symptoms [165].

In this context, pharmacological, genetic and human post-mortem studies demonstrated the involvement of D3R in the pathophysiology of schizophrenia, pointing out that D3R could represent a new target for antipsychotic treatment [166].

However, first- and second-generation antipsychotics, do not exhibit selectivity for D3R over D2R, except for few compounds, including aripiprazole, blonanserin, and cariprazine, that show a D3R-preferring activity [167-169], without the classical side effects, such as extrapyramidal effects or prolactin elevation, caused by D2R blockade [153]. Indeed, *in vivo* human imaging studies shown that three of the most commonly used antipsychotic drugs, clozapine, olanzapine, and risperidone, slightly occupy D3R in the brain of schizophrenic patients [170, 171].

To date, in spite of academic research activity, no selective D3R agonist or antagonist is available for therapeutic use [172].

For instance, the identification of 7-hydroxy-N,N'-di-*n*-propyl-aminotetraline (7-OH-DPAT), an originally-thought selective agonist ligand for D3R, was used to label D3R in the brain [35]. However, further experiment demonstrated that the selectivity of 7-OH-DPAT for D3R under experimental condition were not coherent with in vivo conditions.

Recently, has been proposed that molecules acting as D3R potent antagonist combined to 5-HT_{1A}R partial agonism and 5-HT_{2A}R antagonism may exhibit an enhanced antipsychotic activity [173]. The above-mentioned pharmacological profile widely matches that of buspirone, anxiolytic drugs with an affinity for D2R 5-fold lower than for D3R [174], even if, to our knowledge, the antipsychotic properties of buspirone have not been significantly elucidated.

Dysbindin-1 involvement in schizophrenia

Several studies demonstrated that genetic susceptibility plays an important role in the pathogenesis of schizophrenia, with heritability of which is now estimated to be 82–84% [11, 175].

Indeed, family, twin and adoption studies have been crucial for demonstrating that there is an important genetic contribution in the etiology of schizophrenia [176].

Although a single genetic alteration is not sufficient to explain this heterogeneous and complex disease, understanding the mechanisms through which genetic alteration are associated with an increase in the risk to develop schizophrenia is critical in order to elucidate the molecular mechanisms of this pathology, providing biological basis for the development of more effective treatments as well as new genetic tools for patients stratification and for the improvement of personalized medicine for schizophrenia.

To date, the best-supported susceptibility gene is the dystrobrevin-binding protein-1 (DTNBP1), located within chromosome 6p22.3, which encodes for three main splicing isoforms, the full-length dysbindin-1A and two truncated isoforms, dysbindin-1B and dysbindin-1C [177].

Dysbidin (Dys) is a 40-50 kDa protein that binds to both the component of the dystrophin glycoprotein complex, α- and *β*-dystrobrevin, mainly found in the sarcolemma of the muscle [177], but also in postsynaptic densities in different brain areas, such as the cortex and the hippocampus [178]. As a component of the dystrophin-protein complex, in the CNS, Dys has the important role to maintain the structure and stabilize the neuronal synaptic membrane [179].

However, recent evidence show that Dys is also a component of biogenesis of lysosome-related organelles complex-1 (BLOC-1) [180]

which is involved in the biogenesis of melanosomes and platelet-dense granules, components of the endosomal-lysosomal system [181]. Dysbindin and BLOC-1 components, in the CNS, co-localize with synaptic vesicles and synaptosomes where their main function is to control membrane expression and lysosomal delivery of post synaptic receptors [182]. As a component of BLOC-1, Dys localize mainly in synaptic terminals of the striatum, neocortex, cerebellum and hippocampus [177].

BLOC-1 is a complex that consists of different proteins, including pallidin, muted, cappucino, snapin etc..[183], and the mutation or deletion of dysbindin is known not only to affect these subunits, contributing to synaptic and circuit deficits [184], but also to impair the expression of post-synaptic neurotransmitter receptors [183, 185] involved in the pathogenesis of schizophrenia.

In the last fifteen years, Dysbindin-1 has been largely studied because of evidence demonstrating as variation in the DTNBP1 gene was associated with an increasing in the risk to develop schizophrenia.

Moreover, post-mortem studies show that Dysbindin-1 gene mRNA and protein expression were found down-regulated in different brain regions, such as dorsolateral prefrontal cortex, superior temporal gyrus and hippocampal formation of schizophrenic patients [175, 184, 186-188]. Although these evidence, it remains unclear how variation in Dys expression could contribute to the pathogenesis of schizophrenia.

The actual knowledge about the *in vivo* effects of the disruption of Dys expression is based on the studies performed on the so-called "sandy" (*sdy)* mouse [189], which has a spontaneous genetic deletion ($Dys^{-/-}$ or knocked-out (KO)) of exons 6 and 7 of the DTNBP1 gene [187].

The result of this spontaneous mutation is the loss of all the Dys isoforms.

In several studies, $Dys^{-/-}$ mice have shown several hallmarks related to psychiatric diseases [93, 190-192] such as hyperactive behavior [193], altered sensory motor gating [117, 194], deficits in social behavior [195] and spatial learning and memory deficits in the Morris water maze [193]. Moreover, Dys mutants display several cognitive impairments of working memory in a discrete paired-trial T-maze task [117] and in a delayed non-match-to-position operant task [196].

Recent cell biology studies have begun to focus on the physiological function of dysbindin in neurons, since very little is known about the post synaptic activity of this protein [197].

In vitro studies, performed in rat primary cortical neurons, have shown that Dys down-regulation is found to increase the expression of D2R on the cell surface, but not D1R [197]. Moreover, Papaleo and collaborators demonstrated alterations in the response of pyramidal neurons of the mPFC layer II/III following D2R activation in Dys KO mice and an enhanced behavioral response to D_2-like agonist and antagonist, consistent with a higher membrane density of this receptor [117].

Current antipsychotic drugs have a high affinity for both D_2 and D_3 receptors since they show a structural homology of about 80% [124]. Therefore, it's difficult to distinguish the unique contribution of these receptors in behavioral as well as in physiological functions.

Interestingly, in 2015 Schmieg and co-workers demonstrated that the alteration of Dys expression modulates in a similar, but not identical, manner also D3R. In particular, the authors observed that dysbindin-1 co-expression reduced cell surface density of D3R more markedly than for D2R, suggesting a fundamental role of Dys in D3R trafficking [198].

However, is still unknown the mechanism by which Dys modulation of D_2-like receptor intracellular trafficking might interact with D3R signaling.

Aims of the thesis

Objective of this thesis has been to investigate the involvement of D3R in schizophrenia-related symptoms by using a murine models. More specifically, the main aims were:

1. Study the effects of D3R blockade in an animal model of schizophrenia based on NMDA hypofunction;

2. Test the hypothesis that buspirone, a non benzodiazepine anxiolytic drug behaving as D3R antagonist, may exerts antipsychotic-like properties in a preclinical model of schizophrenia;

3. Investigate the involvement of D3R on the effects of buspirone in $D3R^{-/-}$ mice behavioral paradigms ;

4. Determine, with a genetic approach in both humans and mice, the selective contribution of D3R hypofunction in the context of Dys-dependent alteration of D2R-like intracellular trafficking;

5. Assess if the presence of an epistatic (gene-by-gene) interaction between D3R and Dys (DTNBP1) genes affects mPFC-dependent cognitive function in both schizophrenic patients and genetically modified mice in presence or absence antipsychotic drugs;

6. Idenfy if the interaction between D3R and Dys functional genetic variants produces different molecular outcome in mPFC and striatum.

Chapter II

“Buspirone counteracts MK-801-induced schizophrenia-relevant phenotypes through dopamine D3 receptor blockade”

Buspirone counteracts MK-801-induced schizophrenia-relevant phenotypes through dopamine D3 receptor blockade

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Abstract

Several efforts have been made to develop effective antipsychotic drugs. Currently available antipsychotics are effective on positive symptoms, less on negative symptoms, but not on cognitive impairment, a clinically-relevant dimension of schizophrenia.
Drug repurposing offers great advantages over the long-lasting, risky and expensive, de novo drug discovery strategy. To our knowledge, the possible antipsychotic properties of buspirone, an azapirone anxiolytic drug marketed in 1986 as serotonin 5-HT1A receptor (5-HT1AR) partial agonist, have not been extensively investigated despite its intriguing pharmacodynamic profile, which includes dopamine D3 (D3R) and D4 receptor (D4R) antagonist activity. Multiple lines of evidence point to D3R as a valid therapeutic target for the treatment of several neuropsychiatric disorders including schizophrenia. In the present study, we tested the hypothesis that buspirone, behaving as dopamine D3R antagonist, may have antipsychotic-like activity. Effects of acute administration of buspirone was assessed on a wide-range of schizophrenia-relevant abnormalities induced by a single administration of the non-competitive NMDAR antagonist MK-801, in both wild-type mice (WT) and D3R-null mutant mice (D3R-/-).
Buspirone (3 mg·kg-1, i.p.) was devoid of cataleptogenic activity in itself, but resulted effective in counteracting disruption of prepulse inhibition (PPI), hyperlocomotion and deficit of temporal order recognition memory (TOR) induced by MK-801 (0.1 mg·kg-1, i.p.) in WT mice. Conversely, in D3R-/- mice, buspirone was ineffective in preventing MK-801-induced TOR deficit and it was only partially

effective in blocking MK-801-stimulated hyperlocomotion. Taken together, these results indicate, for the first time, that buspirone, might be a potential therapeutic medication for the treatment of schizophrenia. In particular, buspirone, through its D3R antagonist activity, may be a useful tool for improving the treatment of cognitive deficits in schizophrenia that still represents an unmet need of this disease.

Keywords
Buspirone, dopamine d3 receptor, MK-801, Schizophrenia, Antipsychotics, prepulse inhibition, temporal order recognition

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Introduction

Schizophrenia is a chronic and devastating multifactorial mental illness affecting approximately 0.7-1% of population worldwide (Landek-Salgado et al. 2016). The development of secondgeneration antipsychotics has yielded some advances in terms of efficacy, but only modest improvement in addressing the negative symptoms of schizophrenia. To date, no antipsychotics display robust effects on cognitive deficits or impaired social processing that are the most clinically-relevant dimensions of the disease (Owen et al. 2016). Drug repositioning refers to the process of finding new uses for already approved and commercialized medications and it is thought to offer great advantages over the long-lasting, risky and expensive de novo drug discovery strategy. This is because the pharmacological and toxicological profiles of approved medications are well-characterized (Ashburn and Thor, 2004). It has been suggested that repositioned drugs may represent effective alternative compounds for the treatment of neuropsychiatric disorders for which the classical drug discovery process is hampered by the poor knowledge of the pathophysiological mechanisms (Lee and Kim, 2016). In this context, the azapirone anxiolytic drug buspirone (Buspar®), has been proposed for the treatment of substance use disorder (SUD,Leggio et al. 2016). Regarding schizophrenia, earlier clinical trials suggested that buspirone added to both typical and atypical antipsychotics ameliorates negative symptoms (Ghaleiha et al. 2010; Sheikhmoonesi et al. 2015), while other preclinical and clinical data showed buspirone as scarcely effective in improving cognitive dysfunction (Horiguchi and Meltzer, 2012; Maeda et al. 2014; Piskulić et al. 2009).

At a pharmacological level, buspirone, besides its claimed 5-HT1AR partial agonist activity, is endowed with D3R/D4R antagonist activity and binds to dopamine D2 receptor (D2R) with an affinity 5-fold lower than for D3R (Bergman et al. 2013). Available evidence indicates that D3R can be considered as a new validated pharmacological target for the treatment of several neuropsychiatric disorders, including SUD, Parkinson's disease, depression and schizophrenia (Sokoloff and Le Foll, 2016; Maramai et al. 2016; Leggio et al. 2016). The restricted localization of D_3Rs in the limbic system, particularly in the nucleus accumbens (NAc), has attracted great interest especially for the development of safe and effective medications devoid of the classical side effects (extrapyramidal side effects and prolactin elevation) caused by D_2R blockade (Gross and Drescher, 2012). In fact, antipsychotics targeting D_3R, such as blonanserin and cariprazine, have been demonstrated effective in treating positive and negative symptoms with a good safety profile (Hori et al. 2014; Leggio et al. 2016; Earley et al. 2017). Beside the

high expression in NAc, D_3Rs are expressed specifically in the layer 5 pyramidal neurons of medial prefrontal cortex (mPFC,Lidow et al. 1998), where they control in a peculiar manner neuronal excitability (Clarkson et al. 2017). D_3Rs play a fundamental role in physiological mechanisms underlying mPFC-dependent cognitive functions as well as in crucial pathophysiological processes sub serving mPFC-dependent cognitive dysfunctions (Nakajima et al. 2013). In particular, it seems that selective antagonism on D_3R improves cognitive functions while selective agonism exerts opposite, detrimental effects (Watson et al. 2012) . Recently, it has been proposed that molecules joining $5HT_{1A}$R partial agonism and 5-HT_{2A} antagonism to D_3R antagonism may exhibit stronger antipsychotic effects (Brindisi et al. 2014). As aforementioned, the pharmacological profile of buspirone largely matches that of these potential antipsychotics. However, as far as we know, the antipsychotic properties of buspirone have not yet been extensively elucidated. In the present study, we tested the hypothesis that buspirone, behaving as dopamine D_3R antagonist, may exert antipsychotic-like properties in a preclinical model of schizophrenia, based on NMDAR hypofunction. This pharmacological model, as compared with dopamine-based models, appears to more efficiently recapitulate several symptoms of schizophrenia, particularly those related to cognitive dysfunction (Kantrowitz and Javitt, 2010). The effect of acute administration of buspirone was evaluated on hyperlocomotion, prepulse inhibition (PPI) disruption and temporal order recognition (TOR) memory impairment, elicited by acute administration of the non-competitive NMDAR antagonist MK-801 in WT mice. In order to assess the involvement of D_3R on the effect of buspirone, the same behavioral paradigms, with or without buspirone, were applied to $D_3R^{-/-}$ mice.

Materials and methods

Animals and housing

In these experiments, $D_3R^{-/-}$ mice and their WT littermates (males, 8–12 weeks old), bred by a heterozygous ($D_3R^{+/-}$ x $D_3R^{+/-}$) mating strategy, were tested. Animals were group-housed (2–5 mice per cage), with free access to chow and water, in an air-conditioned room, with a 12-h light– dark cycle. D_3R mutant mice were 10th–12th generation of congenic C57BL/6J mice, generated by a back-crossing strategy (Accili et al, 1996). Genotypes were identified by PCR analysis of tail DNA as previously described (Leggio et al. 2015). The experimenters handled animals on alternate days during the week preceding the behavioral tests. Animals were acclimatized to the testing room at least one hour before the beginning of the tests. All experiments were performed during the dark phase and in according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of the Catania University.

Drugs

(+)MK-801 hydrogen maleate and buspirone hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in saline. Clozapine and haloperidol (Sigma-Aldrich) were dissolved in few drops of 1 N HCl and further diluted with saline; the pH was adjusted to 7 with $NaHCO_3$. All drug solutions were prepared daily and intraperitoneally (i.p.) administered by using an injection volume of 10 ml/kg.

Behavioral testing

Temporal order recognition (TOR) test

The TOR test was carried out as previously described (Barker et al. 2011; Managò et al. 2016) with minor modifications. Animals explored in an evenly illuminated (9 ± 1 lux) square open field (40 x 40 x 40 cm, Ugo Basile, Gemonio, Italy) in which the floor was covered with sawdust. The behavior of animals was recorded using a video camera (Sony Videocam PJ330E) and then scored by an independent observer. The objects presented were made of plastic Duplo blocks (Lego®), different in shape, color, and size (9 x 8 x 7 cm to 12 x 11 x 10 cm) and too heavy to be moved by the mice. After one week of handling, a 4-day pretesting procedure was carried out. Mice were habituated to the empty arena for 10 min on the day 1 and 2. Afterwards, on the day 3 and 4, mice were i.p. injected with saline 20

min before being placed in the arena containing two objects (different from those ones eventually used during the test) for 10 minutes. This pretesting procedure was performed in order to minimize stress-related behavior induced by injections as well as to prevent neophobia during the test. The objects were located in two corners of the arena, 10 cm far from the sidewalls. The test consisted of two sample phases and one test trial (Fig. 1A). During the sample phases, animals were allowed to explore two copies of an identical object for a total of 5 min. Different objects were used for each sample phase, with a delay between the sample phases of 1 h. The test trial was performed 3h after the sample phase 2. During the test trial (5 min duration), animals were exposed to a third copy of the objects from sample phase 1 and a third copy of the objects from sample phase 2. Objects were cleaned with a 10% ethanol solution in between each test in order to avoid olfactory cues. Any feces were removed and the sawdust was shaken in order to equally redistribute any odor cues. If the temporal order memory is intact, animals should spend more time exploring the object from sample 1, the less recently experienced object, compared with the object from sample 2, the more recently experienced object. The objects utilized in each sample phase as well as the positions of the objects during the test were counterbalanced between the animals. Exploratory behavior was defined as the animal directing its nose toward the object at a distance of < 2 cm. Looking around while sitting, climbing or rearing against the objects were not considered as exploration. Animals that failed to complete a minimum of 2 seconds (sec) of exploration in each phase of the task were excluded from the analysis. Discrimination between the objects was calculated using a discrimination ratio (DR) that takes into account individual differences in the total amount of exploration. In particular, data are depicted as DR, calculated as [(less recently experienced object exploration time − more recently experienced object exploration time)/total exploration time]. The higher is the DR, the better is TOR memory.

Acoustic startle response and prepulse inhibition (PPI) test

Acoustic startle response and PPI were measured using four PPI sets from SR-Lab Systems (San Diego Instruments, San Diego, CA, USA). The experimental procedure was adapted from Papaleo et al. (2012). Animals were exposed to a short “matching” startle session before the PPI testing. They were placed in the startle chambers for a 5-min acclimation period with a 65 dB(A) background noise, and then exposed to a total of 17 acoustic startle stimulus (pulse) trials (40 ms — 120 dB (A) noise bursts) that were interspersed with 3 acoustic prepulse plus acoustic pulse trials in which the pulse was preceded

100 ms (onset-to-onset) by a 20 ms noise burst, 10 dB above background. Animals were assigned to each drug dose group based on average PPI% from the matching session to ensure similar baseline PPI levels between experimental groups. The PPI test session, with or without drug treatment, was carried out 5-7 d after the matching session. The animals were placed in the startle chambers for a 5-min acclimation period with a 65 dB(A) 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 five trial types were acoustic prepulse plus acoustic startle stimulus trials. Prepulse tones were 20 ms at 70, 75, 80, 85 and 90 presented 100 ms before the startle stimulus. PPI was calculated by using the following formula: 100 Å~ {[pulse-only units − (prepulse + pulse units)]/(pulse-only units)}.

Open field (OF) test

Animals were tested in the same square open field mentioned above (divided into sixteen quadrants by lines on the floor) over a 30 minute-period. Locomotor activity was 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 numbers of lines crossed (crossings) with all four paws (Accili et al. 1996). The behavior of animals was recorded by using a video camera and eventually analyzed by one observer blinded to genotype/treatment.

Catalepsy test

The catalepsy test was carried out as previously reported with minor changes (Fink-Jensen et al. 2011). The apparatus was made of 2 wooden supports linked by a steel bar (lenght: 7.5 cm; diameter 0.9 cm); The system was stabilized by another wooden support opposite to the steel bar. The catalepsy was evaluated by placing the animals with the forepaws on the horizontal steel bar positioned 4.5 cm above the floor. Animals were tested at different time points: 30, 60, 90 and 120 min after the pharmacological treatment. The latency (cut off time) was 600 sec. The end point of the test was considered when both forepaws were removed from the bar or when the animal moved its head in exploratory manner. Each trial was repeated for three times and the highest time value was taken.

Experimental Design

The behavioral effects of a single injection of buspirone were evaluated on MK-801-induced schizophrenia-like phenotypes both in WT and in $D_3R^{-/-}$ mice. These effects were compared to those of clozapine, the most effective commercially available antipsychotic (Owen et al. 2016), injected at a dose of 1 $mg.kg^{-1}$. This dose has been revealed to be effective in ameliorating cognitive dysfunction (Mutlu et al. 2011; Park et al. 2014). The dose of buspirone (3 $mg.kg^{-1}$) was selected based on our previous experience (Leggio et al. 2014) as well as according to a work by Di Ciano et al. (2017). To avoid effects of test-related anxiety, animals were divided into independent cohorts and subjected to the most stressful tests as the last. Animals were tested as follows: WT,cohort 1, open field test, catalepsy test; WT, cohort 2, TOR test, PPI test; $D_3R^{-/-}$, cohort 2, open field, TOR test. A washout period of at least seven days was given between each experimental procedure.

Experiment 1 - Effect of Buspirone on MK-801-induced TOR memory deficit in WT mice.

Administration of NMDAR antagonists before the sample phase 2 impairs TOR memory affecting both reconsolidation and consolidation mechanism (Warburton et al. 2013). Therefore, buspirone, clozapine and MK-801 were administered 45 min, 30 min and 20 min, respectively, before the sample phase 2. The chosen dose of MK-801 (0.1 $mg.kg^{-1}$) is able to produce cognitive impairment without inducing locomotor disturbance (stereotypies, ataxia; Blot et al. 2015).

Experiment 2 - Effect of buspirone on MK-801-stimulated hyperlocomotion and assessment of catalepsy in WT mice.

Mice received injections of buspirone, clozapine and MK-801 with the same timing of treatment used for the TOR test and then placed into the empty open field. The dose of 0.1 $mg.kg^{-1}$ MK- 801 is effective in stimulating hyperlocomotion (Zhang et al. 2007). For the catalepsy test, animals were injected with buspirone, clozapine and haloperidol (1 $mg.kg^{-1}$) and then tested at different time points (30, 60, 90 and 120 min). The haloperidol-induced catalepsy at the dose of 1 $mg.kg^{-1}$ is a widely-used model for the evaluation of extrapyramidal side effects induced by drugs (Pogorelov et al. 2017).

Experiment 3 - Effect of buspirone on MK-801-induced PPI disruption in WT mice.

Mice were given injections of buspirone, clozapine and MK-801, 45 min, 30 min and 20 min (including the 5-min acclimation period), respectively, before to be placed in the startle chambers for the PPI test. We chose the dose of 0.1 $mg.kg^{-1}$ MK-801 because this dose is sufficient to disrupt PPI (Spooren et al. 2004; Zhang et al. 2007).

Experiment 4 - Effect of buspirone on MK-801-induced TOR memory deficit and hyperlocomotion in $D_3R^{-/-}$ mice.

To figure out whether or not the effects of buspirone were mainly mediated via the blockade of D_3R, we tested $D_3R^{-/-}$ mice (open field and TOR) treated with the same pharmacological treatment carried out in WT mice, both in terms of doses and timing of treatment. Unfortunately, we could not evaluate the effect of buspirone on PPI test because the vast majority of $D_3R^{-/-}$ mice exhibited a very low acoustic startle reactivity during the startle matching session (data not shown). This made difficult the assembling of experimental groups with similar PPI%.

Statistics

Statistical analysis was performed by using graphpad prism 7 (graphpad software La Jolla, CA, USA). In the TOR experiments, one-way ANOVA with treatment as between-subject factor was used to determine the main effect. Acoustic startle reactivity was analyzed by performing a twoway ANOVA with acoustic startle stimulus as a within-subjects factor and treatment as a between-subjects factor. To analyze PPI%, a two-way repeated-measures ANOVA with prepulse intensity as a within-subjects factor and treatment as a between-subjects factor was carried out. Changes in locomotor activity (number of crossings for each time-point) as well as induction of catalepsy were assessed by performing a two-way repeated-measures ANOVA with time-point as a within-subjects factor and treatment as a between-subjects factor. A one-way ANOVA with treatment as between-subject factor was carried out for the assessment of the total number of crossings. For all data analyses, upon confirmation of significant main effects, differences among individual means were assessed using the Newman–Keuls' post hoc test. For all analyses, significance was accepted with a p value less than 0.05. Standard error of the mean (s.e.m.) and variance were found similar between groups. All data are presented as mean ± s.e.m.

Results

Buspirone counteracted MK-801-induced memory deficits in WT mice tested in the TOR paradigm

The discrimination performance of WT mice was significantly affected by pharmacological treatments, during the test phase of the TOR test (main effect of treatment, $F_{(5, 45)} = 5.374$, $P = 0.0006$, n= 8/10 per group). MK-801 induced a strong TOR memory impairment. Indeed, veh + MK-801-treated WT mice exhibited a greater preference in exploring the more recently experienced object in comparison with veh + veh-treated WT mice, which, as expected, spent more time exploring the less recently experienced object (post-hoc analysis: $P < 0.001$ vs veh + veh group; fig 1B). Worthy of note,, bus + MK-801-treated WT mice explored significantly more the less recently experienced object than the more recently one in a similar manner as veh + veh-treated WT mice (post-hoc analysis: $P < 0.01$ vs veh + MK-801 group; $P > 0.05$ vs veh + veh group; fig 1B). Thus, buspirone efficiently prevented MK-801-induced TOR memory impairment. Clo + MK-801-treated WT mice did not show an optimal discrimination performance even though they performed significantly better than veh + MK-801-treated WT mice and not differently from the veh + veh-treated WT mice (post-hoc analysis: $P < 0.05$ vs veh + MK-801 group; $P > 0.05$ vs veh + veh group; fig 1B). Both buspirone and clozapine, when injected alone, had no effect on discrimination performance (post-hoc analysis: $P > 0.05$ vs veh + veh group ; fig 1C).

Buspirone blocked MK-801-stimulated hyperactivity and did not cause catalepsy in WT mice.

The pharmacological treatments significantly modified the locomotor activity of WT mice during each 5-min time-point (main effects of treatment, $F_{(5, 54)} = 10.42$, $P < 0.0001$; time-point, $F_{(5, 270)} = 4.274$, $P = 0.0009$; treatment x time-point interaction, $F_{(25, 270)} = 6.18$, $P < 0.0001$; n = 9/11 per group). In addition, ANOVA showed a significant main effect of treatment ($F_{(5, 54)} = 10.42$, $P < 0.0001$) on the total crossings over 30 min for WT mice. As expected, MK-801 produced a strong hyperlocomotion in WT mice. Indeed, veh + MK-801-treated WT mice performed a significant higher number of crossings compared to veh + veh-treated WT mice (post-hoc analysis: 5-min: $p < 0.01$; from 10-min to 30-min $p < 0.001$ fig 2A, C). Interestingly, buspirone did not alter per se the locomotor activity (post-hoc analysis: all time-points $p > 0.05$ vs veh + veh group; fig 2B, D), but it completely

blocked MK-801-induced hyperactivity. Bus + MK-801-treated WT mice performed a significant lower number of crossings compared to veh + MK-801-treated WT mice (post-hoc analysis: all time-points: $p < 0.001$ vs veh + MK-801 group; fig 2A, C), displaying a locomotor activity similar to that of veh + veh-treated WT mice (post-hoc analysis: All time-points: $p > 0.05$ vs veh + veh group; fig 2B, D). Clozapine did not modify per se the locomotor activity (post-hoc analysis: all time-points: $p > 0.05$ vs veh + veh group; fig 2B, D), but it significantly prevented MK-801-induced hyperactivity only in the first 10 min, loosing progressively its efficacy from the 15-min time point to the end of the test (post-hoc analysis: 5-min: $p < 0.001$; 10-min: $p < 0.05$ vs veh + MK-801 group. 15-min: $p < 0.01$; from 20-min to 30-min $p < 0.001$ vs veh + veh group; fig 2A, C). Regarding the catalepsy test, significant main effects of treatment ($F_{(3, 20)} = 48.11$, $P < 0.0001$, n= 6 per group) and time-point ($F_{(3, 60)} = 21.70$, $P < 0.0001$), together with a significant treatment x time-point interaction ($F_{(3, 60)} = 21.77$, $P < 0.0001$) were found on the duration of catalepsy. As expected, haloperidol caused a severe catalepsy state (post-hoc analysis: all time-points: $P < 0.001$ vs veh group; fig 2E), an effect not induced by clozapine or buspirone (post-hoc analysis: all time-points: $P > 0.05$ vs veh group; fig 2E).

Buspirone blocked MK-801-induced PPI disruption in WT mice.

In the assessment of acoustic startle reactivity, ANOVA revealed a main effect of acoustic startle stimulus ($F_{(1, 110)} = 76.42$, $P < 0.0001$, n= 8/11 per group) but not a main effect of treatment ($F_{(5, 110)} = 1.36$, $P = 0.2450$) or a significant interaction between the factors ($F_{(5, 110)} = 1.048$, $P = 0.3933$). Except for clozapine, which per se significantly decreased the acoustic startle reactivity at 120-dB (post-hoc analysis at120 dB stimulus: $P < 0.05$ vs veh + veh group; fig 3B), all other experimental groups displayed similar acoustic startle reactivity (post-hoc analysis: $P > 0.05$ vs veh + veh group; fig 3A and 3B). With regard to the PPI test, there were significant main effects of treatment ($F_{(5, 55)} = 3.525$, $P = 0.0078$) and prepulse intensity ($F_{(4, 220)} = 49.16$, $P < 0.0001$) but not a significant treatment x prepulse intensity interaction ($F_{(20, 220)} = 1.4$, $P = 0.1238$). As expected, MK-801 significantly disrupted PPI; veh + MK-801-treated WT mice showed a progressively lower PPI% that reached statistical significance at 80 dB prepulse intensity , (posthoc analysis: $P < 0.01$ vs veh + veh group; fig 3C). Interestingly, buspirone, which had no effect on PPI when administered alone (post-hoc analysis: $P > 0.05$ vs veh + veh group;

fig 3D), completely blocked MK-801-induced PPI disruption. Bus + MK-801-treated WT mice exhibited PPI%, significantly greater than veh + MK-801-treated WT mice at 80 dB prepulse intensity, and similar to veh + veh-treated WT mice at all prepulse intensities (post-hoc analysis: $P < 0.01$ vs veh + MK-801 group, $p > 0.05$ vs veh + veh group; fig 3C). Noteworthy, clozapine per se disrupted PPI (post-hoc analysis: 75 and 80 dB prepulse: $p < 0.05$ vs veh + veh group; fig 3D), but did not block MK-801-induced PPI disruption (post-hoc analysis at 80 dB prepulse: $p < 0.05$ vs veh + veh group and $p > 0.05$ vs veh + MK-801 group; fig 3C).

Buspirone was ineffective in preventing MK-801-induced TOR memory deficit and hyperlocomotion in D3R-/- mice.

The memory of $D_3R^{-/-}$ mice was significantly affected by pharmacological treatments, during the test phase of the TOR test (main effect of treatment $F_{(3, 18)} = 7.478$, $P = 0.0019$, n= 5/6 per group). MK-801 produced a marked TOR memory deficit in $D_3R^{-/-}$ mice, comparable to that observed in WT mice. In particularly, veh + MK-801-treated $D_3R^{-/-}$ mice significantly preferred exploring the more recently experienced object than the less recently one in contrast with veh + veh-treated $D_3R^{-/-}$ mice that displayed an intact TOR memory and behaved in the opposite way (post-hoc analysis: $P < 0.01$ vs veh + veh $D_3R^{-/-}$ group; fig 4A). Consistent with the hypothesis that buspirone acts on D_3R receptors, bus + MK-801-treated $D_3R^{-/-}$ mice behaved in a manner similar to veh + MK-801-treated $D_3R^{-/-}$, showing the same TOR memory impairment (post-hoc analysis: $P < 0.001$ vs veh + veh $D_3R^{-/-}$ group, $P > 0.05$ vs veh + MK-801 $D_3R^{-/-}$; fig 4A), i.e. in $D_3R^{-/-}$ buspirone was unable to prevent MK-801-induced TOR memory impairment as it did in WT mice. Notice that at variance with what observed in WT mice, in $D_3R^{-/-}$ buspirone on its own disrupted the discrimination of the experienced objects, though not in a significant manner (posthoc analysis: $P > 0.05$ vs veh + veh $D_3R^{-/-}$ group; fig 4B). In the OF test, MK-801 produced also a robust and persistent hyperlocomotion in $D_3R^{-/-}$ mice; significant main effects of treatment ($F_{(3, 35)} = 11.74$, $P < 0.0001$, n= 8/11 per group) and timepoint ($F_{(5, 175)} = 15.92$, $P < 0.0001$), and a significant treatment x time-point interaction ($F_{(15, 175)} = 5.123$, $P < 0.0001$) were found on locomotor activity of $D_3R^{-/-}$ mice during each 5-min time-point. Moreover, ANOVA revealed a significant main effect of treatment ($F_{(3, 35)} = 11.34$, $P < 0.0001$) on the total crossings that $D_3R^{-/-}$ mice performed throughout the 30 min of the test. Veh + MK-801- treated $D_3R^{-/-}$ mice, compared to veh + veh-treated $D_3R^{-/-}$ mice, carried out a

significant higher number of crossings (post-hoc analysis at 5-min: $p < 0.05$; from 10-min to 30-min: $p < 0.001$; fig 4C, E) . Buspirone, which was devoid of effect when injected alone (post-hoc analysis: all timepoints $p > 0.05$ vs veh + veh $D_3R^{-/-}$group; fig 4D, F), significantly attenuated MK-801-stimulated hyperlocomotion, but its effect diminished from the 10-min time point on. Indeed, Bus + MK-801-treated $D_3R^{-/-}$ mice performed a number of crossings similar to that of veh + veh-treated $D_3R^{-/-}$ mice but significantly lower compared to veh + MK-801-treated $D_3R^{-/-}$ mice during the first 10 minutes (post-hoc analysis at 5-min: $p < 0.001$; from 10-min to 20-min: $p < 0.01$; at 25-min and 30-min: $p < 0.05$ vs veh + MK-801 $D_3R^{-/-}$group. Fig 4C, E).

Discussion

These results provide the first evidence that buspirone counteracts a wide-range of schizophrenia-relevant phenotypes through its antagonism at D_3R. To investigate the antipsychotic properties of buspirone, we chose a pharmacological model based on NMDAR hypofunction triggered by acute administration of the NMDAR antagonist MK- 801. Although not devoid of limitations, this model is extensively employed for the assessment of potential antipsychotic activity of investigational compounds (Bubenikova et al. 2008; Adell et al. 2012). Indeed, NMDAR dysfunction may recapitulate “core” symptoms of schizophrenia, particularly the multiplicity of cognitive deficits, more faithfully than dopamine-based models (Kantrowitz and Javitt, 2010). Cognitive deficits observed in schizophrenic patients have been strongly associated with an abnormal PFC activity (Drisien et al. 2008). Earlier studies indicated that the cognitive impairment induced by MK-801 arises from an intensification of the discharge of mPFC pyramidal neurons, triggered via NMDAR blockade in inhibitory interneurons of mPFC and hippocampus (HP, Homayoun and Moghaddam 2007; Jodo et al. 2005). D_3Rs are expressed specifically in layer 5 pyramidal neurons of mPFC of both primate and rodents (Lidow et al. 1998) and modulate uniquely the neuronal excitability (Clarkson et al. 2017). Consequently, D_3Rs play a fundamental role in prefrontal-dependent cognitive functions (Nakajima et al. 2013). Studies on dopamine receptor-specific reporter gene mice further revealed an abundant expression of D_3Rs in HP (www.gensat.org); furthermore, hippocampal lesions leave single item object recognition memory intact, while impair temporal order memory (Warburton and Brown, 2015). Based on these premises,, we assessed the effect of buspirone in the TOR memory task. This behavioural task depends on interconnections among mPFC, perirhinal cortex (PRH) and HP (Barker et al. 2011; Managò et al.

2016) and is used to measure recency discrimination, a cognitive function impaired in schizophrenic patients (Schwartz et al. 1991; Rizzo et al. 1996). To our knowledge, this is the first study demonstrating that acute systemic administration of MK-801 at the dose of 0.1 mg.kg^{-1}, markedly impairs TOR memory in mice. Therefore, our results confirm the face validity of the pharmacological model based on NMDAR hypofunction triggered by acute administration of the NMDAR antagonist MK-801, being also consistent with earlier findings showing a disruption of TOR memory following intra-PRH or intra-mPFC infusion of the selective NMDAR antagonist AP5 (Warburton et al. 2013).We found that buspirone prevented MK-801-induced TOR memory impairment in WT mice even better than clozapine. Very interestingly, this effect was completely abolished in $D_3R^{-/-}$ mice. Thus, these data provide the first evidence that buspirone may be effective in treating cognitive deficits in schizophrenia, and that its efficacy against MK-801- induced cognitive dysfunction relies exclusively on D_3Rs blockade. These findings are particularly relevant, considering that cognitive dysfunction represents a major challenge in the pharmacological treatment of schizophrenic patients. Furthermore, our results are consistent with previous studies, reporting that some antipsychotics that behave as selective D_3R antagonists or D_3R preferring partial agonists enhance cognitive functions in schizophrenia (Nakajima et al. 2013; Zimnisky et al. 2013). Recently, Barker and colleagues (2017) discovered that the pharmacogenetic deactivation of a specific neuronal circuit originating in the dorsal CA1 region of HP and projecting to mPFC, selectively disrupts TOR memory in mice. Thus, we speculate that a glutamatergic/dopaminergic imbalance in specific neuronal circuits connecting HP and mPFC might disrupt the connection between these two brain areas, leading to memory impairment in mice tested in the TOR paradigm. In this context, D_3R blockade, particularly in mPFC and HP, might prevent the hyperactivity of the dopaminergic system subsequent to NMDARs hypofunction (Snyder and Gao, 2013). However, because $D_3R^{-/-}$ mice appeared to be as sensitive as WT mice to the cognitive effects of acute administration of MK-801, other neurotransmitters and/or dopamine receptor subtypes are likely to be involved, and may represent compensatory mechanisms that prevails over D_3R control in $D_3R^{-/-}$ mice. Hyperactivity is a valuable correlate, easily modelled in rodents, widely associated with positive symptoms and psychomotor agitation in most schizophrenic patients (Jones et al. 2011). Here, we found that buspirone blocked MK-801-stimulated hyperactivity, but did not cause catalepsy in WT mice; moreover, because the preventing effect of buspirone on MK-801-stimulated hyperactivity was absent in $D_3R^{-/-}$ mice, it moust be, at least in part, attributable to D_3R

antagonism. This conclusion is consistent with earlier studies showing D_3R antagonists as effective on MK-801-stimulated hyperactivity (Leriche et al. 2003; Brindisi et al. 2014; Sun et al. 2016). Considering that positive symptoms are not well-managed in a considerable number of patients suffering from schizophrenia (Miyamoto et al. 2012), our observation, together with other published reports, points to D_3R as potential target to treat hyperactivity.; We cannot exclude a contribution of other receptors targeted by buspirone in mediating its antipsychotic-like effects in our experimental paradigms. Buspirone in fact, binds to 5-$HT_{1A}R$, where it behaves as potent partial agonist (Bergman et al .2013), and Several studies have reported that 5-$HT_{1A}R$ antagonists or partial agonists attenuates psychotomimetic effects of MK- 801 (Wedzony et al.2000; Bubenikova-Valesova et al. 2010). Furthermore, buspirone also binds to D_4R with high affinity and behaves as antagonist (Bergman et al. 2013). A highly selective dopamine D_4R antagonist was found to decrease amphetamine-induced hyperlocomotion (Boeckler et al. 2004). Consequently, we cannot exclude a contribute of D_4R in the effects we reported here. PPI is a valuable model to study the sensorimotor gating disruption classically observed in schizophrenia (Papaleo et al. 2012). Because animals and humans are tested in a similar way, this model have face, construct, and predictive validity and is widely employed to identify potential antipsychotic properties of recently developed drugs (Rigdon and Viik, 1991). Our findings demonstrated that buspirone, devoid of effect by itself, completely counteracted PPI disruption dependent on NMDAR hypofunction. These results are partially in agreement with previous studies showing that buspirone weakly counteracts apomorphine-induced PPI disruption (Rigdon and Viik, 1991) while it was without effect on its own (Van den Buuse and Gogos, 2007). The antipsychotic-like effect of buspirone on MK-801-induced PPI disruption might be mainly driven by its antagonist activity at D_3R. Several reports proved that selective D_3R antagonists improve PPI disruption in different preclinical models of schizophrenia (Zhang et al. 2006; Sun et al. 2016; Maramai et al. 2016). Unfortunately we could not directly address the D_3R involvement on the buspirone's effect in PPI by using D3R-/-, because these mice did not exhibit a robust acoustic startle reactivity, suitable for making reliable measurements. However, it is unlikely that the 5 $HT_{1A}R$ partial agonist activity of buspirone could contribute to its efficacy on MK-801-induced PPI disruption. Bubenikova-Valesova and colleagues (2010) found the selective 5-$HT_{1A}R$ partial agonist tandospirone exacerbates MK-801-induced PPI disruption and other groups reported a PPI disruption after 5-$HT_{1A}R$ stimulation (Rigdon and Weatherspoon, 1992; Gogos and Van den Buuse, 2003; Gogos et al. 2006). Again, we cannot exclude the possible involvement of the D_4R blockade also in

the effect of buspirone in MK-801-induced PPI disruption. However, contrasting results have shown positive/negative effects of D_4R antagonists in ameliorating apomorphine-induced PPI disruption (Mansbach et al. 1998; Boeckler et al. 2004; Bristow et al. 1997). Worthy of note, clozapine did not prevent MK-801-induced PPI disruption or MK-801-stimulated hyperlocomotion. Considering that clozapine is one of the most effective antipsychotic drugs, the discrepancy with its poor efficacy in preclinical models point once more to the need for defining "gold pharmacological standards" preclinical models of schizophrenia (Jones et al. 2011), taking into account that doses, strains, behavioural paradigms, all affect the variability, reproducibility and translability to clinical settings. In conclusion, the present study demonstrates that buspirone, a drug currently approved for the treatment of anxious disorders, might be a potential antipsychotic medication and also that D_3R represents a valuable pharmacological target especially for the treatment of cognitive deficits in schizophrenia. Anxious symptoms and cognitive impairment frequently co-occur especially in the prodromal phase of the disease, when the positive symptoms are below the threshold for psychosis (Corigliano et al. 2014). In this scenario buspirone might represent a new pharmacological tool to treat this early phase of the disease. These findings are particularly relevant considering that a substantial number of pharmaceutical industries are turning away from developing antipsychotics for many reasons including costs, unclear disease mechanisms and long-lasting developmental processes. Repositioning of buspirone could represent a novel treatment for schizophrenia. However, further studies are needed to evaluate the efficacy of this drug after chronic treatment in an animal model provided with the three criteria of face, construct and predictive validity.

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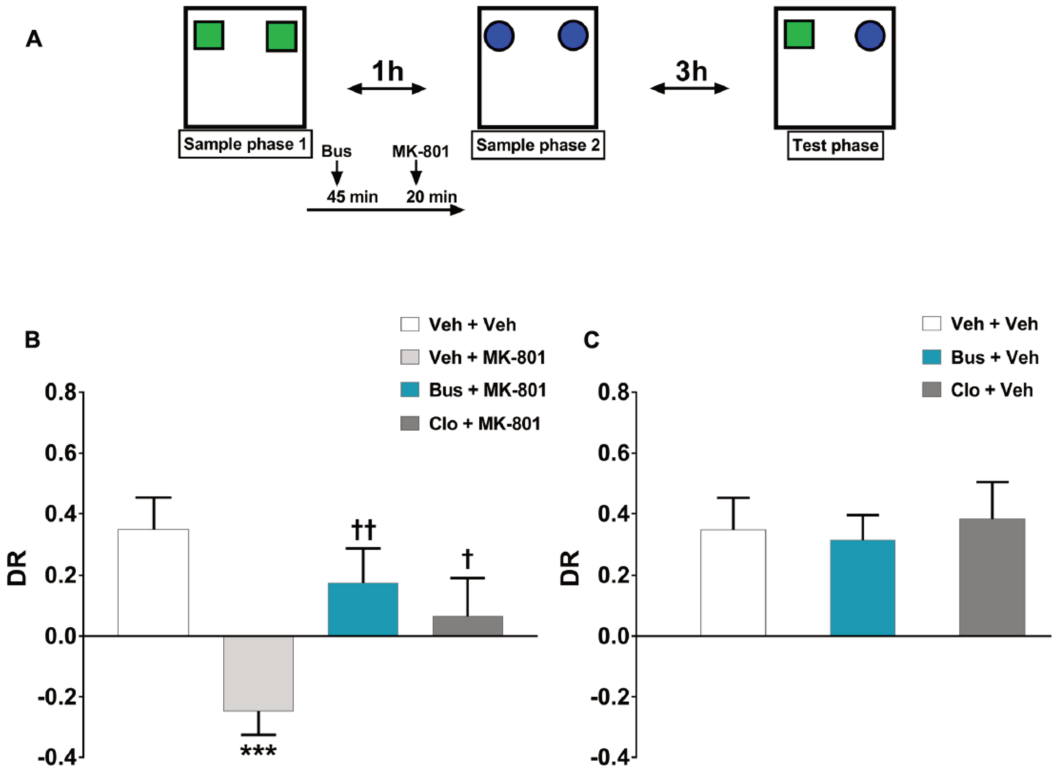

FIGURE 1 | Buspirone counteracted MK-801-induced TOR memory impairment in WT mice.

(**A**) Cartoon illustrating the TOR test and the schedule of treatment. Buspirone (Bus, 3 mg kg^{-1}, i.p.) or vehicle (Veh), clozapine (Clo, 1 mg/kg^{-1}, i.p.) or Veh and MK-801 (0.1 mg kg^{-1}, i.p.) or Veh were injected 45, 30, and 20 min respectively, before the sample phase 2. (**B,C**) Discrimination ratio (DR) displayed by Veh + Veh (n = 10), Veh + MK-801 (n = 10), Bus + MK-801 (n = 9), Clo + MK-801 (n = 9), Bus + Veh (n = 8), Clo + Veh (n = 5) WT mice during the test phase. Data are shown as mean SEM. DR [(less recently experienced object exploration time - more recently experienced object exploration time)/total exploration time]. $p < 0.001$ vs. Veh + Veh WT mice; ††$p < 0.01$ and †$p < 0.05$ vs.

Veh + MK-801 WT mice (One-way ANOVA and Newman–Keuls post hoc test).

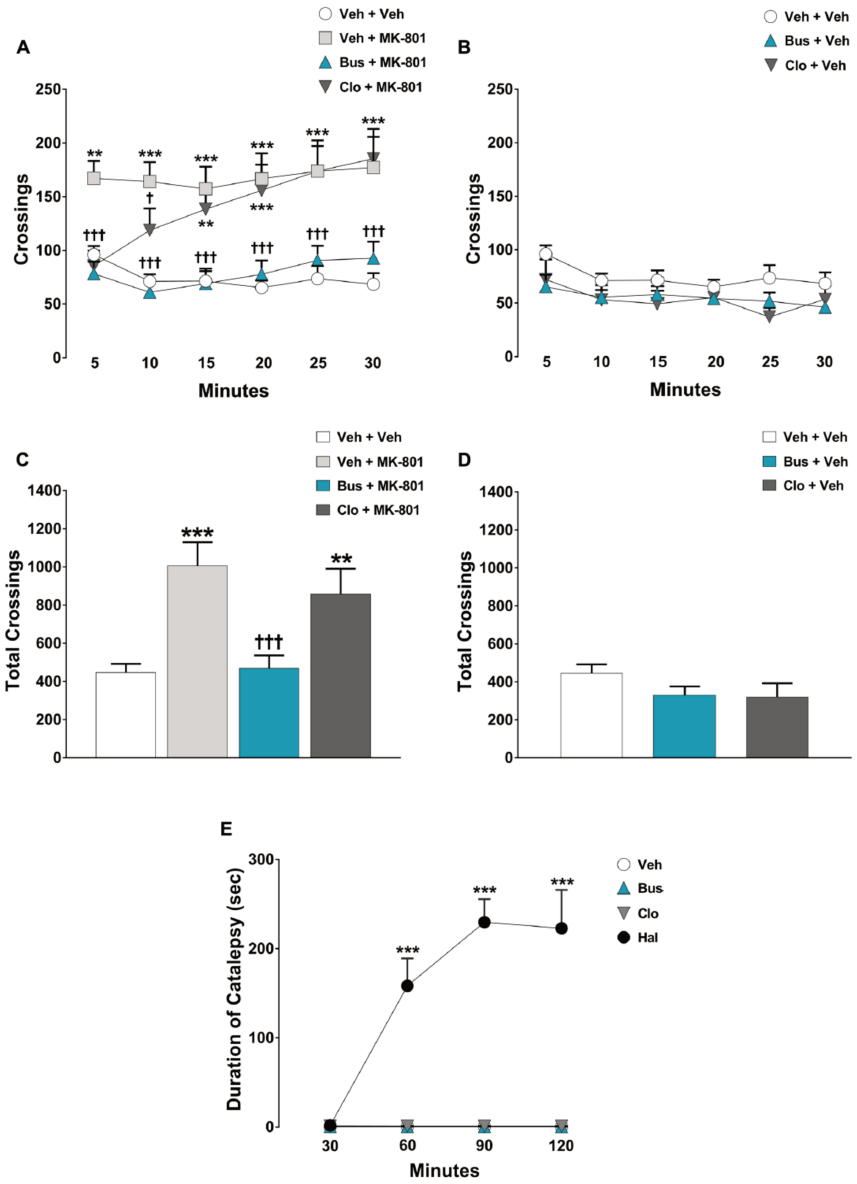

FIGURE 2 | Buspirone blocked MK-801-stimulated hyperlocomotion, but did not cause catalepsy in WT mice.

Buspirone (Bus, 3 mg kg^{-1}, i.p.) or vehicle (Veh), clozapine (Clo, 1 mg kg^{-1}, i.p.) or Veh and MK-801 (0.1 mg kg^{-1}, i.p.) or Veh were injected 45 min, 30 min and 20 min respectively, before the open field. (**A,B**) Locomotor activity (crossings) at each 5-min time point displayed by Veh + Veh (n = 11), Veh + MK-801 (n = 11), Bus + MK-801 (n = 10), Clo + MK-801 (n = 10), Bus + Veh (n = 9), Clo + Veh (n = 9) WT mice. (**C,D**) Locomotor activity (crossings) over a 30-min test period displayed by the same mice. (**E**) Duration of catalepsy state 30, 60, 90, and 120 min after drug injection (n = 6 animals/group). Haloperidol (Hal, 1 mg kg^{-1}) was used as positive control. Data are shown as mean SEM. $p < 0.001$, $p < 0.01$ vs. Veh + Veh WT mice; †††$p < 0.001$ and †$p < 0.05$ vs. Veh + MK-801 WT mice; $p <$ 0.001 vs. Veh (Two-way repeated-measures ANOVA and Newman–Keuls post hoc test).

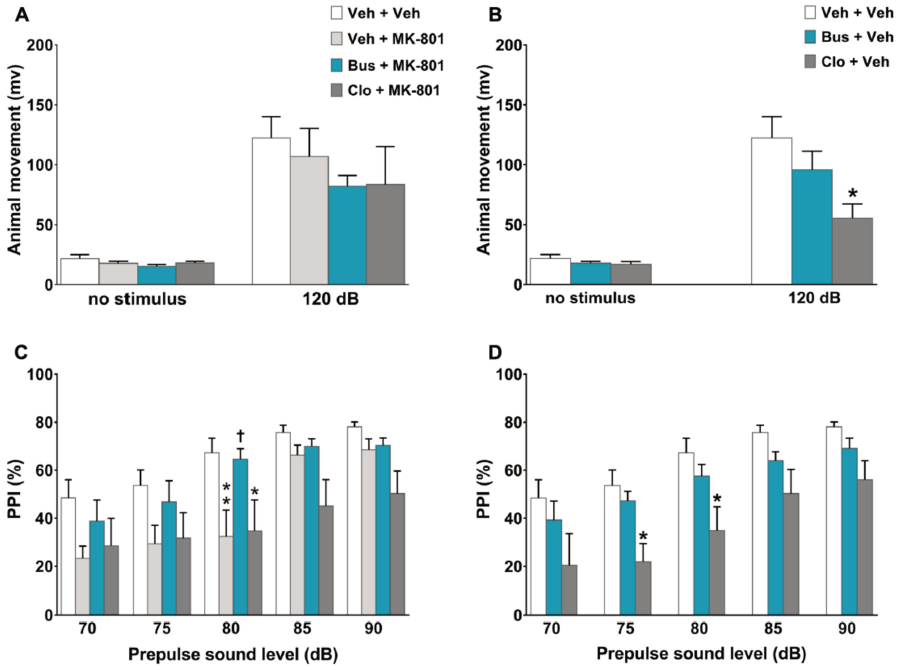

FIGURE 3 | Buspirone blocked MK-801-induced PPI disruption in WT mice. Buspirone (Bus, 3 mg kg^{-1}, i.p.) or vehicle (Veh), clozapine (Clo, 1 mg kg^{-1}, i.p.) or Veh and MK-801 (0.1 mg kg^{-1}, i.p.) or Veh were injected 45, 30, and 20 min before the PPI test, respectively. (**A,B**) Animal movements displayed by Veh + Veh (n = 10), Veh + MK-801 (n = 10), Bus + MK-801 (n = 9), Clo + MK-801 (n = 8), Bus + Veh (n = 13), Clo + Veh (n = 8) WT mice. (**C,D**) PPI% displayed by the same WT mice.
Data are shown as mean SEM. $p < 0.01$,$p < 0.05$ vs. Veh + Veh WT mice; †$p < 0.05$ vs. Veh + MK-801 WT mice; (Two-way ANOVA with or without repeated-measures and Newman–Keuls post hoc test).

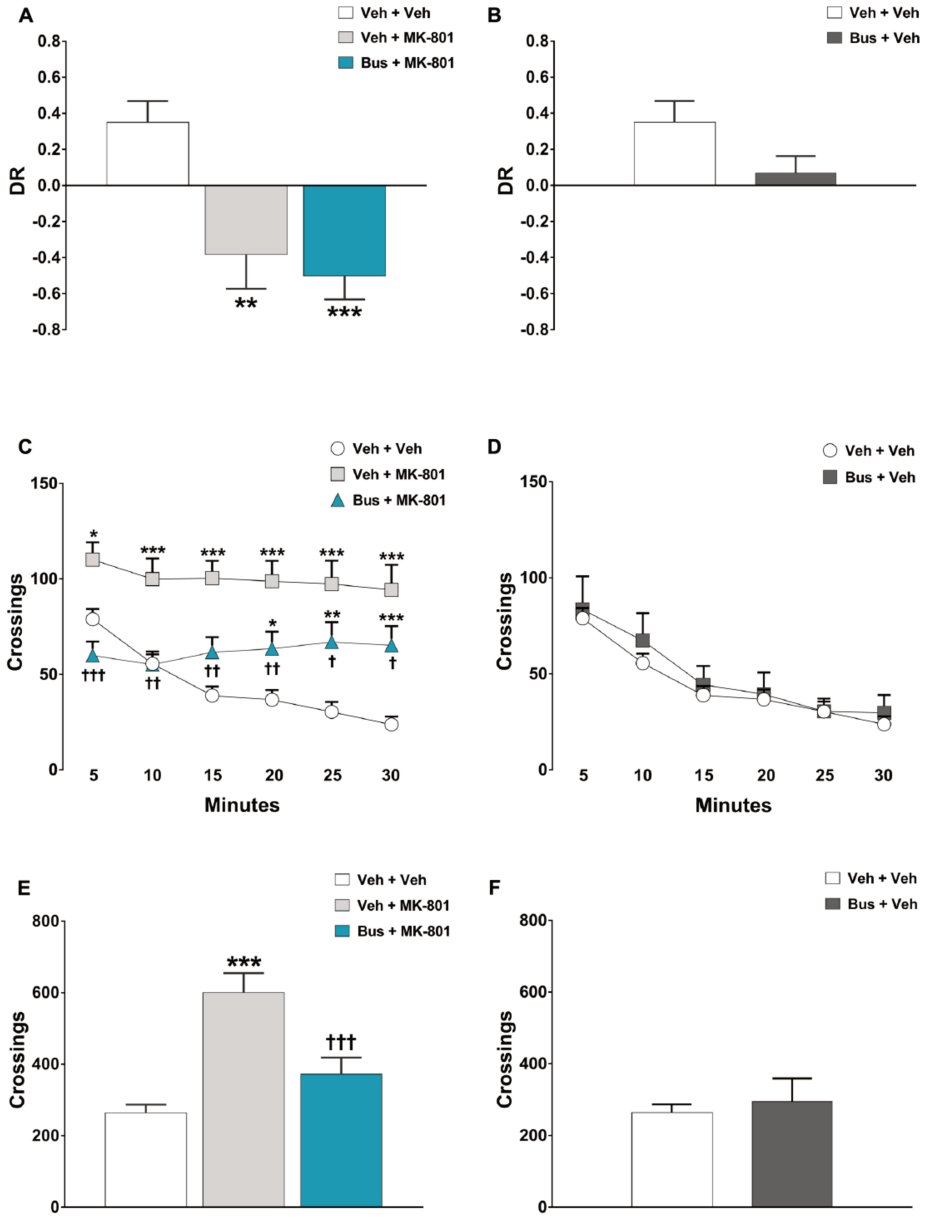

FIGURE 4 | Buspirone was ineffective in preventing MK-801-induced TOR memory deficit and hyperlocomotion. Buspirone (Bus, 3 mg kg^{-1}, i.p.) or vehicle (Veh) and MK-801 (0.1 mg kg^{-1}, i.p.) or Veh were injected 45 min, and 20 min respectively, before the sample phase 2 or the open field test. (**A,B**) Discrimination ratio(DR) displayed by Veh + Veh (n = 5), Veh + MK-801 (n = 6), Bus + MK-801 (n = 5), Bus + Veh (n = 6), D3R= mice during the test phase. DR [(less recently experienced object exploration time - more recently experienced object exploration time)/total exploration time]. (**C,D**) Locomotor activity (crossings) at each 5-min time point displayed by Veh + Veh (n = 11), Veh + MK-801 (n = 11), Bus + MK-801 (n = 11), Bus + Veh (n = 6), D3R= mice. (**E,F**) Locomotor activity (crossings) over a 30-min test period displayed by the same mice. Data are shown as mean SEM. $p < 0.001$, $p < 0.01$, $p < 0.05$ vs. Veh + Veh D3R= mice; †††$p < 0.001$, ††$p < 0.01$ and †$p < 0.05$ vs. Veh + MK-801 WT mice; (One-way or two-way repeated-measures ANOVA and Newman–Keuls post hoc test

Chapter III

“The epistatic interaction between dopamine D3 receptor and Dysbindin-1 modulates higher-order cognitive functions in mice and humans ”

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.

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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 dystrobrevin-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+/− xDys+/−mice), we confirmed the 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+/− x 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+/− x 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+/+ x Dys+/+), selective D3 hypofunction (D3+/− x Dys+/+ single heterozygous mice), Dys hypofunction (D3+/+ x Dys+/− single heterozygous mice), and decreased levels of both D3 and Dys in the same individual (D3+/− x 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 blonanserin (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 0.1M 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) tes*t*

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 x 40 x 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 x 23.5 x 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 $MgCl_2$, 0.7mM $CaCl_2$, and 10mM D-glucose and equilibrated with 95% O_2 and 5% CO_2; 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 infraredsensitive 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 ($D3^{+/+}$ x $Dys^{+/+}$), single ($D3^{+/-}$ x $Dys^{+/+}$ and $D3^{+/+}$ x $Dys^{+/-}$), and double mutant ($D3^{+/-}$ x $Dys^{+/-}$) 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-naive $D3^{+/+}$ x$Dys^{+/-}$ mice (Fig. 2b, c). However, only risperidone and blonanserin improved the TOR performance of all mutant mice compared to vehicle- and drug-treated $D3^{+/+}$ x $Dys^{+/+}$ 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), $D3^{+/+}$Å~$Dys^{+/-}$ naive 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 Tmaze 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 ($D3^{+/+}$ x $Dys^{+/-}$) mice performed worse than wild-type ($D3^{+/+}$ x $Dys^{+/+}$) 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 theWM 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 $D3^{+/+}$ x $Dys^{+/-}$ and $D3^{+/-}$ x $Dys^{+/-}$ 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$^{+/+}$ x Dys$^{+/-}$) and D3 (D3$^{+/-}$ x Dys$^{+/+}$) single heterozygous mice were more active than their wild-type littermates (D3$^{+/+}$ x Dys$^{+/+}$) when tested in an open field arena. In contrast, D3$^{+/-}$ Å~ 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$^{+/+}$ x Dys$^{+/-}$) mice, startle reactivity was increased (Fig. 3g), consistent with previous findings [22]. Conversely, D3 single heterozygous mice (D3$^{+/-}$ x 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$^{+/-}$ x Dys$^{+/-}$) mice (Fig. 3g). 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$^{+/+}$ x Dys$^{+/-}$) mice but similar to D3 single heterozygous (D3$^{+/-}$ x Dys$^{+/+}$) mice, D3$^{+/-}$ x Dys$^{+/-}$ double heterozygous mice exhibited a PPI response higher than that in both wild-type and D3$^{+/+}$ x 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 paralleled by relevant interactions at the molecular level. 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 wildtype levels in $D3^{+/-}$ x $Dys^{+/-}$ double mutant mice (Fig. 4a, b). In contrast, Dys mRNA expression was decreased in the mPFC of both $D3^{+/+}Dys^{+/-}$ and $D3^{+/-}$ x $Dys^{+/-}$ mice (Fig. 4c). However, Dys expression in the striatum was increased in $D3^{+/-}$ mice, but this increase was reversed in $D3^{+/-}$ x $Dys^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 hypofunctioning 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 ($D3^{+/+}$ x $Dys^{+/+}$, $D3^{+/+}$ x $Dys^{+/-}$, $D3^{+/-}$ x $Dys^{+/+}$, and $D3^{+/-}$ x $Dys^{+/-}$). However, increasing the current injection from 50 to 200 pA induced fewer spikes in the pyramidal neurons of $D3^{+/+}$ x $Dys^{+/-}$ 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 ($D3^{+/-}$ x $Dys^{+/-}$) 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 ($D3^{+/+}$ x $Dys^{+/-}$) mice than in wild-type ($D3^{+/+}$ x $Dys^{+/+}$) mice. In contrast, $D3^{+/-}$ x $Dys^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ or wild-type mice ($D3^{+/+}$ x $Dys^{+/+}$; 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 ($D3^{+/-}$ x $Dys^{+/+}$) mice, but restored in double heterozygous ($D3^{+/-}$ x $Dys^{+/-}$; Fig. 5e). Furthermore, following risperidone treatment, quinpirole had again no effects on dopamine levels in single D3 heterozygous ($D3^{+/-}$ x $Dys^{+/+}$; Fig. 5f), but increased dopamine levels in $D3^{+/-}$ x $Dys^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ double heterozygous mice. Taken together, these electrophysiological and neurochemical 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 indicate that in the PFC, the 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 hypofunctioning in the context of reduced Dys expression.

Discussion

This study reveals an epistatic (gene-by-gene) interaction between D3 and Dys (DTNBP1) genes. In particular, Dysdependent 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 mPFC functioning 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 $^{+/-}$ x Dys$^{+/-}$ 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 $D3^{+/-}$ x $Dys^{+/-}$ 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 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 layer 5 pyramidal neurons, which we found in mice with Dys hypofunction, might be related to their increased D3 basal signaling. In support of this hypothesis, D3 hypofunction in $Dys^{+/-}$ mice ameliorated their altered excitability. In addition, postmortem studies revealed a twofold increase in the expression of D3 receptors in the brains of long-term hospitalized drug-free patients with schizophrenia [64], while patients with early psychosis displayed augmented levels of D3 receptor mRNA in T lymphocytes [65]. Thus, based on the present data, it is tempting to suggest that schizophrenia-related phenotypes are associated with a genetic background that increases relative D3 function. Therefore, selective blockade of D3 receptors might shift the D2/D3 balance to favor increased D2 expression, ultimately improving cognitive performance. 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 therapeutic approach.

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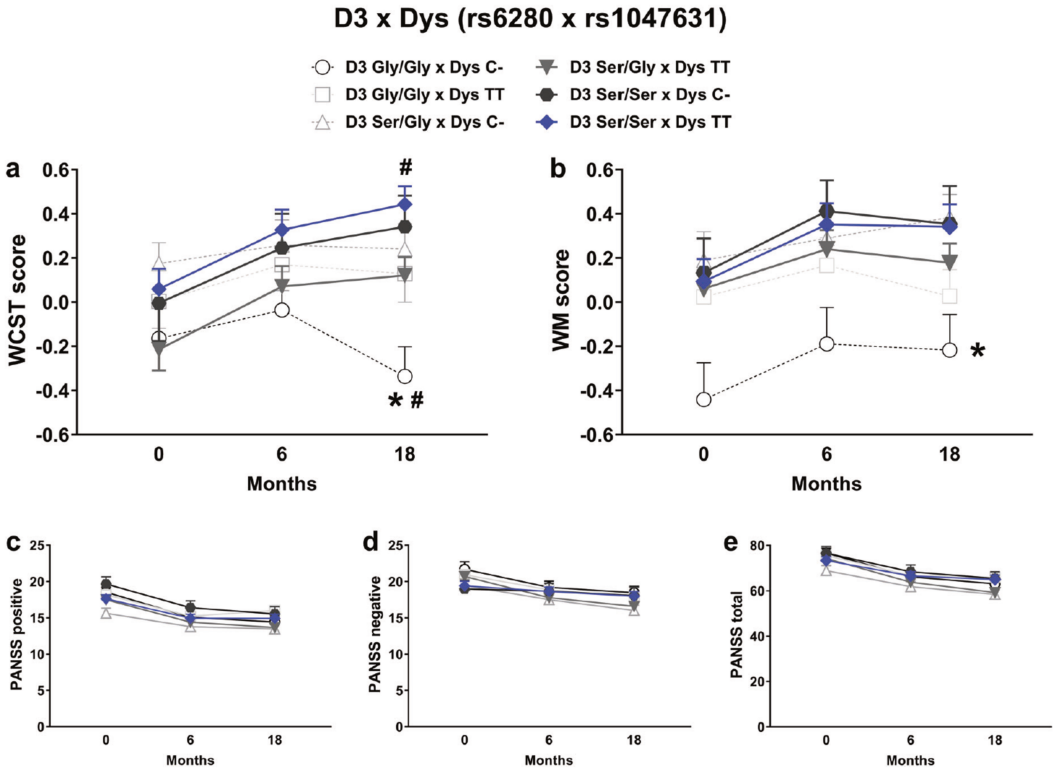


Fig. 1 - Genetic variants that concomitantly and relatively increase both D3 affinity and Dys expression are associated with neurocognitive disadvantages in patients with schizophrenia. **(a)** Wisconsin Card Sorting Test (WCST) performance across genotypes at assessment month 0, 6, and 18. As extracted from the CATIE study [31], the WCST score was calculated by averaging z-scores for perseverative errors and categories achieved. Two-way ANOVA for test performance revealed a significant effect of genotype [$F(5,387) = 2.62$, $p = 0.02$], months of assessment [$F(2,774) = 12.85$, $p < 0.0001$], and a genotype-by-time interaction [$F(10,774) = 2.06$, $p = 0.02$]. Post hoc: *$P < 0.05$ D3 Gly/Gly, Dys C-carrier subjects vs all other groups. #$P < 0.05$ vs 0 or 6 months performance within the same genotype group. Dotted line indicates genotypes not improving with time. **(b)** Working Memory (WM) composite score across genotypes at month 0, 6, and 18 of assessment. As extracted from the CATIE study [31], the WM score refers to the CATIE variable "Memory_S" and it was calculated by standardizing to baseline mean of performance in the letter–number span test and a computerized test of visuospatial working memory. Two-way ANOVA for test performance showed a significant effect of genotype [$F(5,394) = 2.41$, $p = 0.036$], months of assessment [$F(2,788) = 14.91$, $p < 0.0001$], but no genotype-by-time interaction [$F(10,788) = 0.82$, $p = 0.61$]. Post hoc: *$P < 0.05$ D3 Gly/Gly, Dys C-carrier subjects vs all other genotypes groups. Dotted line indicate genotypes not improving with time. Two-way ANOVA for positive scale (**c**: [genotype- by-time $F(10,856) = 1.20$, $p = 0.28$]), negative scale (**d**: [genotype- by-time $F(10,856) = 2.07$, $p = 0.025$], total score (**e**: [genotype-bytime $F(10,856) = 1.38$, $p = 0.19$]). The values are the means ± s.e.m.

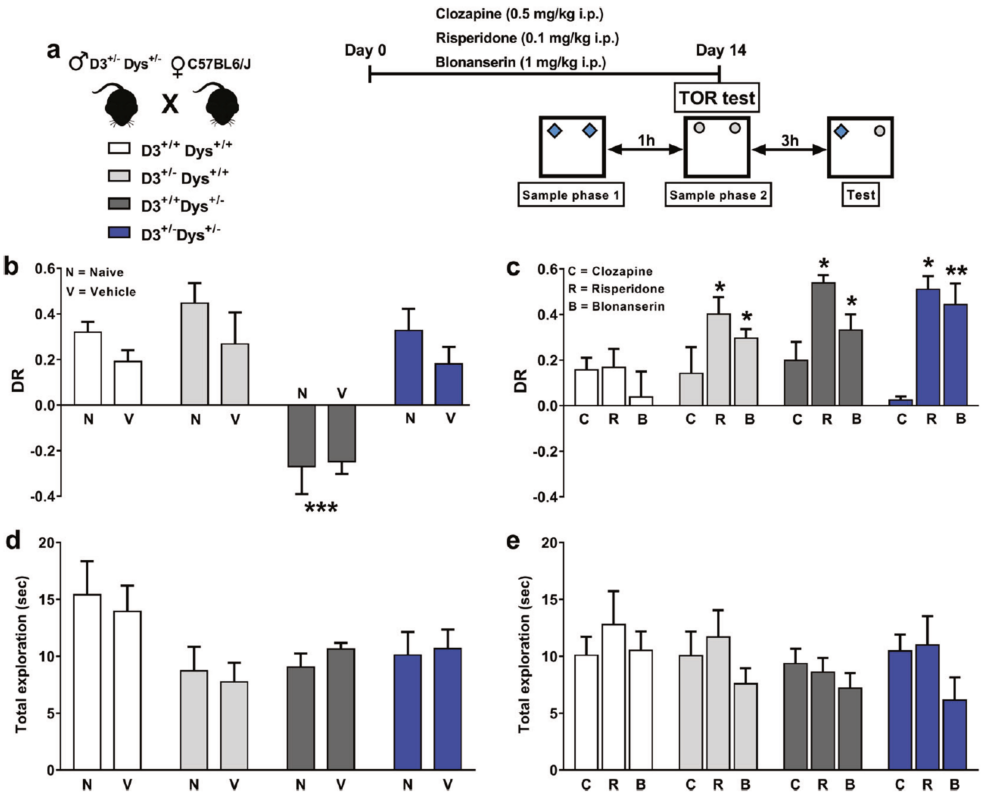


Fig. 2 - D3 hypofunction improves the efficacy of antipsychotics on improving cognitive functions in mice with genetic reduction of Dys expression. **(a)** D3+/− x Dys+/− double mutant (purple bar), D3+/+ x Dys+/− (dark gray bar), D3+/− x Dys+/+ (gray bar) and wild-type D3+/+ x Dys+/+ (white bar) littermate mice were generated by mating D3+/− x Dys+/− males with naive C57BL6/J female mice. The different experimental groups of mice were treated with drug or vehicle once daily for 14 consecutive days before the TOR test. **(b)** Discrimination ratio (DR) displayed by naive [D3+/+ x Dys+/+ (n = 8), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 6), D3+/− x Dys+/− (n = 6)] and vehicle-treated mice [D3+/+ x Dys+/+ (n = 9), D3+/− x Dys+/+ (n = 7), D3+/+ x Dys+/− (n = 7), D3+/− x Dys+/− (n = 7)] during the 5-min test phase of the TOR test. Two-way ANOVAs revealed a genotype effect [$F(3, 47) = 20.26$; $P < 0.0001$]. Post hoc: ***$P < 0.001$ vs naive or vehicle-treated D3+/+ x Dys+/+ mice. **(c)** DR displayed by drug-treated mice [clozapine: D3+/+ x Dys+/+ (n = 8), D3+/− x Dys+/+ (n = 6), D3+/+ x Dys+/− (n = 9), D3+/− x Dys+/− (n = 6); risperidone: D3+/+ x Dys+/+ (n = 6), D3+/− x Dys+/+ (n = 9), D3+/+ x Dys+/− (n = 5), D3+/− x Dys+/− (n = 6); blonanserin: D3+/+ x Dys+/+ (n = 8), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 6), D3+/− x Dys+/− (n = 5)] during the 5-min test phase of the TOR test. Two-way ANOVAs revealed a genotype effect [$F(3, 67) = 5.306$; $P = 0.0024$], a treatment effect [$F(2, 67) = 11.81$; $P < 0.0001$] and a genotype x treatment interaction effect [$F(6, 67) = 2.38$; $P = 0.038$]. Post hoc: **$P < 0.01$, *$P < 0.05$ vs the corresponding drugtreated D3+/+ x Dys+/+ mice. **(d)** Total time spent by naive and vehicle treated mice exploring the two objects presented during the 5-min test phase. **(e)** Total time spent by drug-treated mice exploring the two objects presented during the 5-min test phase. The values are the means ± s.e.m. N naive, V vehicle, C clozapine, R risperidone, B blonanserin

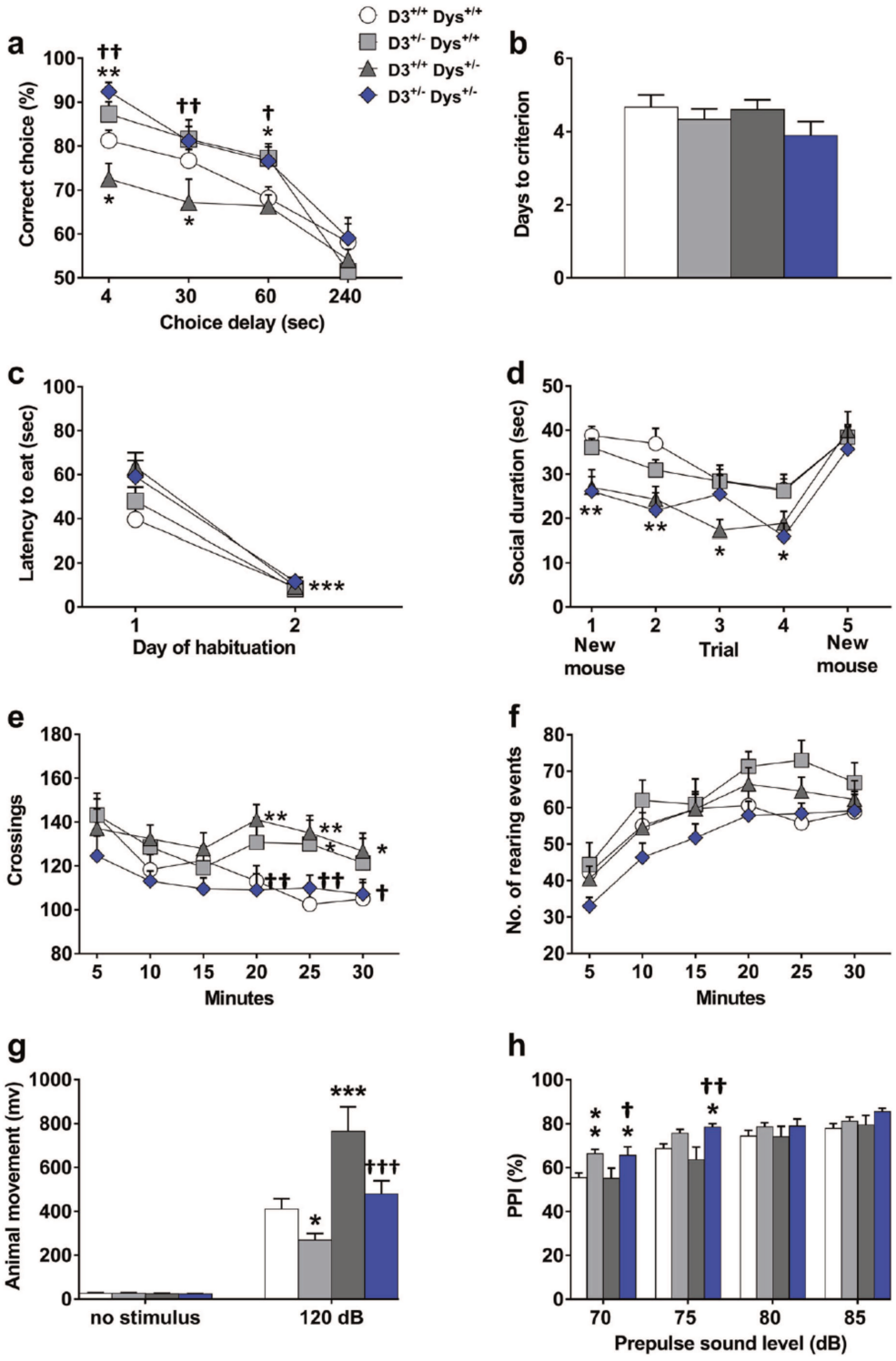
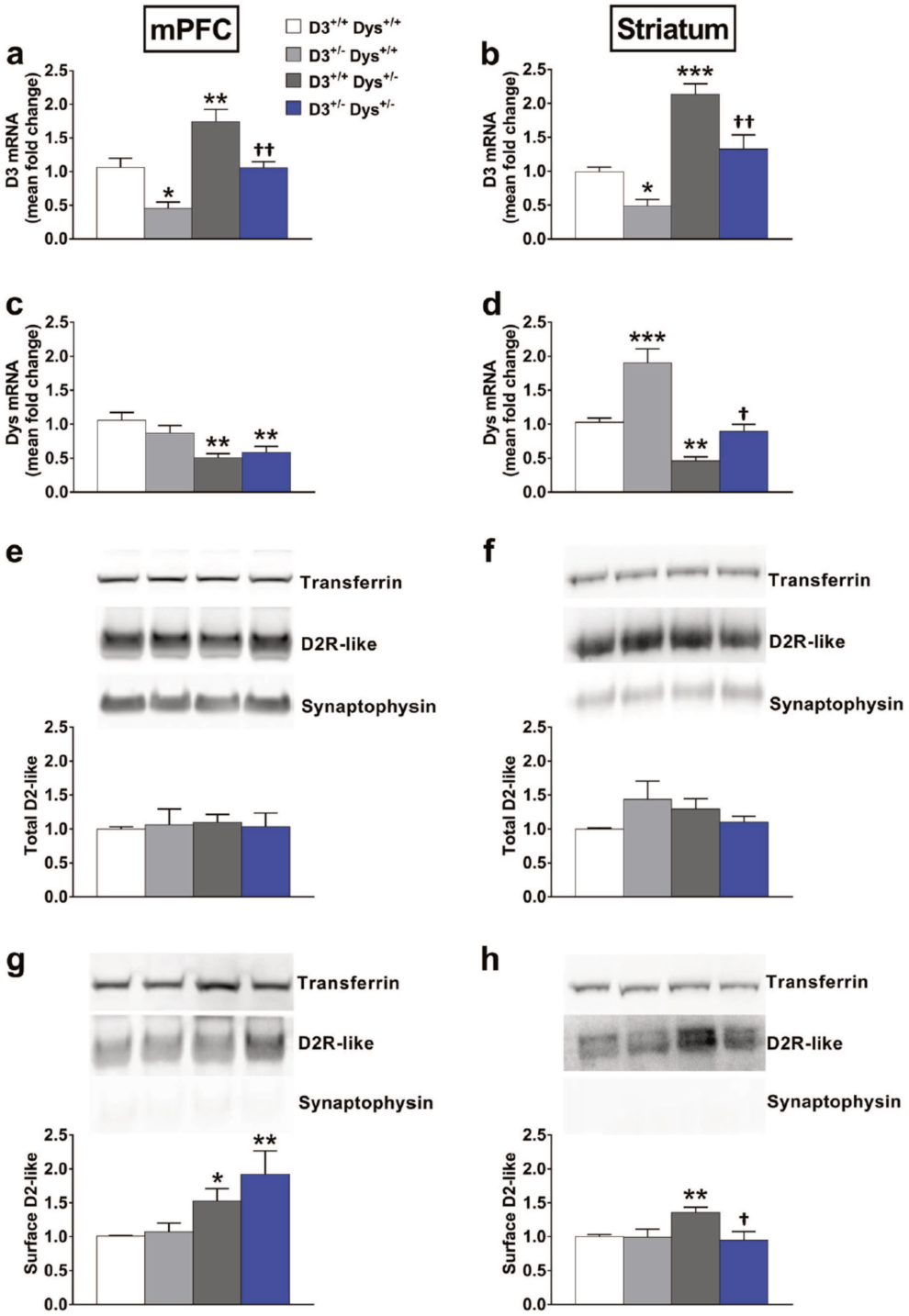

a
b
c
d
e
f
g
h
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
Correct choice (%)
Choice delay (sec)
Days to criterion
Latency to eat (sec)
Day of habituation
Social duration (sec)
New mouse
Trial
New mouse
Crossings
Minutes
No. of rearing events
Minutes
Animal movement (mv)
no stimulus
120 dB
PPI (%)
Prepulse sound level (dB)

Fig. 3 Partial deletion of the D3 gene rescues the working memory (WM) deficits of Dys mutant mice, while having marginal effects in social behavior, locomotor activity, startle, and PPI responses. **(a)** Percentage of correct choices displayed by D3+/+ x Dys+/+ (n = 12), D3+/− x Dys+/+ (n = 9), D3+/+ x Dys+/− (n = 10), and D3+/− x Dys+/− (n = 9) mice during the discrete paired-trial variable-delay T-maze test with different randomly presented intratrial delays (4, 30, 60, and 240 s) and an intertrial delay of 20 s. The 50% value corresponds to chance levels of correct choices. Repeated measures ANOVAs revealed a genotype effect [$F(3, 36) = 8.351$, $P = 0.0002$] and a delay effect [$F(3, 108) = 50.35$, $P < 0.0001$]. **(b)** Days needed to meet the criterion and **c** latency to retrieve the hidden food pellet during the discrete paired trial T-maze task. No differences were found among genotypes in task acquisition [one-way ANOVA, genotype effect: $F(3, 36) = 1.152$, $P = 0.3413$] or food retrieval [two-way ANOVA, genotype effect: $F(3, 36) = 2.542$, $P = 0.0716$; and day effect: $F(1, 36) = 143.3$, $P < 0.0001$]. **(d)** Time spent by D3+/+ x Dys+/+ (n = 15), D3+/− x Dys+/+ (n = 16), D3+/+ x Dys+/− (n = 11), and D3+/− x Dys+/− (n = 9) mice in investigating the same unfamiliar male mouse during each of four successive 1-min trials. A fifth 'dishabituation' trial shows the social investigation activity of the subject mice to the presentation of a new unfamiliar male mouse. Repeated measures ANOVAs revealed a genotype effect [$F(3, 47) = 3.793$; $P = 0.0162$] and a trial effect [$F(4, 188) = 25.71$; $P < 0.0001$]. **(e, f)** D3+/+ x Dys+/+ (n = 14), D3+/− x Dys+/+ (n = 10), D3+/+ x Dys+/− (n = 15), and D3+/− x Dys+/− (n = 13) mice were tested in an open field arena for 30 min. Repeated measures ANOVAs revealed a genotype effect [$F(3, 48) = 3.374$; $P = 0.0258$] and a time effect [$F(5, 240) = 6.026$; $P < 0.001$] on locomotor activity (crossings, **e**), but did not show a genotype effect [$F(3, 48) = 1.742$; $P = 0.171$] on rearing behavior **(f)**. **(g)** Movement by D3+/+ x Dys+/+ (n = 24), D3+/− x Dys+/+ (n = 23), D3+/+ x Dys+/− (n = 17) and D3+/− x 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 [$F(3, 146) = 10.73$; $P < 0.001$]. **(h)** Percent PPI of the acoustic startle response exhibited 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+/+ x Dys+/+ mice and †††$P < 0.001$, ††$P < 0.01$, †$P < 0.05$ vs D3+/+ x Dys+/− mice. The values are the means ± s.e.m.


mPFC
Striatum
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
a
b
c
d
e
f
g
h
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
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††

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+/+ x Dys+/+ (n = 5), D3+/− x Dys+/+ (n = 6), D3+/+ x Dys+/− (n = 6), D3+/− x Dys+/− (n = 6)] and striatum [D3+/+ x Dys+/+ (n = 5), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 6), D3+/− x Dys+/− (n = 6)] measured by quantitative RT-PCR. **(c, d)** Abundance of Dys in the mPFC [D3+/+ x Dys+/+ (n = 11), D3+/− x Dys+/+ (n = 10), D3+/+ x Dys+/− (n=10), D3+/− x Dys+/− (n=9)] and striatum [D3+/+ x Dys+/+ (n=9), D3+/− x Dys+/+ (n=10), D3+/+ x Dys+/− (n=11), D3+/− x Dys+/− (n = 10)] measured by quantitative RT-PCR. Mean fold changes are expressed relative to transcript levels in control mice(D3+/+ x Dys+/+). One-way 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 striatum [$F(3,36) = 25.02$; $P < 0.001$]. **(e, f)** Western blot and densitometric analysis of total expression of D2-like receptors (52 kDa) in the mPFC [D3+/+ x Dys+/+ (n = 8), D3+/− x Dys+/+ (n = 6), D3+/+ x Dys+/− (n = 5), D3+/− x Dys+/− (n = 6)] and striatum [D3+/+ x Dys+/+(n = 9), D3+/− x Dys+/+ (n = 6), D3+/+ x Dys+/− (n = 7), D3+/− x Dys+/− (n = 7)]. **(g, h)** Western blot and densitometric analysis of surface expression of D2-like receptors (52 kDa) in the mPFC [D3+/+ x Dys+/+ (n=9), D3+/− x Dys+/+ (n=6), D3+/+ x Dys+/− (n=6), D3+/− x Dys+/− (n=6)] and striatum [D3+/+ x Dys+/+ (n = 9), D3+/− x Dys+/+ (n = 6), D3+/+ x Dys+/− (n = 7), D3+/− x Dys+/− (n = 7)]. The results presented are normalized to transferrin receptor protein (95 kDa) levels and to the D3+/+ x Dys+/+ 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 [$F(3, 23) = 5.382$, $P = 0.0059$] and striatum [$F(3, 25) = 4.4296$, $P = 0.0125$]. Post hoc: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs D3+/+ Å~ Dys+/+ mice. ††$P < 0.01$, †$P < 0.05$ vs D3+/+Dys +/− mice. Each histogram shows the mean ± s.e.m.

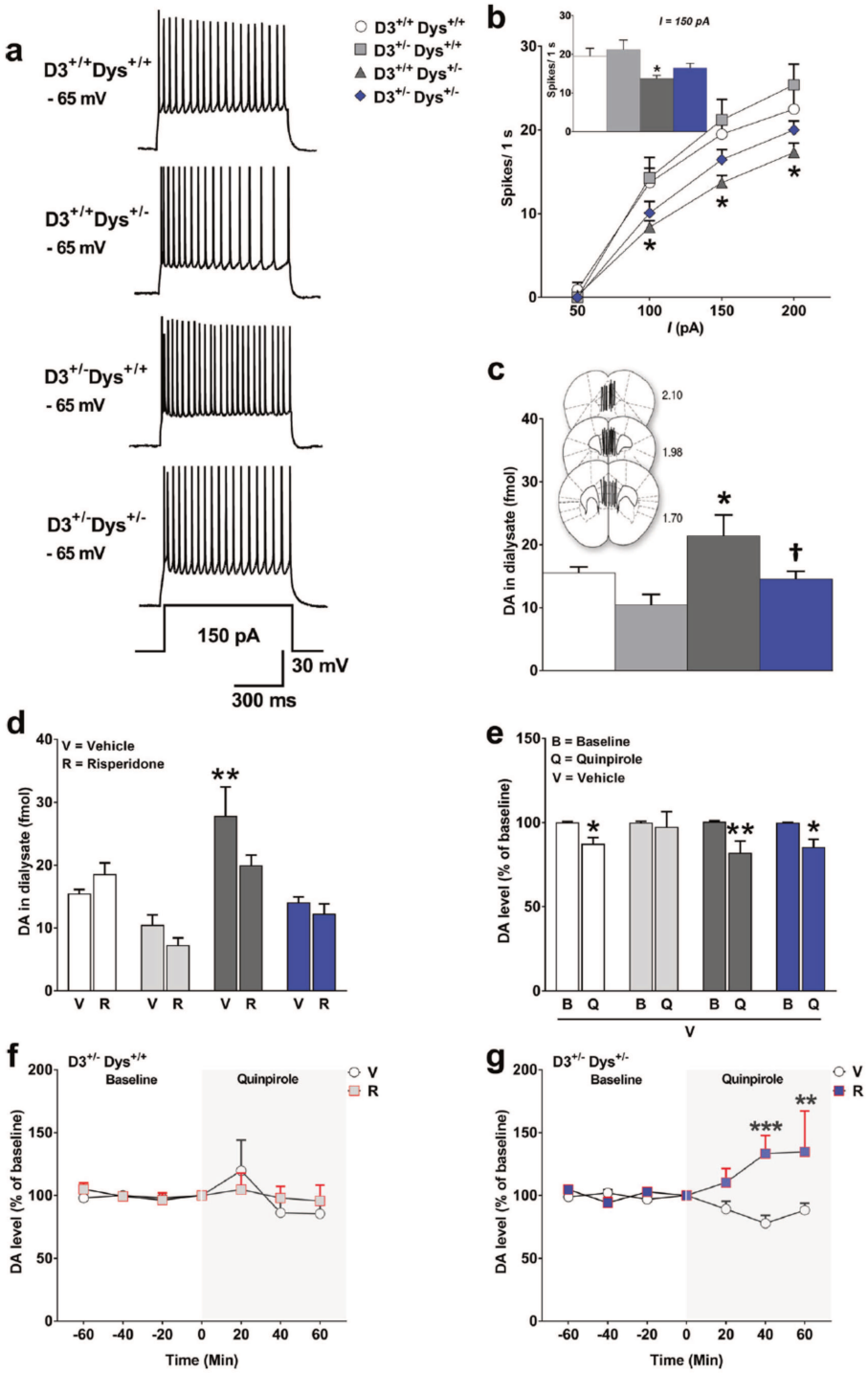

a
D3+/+Dys+/+
- 65 mV
D3+/+Dys+/-
- 65 mV
D3+/-Dys+/+
- 65 mV
D3+/-Dys+/-
- 65 mV
150 pA
30 mV
300 ms
D3+/+ Dys+/+
D3+/- Dys+/+
D3+/+ Dys+/-
D3+/- Dys+/-
b
I = 150 pA
Spikes/ 1 s
I (pA)
c
2.10
1.98
1.70
DA in dialysate (fmol)
d
V = Vehicle
R = Risperidone
DA in dialysate (fmol)
V R V R V R V R
e
B = Baseline
Q = Quinpirole
V = Vehicle
DA level (% of baseline)
B Q B Q B Q B Q
V
f
D3+/- Dys+/+
Baseline
Quinpirole
V
R
DA level (% of baseline)
Time (Min)
g
D3+/- Dys+/-
Baseline
Quinpirole
V
R
DA level (% of baseline)
Time (Min)

Fig. 5 Partial deletion of the D3 gene reverses the decreased excitability of pyramidal neurons, as well as the increased levels of DA in the mPFC of heterozygous Dys mutant mice. Representative traces **(a)** 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+/+ x Dys+/+ (n = 10); D3+/− x Dys+/+ (n = 10); D3+/+ x Dys+/- (n = 13); and D3+/− x Dys+/− (n = 11). 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 an intensity x genotype interaction [F(9, 120)= 3.254, P = 0.0014] and intensity effect [F(3, 120) = 3 97.7, P < 0.0001], as well as a genotype effect [F(3, 40) = 3.833, P = 0.0167]. **(c)** 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+/+ x Dys+/+ (n = 8), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 7), and D3+/− x Dys+/− (n = 6) mice. 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+/−. **(d)** Extracellular dopamine levels in the mPFC of D3+/+ x Dys+/+, D3+ /− x Dys+/+, D3+/+ x Dys+/− D3+/− x Dys+/− following chronic treatment (14 days) with risperidone (R) [D3+/+ x Dys+/+ (n = 7), D3+/− x Dys+/+ (n = 5), D3+/ + x Dys+/− (n = 5), D3+/− x Dys+/− (n = 9)] or vehicle (V) [D3+/+ x Dys+/+ (n = 11), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 12), D3+/− x Dys+/− (n = 10)]. Two-way ANOVAs revealed a genotype effect [F(3, 53) = 7.004, P = 0.0005]. Post hoc: **P < 0.01 vs D3+/+Dys+/+ vehicle treated mice. **(e)** Quinpirole-induced dopamine release in the mPFC of D3+/+ x Dys+/+ (n = 10), D3+/− x Dys+/+ (n = 5), D3+/+ x Dys+/− (n = 9), D3+/− x Dys+/− (n = 12) following (14 days) with vehicle (V). Two-way ANOVAs treatment effect F(1, 62) = 15.28; P = 0.0002]. Post hoc: *P < 0.05 and **P < 0.005 vs baseline within each genotype group. **(f)** Quinpirole-induced dopamine release in the mPFC of D3+/− x Dys+/+ (vehicle n = 5, risperidone n = 5) and **(g)** D3+/− x Dys+/− (vehicle n = 12, risperidone n = 9) 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+/− x Dys+/− vehicletreated mice. Values are the means ± s.e.m. V vehicle, R risperidone, B baseline, Q quinpirole, W washout

SUPPLEMENTARY INFORMATION

Supplementary materials and methods

Human subjects

Subjects were genotyped for 492,900 single-nucleotide polymorphisms (SNPs) using an Affymetrix 500K two-chip genotyping platform plus a custom 164K fill-in chip following multiple quality control steps for both subjects and SNPs[1]. SNPs of interest were extracted using PLINK software for Windows (http://pngu.mgh.harvard.edu/purcell/plink/), and the Hardy–Weinberg equilibrium test (HWE) was performed for each SNP.

CATIE study setting and design

The Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE, ClinicalTrials.gov Identifier: NCT00014001) study was initiated by the NIMH to compare the effectiveness of antipsychotic drugs[2]. The protocol was made available to the public for comment, and a committee of scientific experts, health care administrators, and consumer advocates critiqued the study under the auspices of the NIMH. The study was conducted between January 2001 and December 2004 at 57 clinical sites in the United States. Patients were initially randomly assigned to receive olanzapine, perphenazine, quetiapine, or risperidone under double-blind conditions and followed for up to 18 months or until treatment was discontinued for any reason (phase 1). The present report is limited to phase 1 results. Eligible patients were 18 to 65 years of age; had received a diagnosis of schizophrenia, as determined on the basis of the Structured Clinical Interview of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Patients were excluded if they had received a diagnosis of schizoaffective disorder, mental retardation, or other cognitive disorders; had a history of serious adverse reactions to the proposed treatments; had only one schizophrenic episode; had a history of treatment resistance, defined by the persistence of severe symptoms despite adequate trials of one of the proposed treatments or prior treatment with clozapine; were pregnant or breast-feeding; or had a serious and unstable medical condition. (Detailed information about interventions are available here[3]). In this study we included only patients for which genotypic data for DTNBP1 and D3 was also available.

Mice

Animals were group-housed (2–4 mice per cage) with free access to food and water in an airconditioned room (22±2°C), with a 12-h light–dark cycle. Genotypes were identified by PCR analysis of tail DNA as previously described[4, 5]. The experimenter handled animals on alternate days during the week preceding the first behavioral test. Animal sample size was chosen based on studies using related methods and is similar to what is generally employed in the field. Randomisation was not used to assign animals to experimental groups, and the investigator was blinded to the genotype of animals. All experiments were carried out according to EU Directive 2010/63/EU and the Institutional Animal Care and Use Committees of both Catania University and the Istituto Italiano di Tecnologia (IIT).

Drugs and treatments

Animals were treated with drug or vehicle once daily for 14 consecutive days before the temporal order recognition (TOR) test (Figure 2A). On the day of the test, drug or vehicle was administered 3 h prior to sample phase 1. The dose of risperidone (0.1 mg/kg) was selected based on our previous experience (Scheggia et al., 2018). Clozapine and blonanserin were administered at doses of 0.5 mg/kg and 1 mg/kg, respectively. These doses have been shown to be effective for improving cognitive dysfunction[6, 7].

Behavioral tasks

TOR test

Mice explored an evenly illuminated (9 ± 1 lux) square open field (40 x 40 x 40 cm, Ugo Basile, Gemonio, Italy) with a sawdust-covered floor. The behavior of the mice was recorded using a video camera (Sony Videocam PJ330E) and was then scored by an independent observer. The objects presented were different in shape, color, and size (4 x 4 x 4 cm to 6 x 6 x 6 cm) and too heavy to be moved by the mice. After 1 week of handling, a 4-day pretest procedure was carried out. On days 1 and 2, mice were placed in the empty arena for 10 min. Next, on days 3 and 4, mice were placed in the arena containing two objects (different from those eventually used during the test) for 10 min. This pretest procedure was performed to acclimatize the animals to the arena as well as to prevent neophobia during the test. The objects were located in two corners of the arena, 10 cm from the side walls. The test consisted of two sample phases and one test phase (Figure 2A). During the sample phases, animals were allowed to

explore two copies of an identical object for a total of 5 min. Different objects were used for each sample phase, with a 1-h delay between the sample phases. The test phase was performed 3 h after sample phase 2. During the test (5-min duration), animals were exposed to a third copy of the objects from sample phase 1 and a third copy of the objects from sample phase 2. All objects were cleaned with a 10% ethanol solution between each test to avoid olfactory cues. Any feces were removed, and the sawdust was shaken to equally redistribute any odor cues. If temporal order memory is intact, animals should spend more time exploring the object from sample 1, the less recently experienced object, than the object from sample 2, the more recently experienced object. The objects utilized in each sample phase as well as the positions of the objects during the test were counterbalanced between mice. Exploratory behavior was defined as the mouse directing its nose toward the object at a distance of ≤ 2 cm. Looking around while sitting, climbing the objects and rearing against the objects were not considered exploratory behaviors. Mice that failed to complete a minimum of 2 seconds (sec) of exploration in each phase of the task were excluded from analysis. Discrimination between the objects was calculated using a discrimination ratio (DR) that accounts for individual differences in the total amount of exploration. Specifically, data are presented as the DR, calculated as follows: (less recently experienced object exploration time – more recently experienced object exploration time)/total exploration time. The higher the DR, the better is the performance on the TOR memory test.

Discrete paired-trial variable delay T-maze task

The T-maze apparatus was built from transparent plexiglass (0.5 cm thick; alley dimensions of 40x10.2x17.5 cm; light levels of 20±2 lux in the main alley and 10±2 lux in the 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 the 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 subjected 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 min/day for 2 consecutive days, with both arms of the maze open. The next day, animals were exposed to 10

forced alternation runs: in each run, animals were placed into the T-maze with one goal arm closed off and were allowed to locate and eat the food reinforcement in the open arm for up to 2 min; after consuming the food pellet, the mice were rested for an inter-trial period of at least 20 min in the home cage and were then placed back into the maze for another forced run with the food reinforcement 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, and 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 min, animals were placed back in the maze for another forced run/ choice run paired trial. The test included ten paired runs per day and was repeated daily until the mouse successfully performed 8 correct choices in 10 daily trials (80% choice accuracy) for 3 consecutive days. Animals that did not meet this criterion within 20 days were eliminated. A different pseudorandomly 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 with the 4-second intra-trial delay, testing at three additional intra-trial delays (30, 60 and 240 seconds in a random order, with a 20-second inter-trial delay) was carried out. Mice were subjected to 4 trials with each delay on 4 consecutive days of testing, for a total of 16 trials per delay. After each trial, the apparatus was cleaned with water and 10% ethanol, with special attention to the choice point of the T-maze.

Acoustic startle response and prepulse inhibition (PPI) test

The PPI test session began by placing animals in the plexiglass holding cylinder for a 5-min acclimation period with a 65-dB background noise. Animals were then subjected to a series of trial types 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 measured the startle stimulus alone (acoustic amplitude), which was a 40-ms 120-dB sound burst. The other four trial types were acoustic prepulse plus acoustic startle stimulus trials. Prepulse tones lasted 20 ms at 70, 75, 80 and 85 dB and were presented 100 ms before the startle stimulus. PPI was calculated with the following formula: 100 x [pulse-only units − (prepulse + pulse units)]/(pulse-only units).

Open field test

The apparatus was cleaned with a 10% ethanol solution between each test to avoid olfactory cues. Locomotor activity was quantified by counting the number of crossings (number of squares entered) with all four paws. The behavior of the animals was recorded with a video camera (Sony Videocam PJ330E) and analyzed by one observer blinded to the genotype.

Habituation/dishabituation social interaction test.

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 for a 3-min ITI. In trial 2, the same stimulus mouse was reintroduced 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: anogenital sniffing (direct contact with the anogenital area), body sniffing (sniffing or snout contact with the flank area), and nose-to-nose sniffing (sniffing or snout contact with the head/neck/ mouth area). Scoring was performed by one observer blinded to the genotype.

RNA isolation and real-time PCR

Total RNA was extracted from isolated brain areas with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was dissolved in 30 µl of RNase-free water, and the optical density at 260 and 280 nm was assessed to evaluate the RNA concentration and purity. Total RNA (2 µg) was converted to first-strand cDNA in a 20 µl reaction volume with 200 U of SuperScript III, 50 ng of random hexamer primers, 0.5 mM dNTP mix, 0.01 mM dithiothreitol, 20 mM Tris–HCl (pH 8.3), 50 mM KCl, and 5 mM MgCl2 (Invitrogen Life Technologies). Reactions were carried out at 50°C for 50 min and stopped by heating at 85°C for 5 min. Aliquots of 100 ng of cDNA were amplified in parallel reactions using specific primer pairs for the D3 (Fw: 5'-GGGGTGACTGTCCTGGTCTA-3'; Rv: 5'-AAGCCAGGTCTGATGCTGAT-3'; product length110 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 the reference housekeeping gene (Fw: 5'-CAACTCACTCAAGATTGTCAGCAA-3'; Rv: 5'-GGCATGGACTGTGGTCATGA-3'; product length 118 bp; acc. Num. NM001289726.1). Each PCR (20-µl final volume) contained 0.4 µM primers, 1.6 mM Mg2+, 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 was quantified by the comparative ΔΔCt method.

Slice surface biotinylation

Before starting the surface biotinylation reaction, tissues were washed twice for 5 min in icecold Hanks' balanced salt solution (HBSS) buffer to ensure 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 HBSS buffer containing 200 mM lysine (Sigma, Saint Louis, MO, USA) to block all excess reactive NHSLC-biotin. The tissues were washed twice with ice-cold HBSS and immediately placed on ice for mechanically disruption in 500 µl of lysis buffer (1% Triton X-100, PBS, and protease inhibitor cocktail, Sigma). To remove extra debris, homogenates were centrifuged for 5 min at 4°C and 13,000 r.p.m., and supernatants were collected. To precipitate biotinylated proteins from the homogenates, 50 µl of

immobilized streptavidin beads (Pierce) were added to the samples, and the mixture was rotated for three hours at 4°C. Precipitates were collected by brief centrifugation, mixed with 50 µl of SDS-PAGE loading buffer, boiled for 5 min and stored at -80°C until use. Equal amounts of protein (100 µg) were loaded onto 10% SDS/PAGE gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA).
Western blot 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 an anti-actin antibody obtained from Sigma. Western blot analyses of brain samples were performed as previously described[8]. Quantification was performed using NIH ImageJ (version 1.42q, Bethesda, MD, USA) software. All data were obtained in a blinded manner to the treatment and the genotype of the animals.

Electrophysiology

Slice preparation

After decapitation of mice, 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 NaH2PO4, 1.2 MgSO4, 2 CaCl2, 26 NaHCO3, and 10 D-glucose at pH 7.3. The ACSF was oxygenated, and the pH was buffered by constant bubbling with a gas mixture of O2 (95%) and CO2 (5%). Brains were glued onto a Leica VT1200 vibratome specimen holder and cut in 300-µm-thick coronal sections in modified ACSF with the following composition (in mM): 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 0.5 CaCl2, 4.0 MgCl2 and 250 sucrose; equilibrated with O2 (95%) and CO2 (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 for up to 2.5 h prior to experiments.

Whole-cell patch-clamp recordings

Patch pipettes were filled with an intracellular solution with the following composition (in mM): 115 K-gluconate, 20 KCl, 2 EGTA, 10 HEPES, 2 Mg- ATP, and 0.3 Na2-GTP, pH 7.25, adjusted with KOH. The tip resistance range of pipettes
filled with this 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, the resting potential was set at -65 mV, and depolarizing steps were applied at 0 to 200 pA with a 50-pA interval. Data were analyzed in pClamp 10 (Molecular Devices, Sunnyvale, CA, USA).

In vivo microdialysis

Microdialysis sessions started 24 h after surgical procedures. Probes were perfused with Ringer's solution (composition (in mM): 147 NaCl, 2.2 CaCl2 and 4.0 KCl) at a constant flow rate of 1 µl/min. Collection of basal dialysate samples (20 µl) started 30 min after implantation. After 60 min of basal sampling, a solution of 25 nM quinpirole (Sigma, Dorset, UK) was administered through the probe for another hour of sample collection. Dialysate samples were injected into an HPLC equipped with a reverse phase column (C8 3.5 µm, Waters, Milford, MA, USA), and the DA level was quantified by a coulometric detector (Coulochem II, ESA, Bedford, MA). At the end of the experiment, mice were anesthetized with isoflurane and euthanized. Brains were removed, and serial coronal sections of the mPFC were cut with a vibratome to confirm the correct location of the probes. All measurements were performed in a manner blinded to the genotype.

Statistical analysis

A D'Agostino-Pearson omnibus normality test was performed to assess data distribution. A Levene's test was also applied to verify equality of variances. All data assumed a normal distribution and then they were subjected to parametric tests (one- or two-way analysis of variance (ANOVA) and two-way ANOVA with repeated measures when appropriate). For all data analyses, differences among individual means were assessed using Newman–Keuls post hoc tests and the Holm–Sidak method in the "multcomp" package (Bretz, Hothorn, Westfall, 2010). The Grubbs test was performed to identify outliers. P values of < 0.05 were considered significant. The estimate of dispersion is shown as the standard error of the mean (s.e.m.), and variances were found to be similar among groups. All data are presented as the means ± s.e.m.

Supplementary results

WCST

	Repeated Measures Analysis of Variance Sigma-restricted				
Effect	SS	Degr. of	MS	F	p
Intercept	10.1047	1	10.10466	5.55379	0.018938
genotype	23.8393	5	4.76786	2.62055	0.023990
Error	704.1138	387	1.81942		
MONTHS	7.7785	2	3.88923	12.85154	0.000003
MONTHS*genotype	6.2416	10	0.62416	2.06248	0.025193
Error	234.2336	774	0.30263		

	Repeated Measures Analysis of Variance with Effect Sizes and Powers Sigma-restricted parameterization							
Effect	SS	Degr. of	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0,05)
Intercept	10.1047	1	10.10466	5.55379	0.018938	0.014148	5.55379	0.652048
genotype	23.8393	5	4.76786	2.62055	0.023990	0.032748	13.10273	0.803179
Error	704.1138	387	1.81942					
MONTHS	7.7785	2	3.88923	12.85154	0.000003	0.032141	25.70308	0.997051
MONTHS*genotype	6.2416	10	0.62416	2.06248	0.025193	0.025955	20.62477	0.897415
Error	234.2336	774	0.30263					

WM

	Repeated Measures Analysis of Variance Sigma-restricted parameterization				
Effect	SS	Degr. of	MS	F	p
Intercept	16.9364	1	16.93637	7.74205	0.005654
genotype	26.3325	5	5.26651	2.40746	0.036155
Error	861.9071	394	2.18758		
MONTHS	7.4253	2	3.71264	14.91354	0.000000
MONTHS*genotype	2.0419	10	0.20419	0.82024	0.609180
Error	196.1681	788	0.24894		

	Repeated Measures Analysis of Variance with Effect Sizes and Powers Sigma-restricted parameterization							
Effect	SS	Degr. of	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0,05)
Intercept	16.9364	1	16.93637	7.74205	0.005654	0.019271	7.74205	0.792666
genotype	26.3325	5	5.26651	2.40746	0.036155	0.029646	12.03728	0.763324
Error	861.9071	394	2.18758					
MONTHS	7.4253	2	3.71264	14.91354	0.000000	0.036471	29.82708	0.999151
MONTHS*genotype	2.0419	10	0.20419	0.82024	0.609180	0.010302	8.20237	0.440864
Error	196.1681	788	0.24894					

PANSS POSITIVE	F	DF	P	h^2
Time	76.23	2	>0.0001 ***	0.15
Genotype	2.75	5	0.018*	0.03
Time x Genotype	1.20	10	0.28	0.01

PANSS NEGATIVE	F	DF	P	h^2
Time	41.89	2	>0.0001 ***	0.08
Genotype	1.03	5	0.40	0.01
Time x Genotype	2.07	10	0.024*	0.023

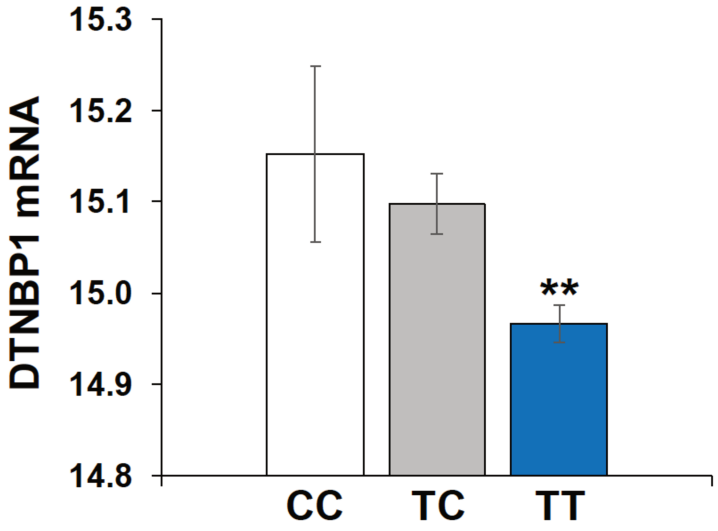
PANSS TOTAL	F	DF	P	h^2
Time	105.00	2	>0.0001 ***	0.20
Genotype	2.04	5	0.07	0.023
Time x Genotype	1.38	10	0.18	0.016

Supplementary Tables 1. Complete statistical analyses including effects sizes and powers for cognitive functions (WCST and WM) and Positive and Negative Syndrome Scale (PANSS) at baseline (month 0), at month 6, and at the end of the study (month 18) stratified by the D3 and DNTBP1 genotypes.

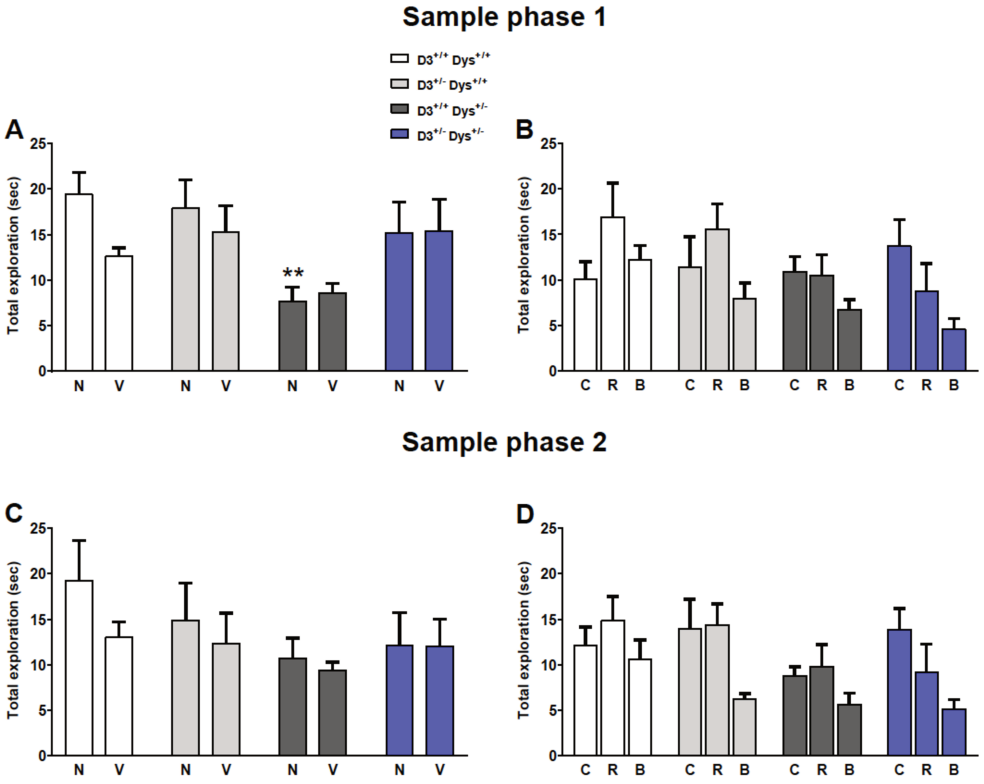
Rs1047631xRs6280 Month0	N	Age	Sex	Education
Gly/Gly & C-carriers	50	42.76(1.39)	36M/14F	11.82(0.30)
Gly/Gly & TT	112	38.60 (0.95)	76M/36F	12.30(0.18)
Ser/Gly & C-carriers	91	40.19(0.93)	67M/24F	12.36(0.22)
Ser/Gly & TT	196	39.97(0.82)	152M/44F	12.02(0.14)
Ser/Ser & C-carriers	62	43.46(1.54)	37M/25F	12.41(0.36)
Ser/Ser & TT	151	42.39(0.91)	120M/31F	12.34(0.18)
Statistics		F(2,656) p=0.77		F(2,656) p=0.45

Rs1047631xRs6280 Month6-Month18	N	Age	Sex	Education
Gly/Gly & C-carriers	34	45.17(1.52)	25M/9F	12.08(0.30)
Gly/Gly & TT	64	39.12 (1.26)	46M/18F	12.33(0.27)
Ser/Gly & C-carriers	55	40.30(1.30)	48M/7F	12.35(0.26)
Ser/Gly & TT	110	40.78(1.01)	85M/25F	11.97(0.20)
Ser/Ser & C-carriers	36	44.52(1.80)	25M/11F	12.49(0.49)
Ser/Ser & TT	94	42.06(1.10)	73M/21F	12.10(0.24)
Statistics		F(2,387) p=0.17		F(2,387) p=0.84

Supplementary Tables 2. Demographic information on the participants stratified by the D3 and DNTBP1 genotypes. Differences in age and education level among the genotypes were analyzed with ANOVA. The values are the means (s.e.m.).



Supplementary Figure 1. RNA sequencing (RNAseq) expression data from the dorsolateral prefrontal cortex (DLPFC) of a group of 594 postmortem human brains. The effect of the Dys SNP (rs1047631) functional genetic variation; C-carriers = individuals with genetic variants increasing Dys expression; TT-carriers = individuals with genetic variants decreasing Dysexpression. One-way ANOVA, $F(2, 591) = 5.70$, $p < 0.005$).



Supplementary Figure 2. Total time spent by naïve and vehicle-treated mice (**A, C**) and drug-treated mice (**B, D**) exploring the two objects presented during the 5-min sample phases 1 and 2 of the TOR test. The values are the means ± s.e.m. N = naïve, V = vehicle, C = clozapine, R = risperidone; B = blonanserin. Two-way ANOVAs revealed a genotype effect [$F(3, 47) = 4.98$; $P < 0.01$]. Post hoc: **$P < 0.01$ vs naïve D3+/+*Dys+/+ mice.

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Chapter IV

General discussion

Buspirone counteracts MK-801- induced schizophrenia -like phenotypes through dopamine D_3 receptor blockade

The results obtained in the first part of this thesis provide the first evidence that buspirone, acting as a D3R antagonist, counteracts a wide-range of schizophrenic phenotypes.

To study the antipsychotic properties of buspirone, we adopted a pharmacological animal model of schizophrenia based on NMDAR hypofunction.

The pharmacological model of schizophrenia triggered by NMDAR antagonist, MK-801, is a well-established model widely used for the evaluation of antipsychotic activity of experimental molecules[199, 200].

Indeed, glutamatergic models provide an alternate approach for understanding the brain abnormalities associated with schizophrenia. Indeed, NMDAR antagonists produce negative and cognitive symptoms of schizophrenia, along with positive symptoms, and induce neuropsychological and sensory processing deficits that are extremely similar to those observed in schizophrenic patients[201].

Several studies demonstrated that reduced NMDA function, induced by MK-801, produces cortical excitation and impair PFC-dependent cognitive functions by disrupting the inhibitory control of PFC-pyramidal neurons[202-204]. Furthermore, mice with hippocampal lesions show no differences in the single item object recognition memory but impaired temporal order memory [205].

D3R has a key role in modulating prefrontal-dependent cognitive functions [148], due to its restricted localization in layer 5 pyramidal

neurons of mPFC [206] and hippocampus, as reported by studies on receptor-specific reporter gene mice.

In the light of these evidence we investigated the antipsychotic effects of buspirone in a behavioral paradigm which depends on the interconnection between mPFC and hippocampus [114, 115], such as the Temporal Order Recognition (TOR) test. In addition, this behavioral task allowed us to measure the “recency discrimination", a cognitive function known to be altered in schizophrenic patients [207, 208].

The results show that MK-801 induced an impairment in TOR memory in mice with a single administration at the dose of 0.1 mg/Kg I.p., confirming the face validity of the model adopted. Moreover, buspirone at the dose of 3 mg/Kg was effective, more than clozapine, in preventing MK-801 induce TOR memory impairment in WT mice. Interestingly, this effect was totally abolished in $D3^{-/-}$ mice.

These data are in line with previous studies reporting that some antipsychotic, acting as D3R selective antagonist or D3R preferring partial agonist improve cognitive performance in schizophrenia [148, 209].

Hyperactivity is a symptom widely associated with positive symptoms and psychomotor agitation in schizophrenic patients [210]. Here, our results show that buspirone, at the dose of 1mg/Kg, blocked MK-801-induced hyperactivity, without causing catalepsy in WT mice. In $D3R^{-/-}$ the effect of buspirone was not very robust.

Another feature typical of schizophrenia is a disruption of the sensorimotor gating. In mice, a valuable model to study this symptom is the pre-pulse inhibition test (PPI). Our findings indicate that buspirone totally counteracted the MK-801-induced PPI disruption in WT mice and had not effect by itself. This finding is in line with previous evidence showing that buspirone weakly counteracts

apomorphine-induce PPI deficit [211] while as devoid of effect on its own [212].

Several study report that selective D3R antagonist ameliorate PPI deficits in different model of schizophrenia [213-215], suggesting that the antipsychotic-like effect of buspirone may be due to its activity on D3R.

Unfortunately it was not possible to measure the effect of buspirone on $D3R^{-/-}$ to study the direct involvement of D3R in PPI since these mice do not exhibit a strong acoustic startle reactivity.

Interestingly, clozapine, one of the most effective antipsychotic drugs, did not prevent Mk-801-induced PPI disruption or hyperlocomotion.

A limit of this study lies in the contribution of other receptors targeted by buspirone, such as 5-HT1AR and D4R, for which buspirone has a high affinity as a partial agonist and antagonist, respectively.

Taken together, these data indicate that buspirone might be a potential therapeutic medication for the treatment of cognitive deficits of schizophrenia through its D3R antagonist activity. Furthermore, the improvement in cognition demonstrated by D3R, reinforce the hypothesis that D3R could be considered a valuable pharmacological target for the treatment of schizophrenia.

Moreover, the repositioning of drugs endowed with D3R antagonist activity could promote the development of a new class of antipsychotic drugs for the treatment of cognitive deficits in schizophrenic patients.

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

In the second part we focused on the genetic modulation of D3R. In particular, we adopted a genetic approach to distinguish phenotypes regulated by epistasis (gene-by-gene interaction) from phenotype for which D3R and Dys have independent or no effect.

We first analyzed data from patient with schizophrenia extracted form the CATIE trial in order to investigated if an epistatic interaction would be detectable in humans clinical behavior. In particular we investigated the interaction between D3R Ser/Gly rs6280 and the Dys rs1047631 genetic variants. The results obtained suggest the presence of an epistatic interaction between D_3 and Dys genetic variants and that this affects cognitive function in patients with schizophrenia. Indeed the cognitive deficits, measured by WCST and WM task , are described as core features of schizophrenia and are related to dopamine signaling within the PFC [216-220].

To selectively address the D_3-Dys interaction, we established a new mouse line with concomitant hypofunction of both genes, D_3 and Dys ($D_3^{+/-}$ Dys $^{+/-}$; double heterozygous).

Mice were tested in the TOR and the WM task, behavioral tests that, like WCST and WM, relies on mPFC functioning and dopaminergic modulation [115, 117, 220, 221]. Data obtained, in mice with the social behavior and in humans with the negative and positive PANSS, show no significant effect of the D_3-by-Dys interaction for general clinical abilities. On the contrary, this effect was stronger in cognitive functions relevant to schizophrenia. This results are in line with previous findings showing that D3R blockade enhance cognitive

functions [148, 209] without inducing extrapyramidal or motor side effects typical of D2R-selective antipsychotic drugs [222, 223].

PPI response to an acoustic stimulus is a sensorimotor gating ability usually decreased in patients with schizophrenia as well as in mouse models relevant to the disease [93, 115, 224]. Consistent with previous findings [117], we found a different impact of D_3/Dys genotypes in PPI measures. Indeed, D_3-Dys double heterozygous mice exhibited a PPI response higher than in both wild-type and $D_3^{+/+}$ $Dys^{+/-}$ mice, suggesting that the beneficial cognitive effects of D_3 blockade should be considered in combination with gene-gene interaction between D_3 and Dys.

Encouraged by the behavioral effect of D_3-Dys genetic interaction we investigated if this was followed by an interaction at the molecular level. Our *in vitro* data show that increased D_3 mRNA expression levels in $Dys^{+/-}$ mice in mPFC and striatum were restored to the wild-type condition in both brain areas in $D_3^{+/-}$ $Dys^{+/-}$. This results are in line with recent evince showing that Dys could influence the expression of D3R [198]. Moreover, we found decreased Dys levels in the mPFC of both $Dys^{+/-}$ and double heterozygous. On the contrary, D_3/Dys genetic interaction rescued Dys expression to the WT level in striatum but not in mPFC.

As already shown, alterations in Dys expression can modify the recycling of D2R [117, 225]. The analysis of the total and surface levels of D2R-like showed that total levels of D2R-like expression were unchanged in both mPFC and striatum by alteration in Dys or D3R genotype. Moreover, while Dys hypofunction increased D2R-like expression on cell surface of both mPFC and striatum, this was unchanged in $D3R^{+/-}$ mice. Interestingly, D_3/Dys double heterozygous show a larger increase of D2R-like expression in the mPFC, in

contrast to the striatum where the expression of D2R-like returned to the wild type level.

These data are in line with several studying suggesting that Dys expression levels are linked to D_2-like receptor trafficking [117, 225, 226].

The different effect of D3R/Dys interaction on D2R/D3R expression in mPFC and striatum suggest a region-specific effect which require further investigation.

However, unlike the striatum, which contains two main classes of medium spiny neurons co-expressing D2R and D3R, in the PFC D3R is expressed in a sub-type of layer 5 pyramidal neurons, different form D1R-D2R- expressing cells [144].

In addition, while D2R-expressing cell project from the PFC to subcortical brain regions, D3R-expressing pyramidal neurons principally project cortically [227, 228].

Therefore, in the light of our electrophysiology results, we assume that D3R hypofunction ameliorates the disrupted excitability of layer 5 pyramidal neurons that we found in $Dys^{+/-}$, since the phenotype showed by $Dys^{+/-}$ was ameliorated in double mutant ($D_3^{+/-}$ $Dys^{+/-}$).

In conclusion, the present study supports D3R as a valuable target for the improvement of psychiatric-related cognitive function.

Moreover, our data suggest that the epistatic interaction between D3R and Dys might represent a new tool for patients stratification for the development and application of a more personalized therapeutic approach for the treatment of schizophrenia.

Conclusions

1. Buspirone prevented MK-801-induced TOR memory impairment and hyperlocomotion in WT mice, through its antagonism at D3R;

2. Contrary to clozapine, Buspirone counteracted MK-801-induced PPI disruption in WT mice;

3. In D3R$^{-/-}$ mice, buspirone was ineffective in preventing MK-801-induced TOR memory deficits and hyperlocomotion;

4. Buspirone, through its antagonism at D3R, may be a useful pharmacological tool for the improvement of the treatment of cognitive deficits in schizophrenia;

5. The repositioning of buspirone and/or other drugs with a pharmacological profile characterized by D3R antagonist activity, might be used for the development of new potential antipsychotic medication.

6. D3R represent a valuable pharmacological target for the treatment of cognitive deficit in schizophrenia;

7. The epistatic interaction between functional variants of D3R and Dys, affects the expression levels of these proteins and the cognitive performance in schizophrenic patients enrolled in the CATIE study;

8. The concurrent D3R/Dys hypofunction ameliorates the efficacy of antipsychotics improving cognitive functions related to Dys hypofunction;

9. Partial deletion of D3R improves PFC-dependent working memory deficits in $Dys^{+/-}$ mice;

10. D3R/Dys interaction produce a D2R/D3R imbalance in PFC with the consequent increase of D2R neuronal surface level, which improves cognitive function;

11. D3R hypofunction ameliorates Dys-dependent neuronal and dopaminergic basal abnormalities in PFC;

12. D3R represent a valid target for improving psychiatry-related cognitive deficits;

13. D3R/Dys epistatic interaction may represent a valuable tool for patients' stratification in schizophrenia;

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