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RESEARCH ARTICLE

Memantine-induced functional rewiring of the glutamate synapse in the striatum of dopamine transporter knockout rats

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Abstract

Background and Purpose: Slow-acting biogenic amines, such as dopamine, are known to modulate fast neurotransmitters e.g. glutamate. In the striatum, dopamine (DA) interacts with glutamate, influencing neural excitability and promoting synaptic plasticity. The exact mechanism of such interaction is not fully understood. This study investigates, in detail, how dopamine overactivity in dopamine transporter knockout ($DAT^{-/-}$) rats, alters the homeostasis of the striatal glutamate synapse from a molecular, behavioural and functional point of view.

Experimental Approach: The expression, localisation, retention and electrophysiological properties of N-methyl-D-aspartate (NMDA) receptors as well as dendritic spine density and morphology were investigated in the striatum of $DAT^{-/-}$ rats, at baseline and after treatment with the non-competitive NMDA receptor antagonist memantine (30 mg kg^{-1}).

Key Results: Dopamine overactivity dramatically reorganises the striatal glutamate synapse, redistributing NMDA receptors in the synapse as typified by reduced synaptic availability and reduced expression of NMDA scaffolding proteins, as well as by increased GluN2B-containing NMDA receptors in the extra synapse. Such changes are accompanied by reduced spine density, suggesting dopamine-induced structural rearrangements. These results converge into a compromised plasticity, as shown by the impaired ability to promote long-term depression (LTD) in the striatum of $DAT^{-/-}$rats. Notably, memantine counteracts hyperlocomotion, reverses spine alterations and abolishes the extrasynaptic movements of NMDA receptors in the striatum of $DAT^{-/-}$ rats, thus restoring functional LTD.

Abbreviations: CREB, cAMP response element-binding protein; fEPSP, field excitatory postsynaptic potential; LTD, long-term depression; PSD95, postsynaptic density protein 95; SAP102, synapse-associated protein 102; xCT^{-}, cystine/glutamate transporter (SLC7A11).

Lucia Caffino and Giorgia Targa contributed equally to the manuscript and can be both considered as first author.

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Conclusion and Implications: A hyperdopaminergic condition seems to alter striatal homeostasis by increasing extrasynaptic NMDA receptors. These findings may be relevant to manipulate disorders characterised by elevated dopaminergic activity.

KEYWORDS
dopamine, dopamine transporter, glutamatergic neurotransmission, memantine, striatum

1 | INTRODUCTION

It is established that dopaminergic input from the midbrain is crucial for the physiological development and/or maintenance of a properly organised and functional striatum (Küppers et al., 2000; Zhou & Palmiter, 1995). In the rat striatum, the vast majority of neurons (more than 95%) is represented by medium spiny neurons (MSNs) (Kreitzer, 2009), which receive at the same time dopaminergic inputs from the ventral tegmental area or substantia nigra pars compacta, and glutamatergic projections from the medial prefrontal cortex and thalamus (Tepper & Lee, 2007). Consistent with such projections, glutamate receptors are densely present in striatal neurons (Götz et al., 1997).

Glutamate and **dopamine** (DA) interact at the dendritic spines of these neurons, through manifold connections (Nair et al., 2014), where glutamate receptors densely co-localise with striatal **dopamine receptors** in the postsynaptic medium spiny neurons and regulate excitatory currents (Tarazi & Baldessarini, 1999). It is well known that the slow-acting neurotransmitter dopamine modulates both presynaptic and postsynaptic fast glutamate neurotransmission at the striatal level (Beaulieu & Gainetdinov, 2011; Svenningsson et al., 2004). Particularly, **N-methyl-D-aspartate (NMDA)** and dopamine receptors interact (Cepeda & Levine, 1998; Lee et al., 2002; Zhang et al., 2009) to mediate motor functions (Chase & Oh, 2000) and spatial memory (Ferretti et al., 2005). Notably, striatal NMDA–dopamine interaction has been shown in humans as well (Breier et al., 1998).

Glutamate neurotransmission occurs in the highly specialised postsynaptic density, a well-organised cytoskeletal structure located within the postsynaptic neuron at sites of excitatory synapses, containing **NMDA and non-NMDA glutamate receptors** as well as other proteins involved in receptor anchoring, signalling and modulation (Sheng & Hoogenraad, 2007). These cytoskeletal and regulatory proteins interact to coordinate activity-dependent changes in the postsynaptic density, including long-term potentiation (LTP) and long-term depression (LTD), critical for learning and memory (Malenka & Bear, 2004). While evidence exists showing that dopamine influences the trafficking of striatal NMDA receptors between subcellular compartments to postsynaptic sites (Dunah & Standaert, 2001), no evidence exists as to whether dopamine influences NMDA receptor movements toward extrasynaptic sites, as it is established that, in the plasma membrane, NMDA receptors are quite mobile as they can laterally move between synaptic and extrasynaptic sites (Groc et al., 2009). The investigation of the location of NMDA receptors on the plasma membrane is relevant because it affects their physiological function. In fact, mis-localisation of NMDA receptors is a critical contributing factor to glutamatergic dysfunction in several disorders of the central nervous system (Gladding & Raymond, 2011). Further, when dopamine neurotransmission is altered, changes in the levels and functions of glutamate receptors occur, as shown in experimental models of Parkinson's disease (Mellone & Gardoni, 2018), schizophrenia (Mohn et al., 1999) and addiction (Gardoni & Bellone, 2015).

The **dopamine transporter (DAT)** plays an active modulatory role in synaptic dopamine homeostasis. Knockout of DAT in mice leads to sustained elevation of extracellular dopamine concentrations and several other changes in the dopaminergic synapse (Giros et al., 1996) that cause significant alterations in striatal glutamate critical determinants and synaptic plasticity (Yao et al., 2004). Recently, we have developed a rat lacking the DAT ($DAT^{-/-}$ rat), whose features overlap with those of $DAT^{-/-}$ mice, showing markedly elevated

What is already known?

- Rats lacking the dopamine transporter (DAT) show increased levels of extracellular dopamine.
- Dopamine interacts with glutamate, influencing neural excitability and synaptic plasticity. However, the mechanism is unknown.

What this study adds?

- Dopaminergic overactivity reorganises the striatal glutamate synapse redistributing GluN2B-containing NMDA receptors in the extra synapse.
- Memantine normalises behavioural, molecular and structural alterations in $DAT^{-/-}$ rats, restoring functional glutamate homeostasis.

What is the Clinical Significance?

- We provide a mechanism of dopamine-associated dysregulation of glutamate neurotransmission for disorders characterised by hyperdopaminergia.

extracellular dopamine levels and profound alterations in presynaptic and postsynaptic components of dopamine transmission together with elevated locomotor activity at baseline (Leo et al., 2018). Thus, the DAT$^{-/-}$ rat represents a unique model to investigate how the glutamate synapse copes with an overactivated dopamine transmission.

In this study, we have investigated the hypothesis that dopamine overactivity, as shown in the striatum of DAT$^{-/-}$ rats, might alter the homeostasis of the glutamate synapse, with the aim of further deepening the peculiar features of dopamine–glutamate interaction.

2 | METHODS

2.1 | Animals

Zinc-finger nucleases (ZFNs) design, construction, *in vitro* validation, microinjection and founder selection were performed as previously described (Carbery et al., 2010; Geurts et al., 2009). The zinc-finger nucleases target site was CTCATCAACCCGCCACAGAccaccaGTGGAGGCTCAAGAG in the Exon 2 of *Slc6a3* (DAT gene; NCBI Gene ID: 24898; Genomic NCBI Ref Seq: NC_005100.3; mRNA NCBI Ref Seq: NM_012694.2). The knockout rat lines (DAT$^{-/-}$) were created in the outbred Wistar Han background at SAGE Labs. Adult littermate rats were housed by genotype in groups of three to four with free access to tap water and standard pellet food (Tecniplast GR900 cages). They were kept at 22°C and on a 12/12 h light/dark cycle (lights on 0700–1900 h). All experiments were conducted following the guidelines established by the European Community Council (Directive 2010/63/EU of 22 September 2010) and were approved by the Belgian Ministry of Health (Neurosciences LA1500024). All efforts were made to minimise animal suffering and to reduce the number of animals used. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

In each experiment performed, 3-month-old male DAT$^{-/-}$ and DAT$^{+/+}$ rats have been used. Genotyping was performed by polymerase chain reaction (PCR) followed by enzymatic digestion with BtsI MutI (New England Biolabs (Milan, Italy) as previously described (Leo et al., 2018).

2.2 | Drugs

Memantine was dissolved in 0.9% saline. Experimental procedures on DAT$^{-/-}$ and their wild-type counterpart (DAT$^{+/+}$) animals were conducted 30 min after intraperitoneal injection (i.p. 30 mg kg^{-1}).

2.3 | Preparation of protein extract and Western blot assays

Proteins in the whole homogenate, postsynaptic, extra synaptic and nuclear fractions of the striatum were analysed as previously described with minor modifications (Caffino et al., 2019). Briefly, rats were decapitated without anaesthesia in order to avoid the effects of anaesthetics on gene and protein expression. Striata from adult male rats DAT$^{+/+}$ and DAT$^{-/-}$ naïve in Experiment 1 (DAT$^{+/+}$ ($n = 5$), DAT$^{-/-}$ ($n = 5$)), and DAT$^{+/+}$ and DAT$^{-/-}$ treated with saline or memantine (dissolved in 0.9% saline) in Experiment 2 (DAT$^{+/+}$ $n = 6$, DAT$^{-/-}$ $n = 6$, DAT$^{+/+}$ treated with memantine $n = 6$, DAT$^{-/-}$ treated with memantine $n = 6$) were homogenised in a Teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$ and 0.1 mM PMSF, in the presence of commercial cocktails of protease and phosphatase inhibitors. An aliquot of each homogenate was then sonicated and stored at −20°C. The remaining homogenate was centrifuged at 800*g* for 5 min; the resulting pellet (P1), corresponding to the nuclear fraction, was resuspended in a buffer (HEPES, 20 mM; dithiothreitol, 0.1 mM; EGTA, 0.1 mM) with protease and phosphatase inhibitors, whereas the obtained supernatant was then centrifuged at 13000*g* for 15 min, obtaining a pellet. This pellet was resuspended in a buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100,000*g* for 1 h. The resulting supernatant, referred to as Triton X-100 soluble fraction (TSF, extrasynaptic fraction), was stored at −20°C; the pellet, referred to as PSD or Triton X-100 insoluble fraction (TIF, postsynaptic density), was homogenised in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at −20°C in the presence of glycerol 30%. Total proteins have been measured in the homogenate, P1, triton-insoluble fraction and triton-soluble fraction fusing the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as the calibration standard.

Equal amounts of proteins of the homogenate (10 μg), nuclear fraction (15 μg), triton-insoluble fraction (8 μg) and triton-soluble fraction (50 μg) were run on a sodium dodecyl sulphate 8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). The entire nitrocellulose blot was cut close to the molecular weight at which protein bands are expected to be detected, as suggested by their specific molecular weight and the information depicted in the datasheet of the antibody. Blots were blocked for 1 h at room temperature with I-Block solution (Life Technologies Italia, Italy) in TBS 0.1% Tween-20 buffer and incubated with antibodies against the proteins of interest.

The conditions of the primary antibodies were the following:- anti-GLT-1 (1:2000, Abcam, Cat# Ab41621, RRID:AB_941782), anti-vGluT1 (1:1000, Cell Signalling Technology, Cat# 12331, RRID:AB_2797887), anti vGluT2 (1:1000, Cell Signalling Technology, Cat# 71555, RRID:AB_2799805), anti xCT^{-}[**SLC7A11**] (1:1000, Abcam, Cat# ab216876), anti phospho-Synapsin1 Ser603 (1:1000, Cell Signalling Technology, Cat# 88246, RRID:AB_2800119), anti Synapsin1 (1:2000, Santa Cruz Biotechnology, Cat# sc-8295, RRID:AB_677472), anti phospho-αCaMKII Thr286 (1:1000, Thermo Fisher Scientific, Cat# MA1047, RRID:AB_325402), anti αCaMKII (1:2000, Millipore, Cat# 05-532, RRID:AB_309787), anti GluN1 (1:1000, Cell Signalling Technology, Cat# 5704, RRID:AB_1904067), anti GluN2A (1:1000, Cell Signalling Technology, Cat# 4205, RRID:

AB_2112295), anti GluN2B (1:1000, Cell Signalling Technology, Cat# 14544, RRID:AB_2798506), anti SAP102 (1:1000, Cell Signalling Technology, Cat# 47421, RRID:AB_2799325), anti Rabphilin (1:2000, Proteintech, Cat# 11396-1-ap, RRID:AB_2181145), anti-PSD95 (1:2000, Cell Signalling Technology Cat# 3450, RRID:AB_2292883), anti phospho-CREB Ser133 (1:1000, Cell Signalling Technologies, Cat# 9198, RRID:AB_2561044), anti CREB (1:1000, Cell Signalling Technologies, Cat# 9197, RRID:AB_331277), anti phospho-NL-1 Thr739 (1:1000, Sigma-Aldrich, Cat# ABN1707), anti-NL-1 (1:1000, Synaptic System, Cat# 129003, RRID:AB_887746), anti-Arc/Arg3.1 (1:500, BD Biosciences, Cat# 612603, RRID:AB_399886) and anti β-actin (1:5000, Sigma-Aldrich, Cat# A5441, RRID:AB_476744). Results were standardised using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Immunocomplexes were visualised by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories, RRID: SCR_019037), analysed with Image LabTM software (Bio-Rad, RRID: SCR_014210) and representative immunoblots for each protein are shown in Figures 1g, 2l and 3g. Because gels were run at least two times, the obtained results were averaged with a correction factor: Correction factor gel B = average of (OD protein of interest/OD β-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel B) (Caffino et al., 2020). Cropped immunoblots related to the protein expression levels measured in the whole homogenate, postsynaptic and extrasynaptic density, and nuclear fraction of striatum are presented in Figures S2–S5.

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.4 | mRNA extraction and real-time PCR analysis

Total RNA from striatal tissues (experiment 2) of $DAT^{+/+}$ ($n = 8$) and $DAT^{-/-}$ ($n = 8$) were isolated by single-step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer instructions, quantified by means of the Nanodrop spectrophotometric analysis and stored at −20°C until further processing.

Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real-time RT-PCR) to assess mRNA levels, as previously described (Mottarlini et al., 2022). Briefly, an aliquot of each sample was treated with DNase (DNase I, RNase-free buffer di MnCl2-Thermo Scientific) to remove any genomic DNA still present, for 30 min at 37 °C and, afterward, incubated at 65°C for 10 min with ethylenediaminetetraacetic

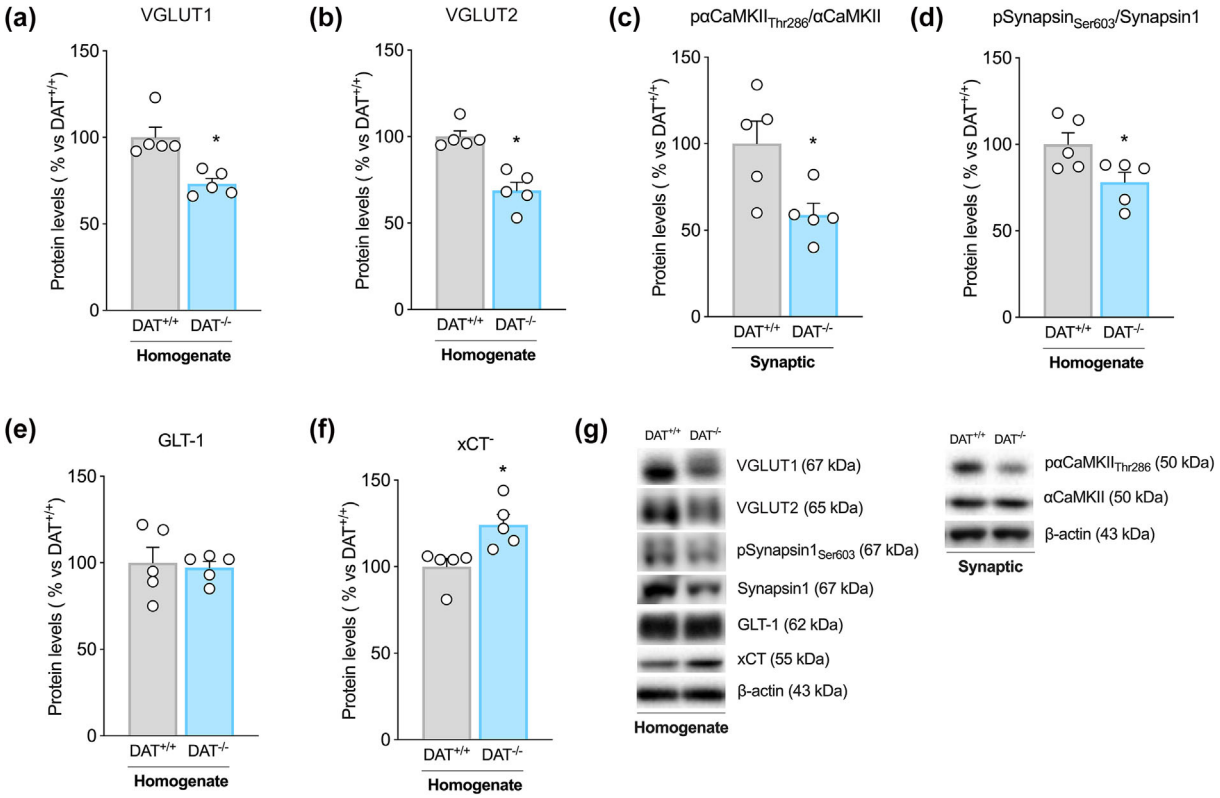

FIGURE 1 Presynaptic changes in the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. Protein levels of (a) VGLUT1, (b) VGLUT2, the ratio of (d) pSynapsin$_{Ser603}$/Synapsin1, (e) GLT-1 and (f) xCT^{-} were measured in the whole homogenate of the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. The ratio (c) pαCaMKII$_{Thr286}$/αCaMKII was investigated in the postsynaptic density. (g) Representative immunoblots are shown for VGLUT1, VGLUT2, pαCaMKII$_{Thr286}$, αCaMKII, pSynapsin$_{Ser603}$, Synapsin1, GLT-1, xCT^{-} and β-actin. Results are expressed as mean percentage ± mean standard error from five independent determinations for each experimental group. Unpaired Student's *t* test *$P < 0.05$ versus $DAT^{+/+}$ rats.

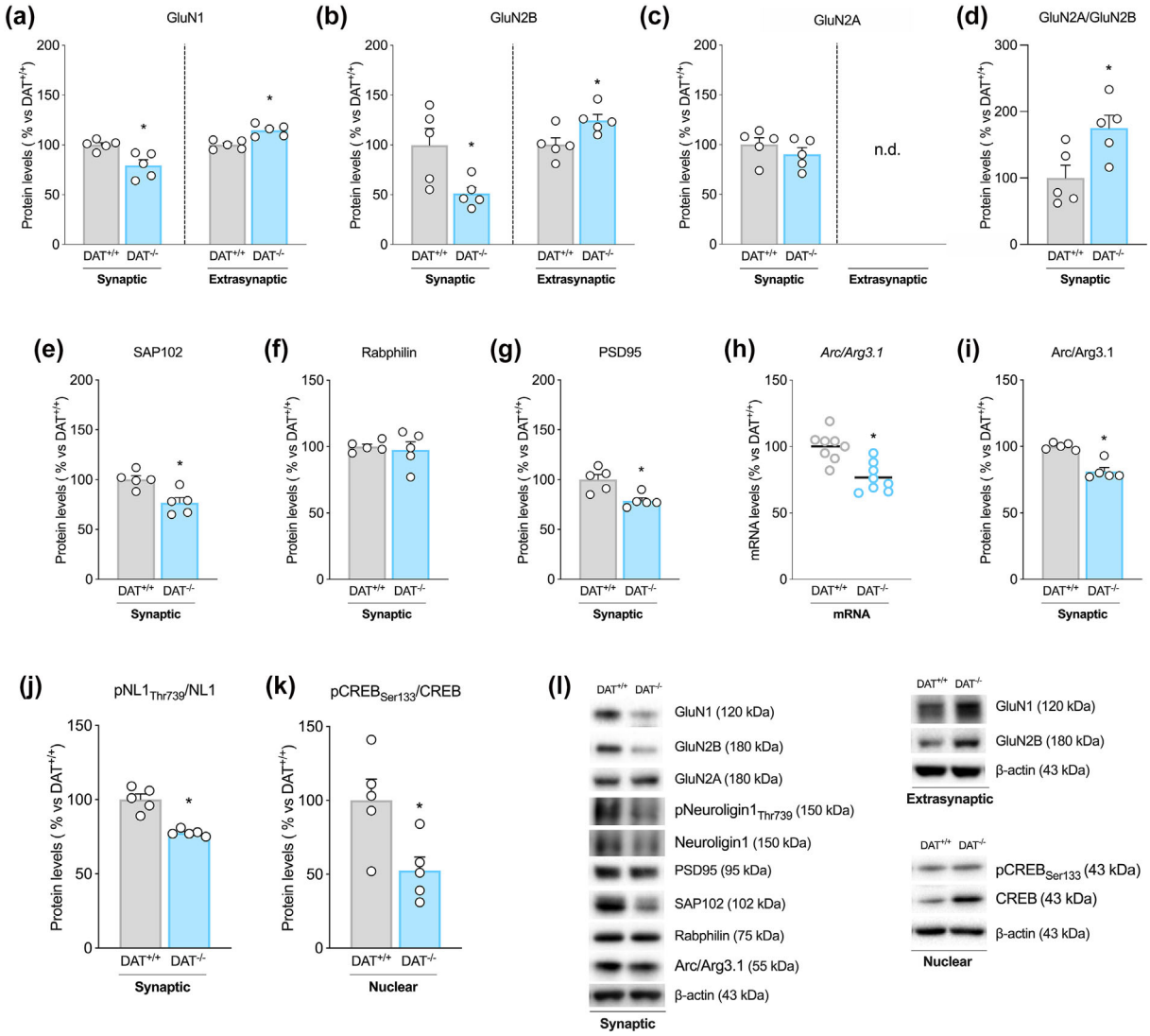

FIGURE 2 NMDA receptor subunits and their related scaffolding protein, adhesion molecule neuroligin 1 (NL-1) and transcription factor CREB protein alterations in the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. (a) GluN1 and (b) GluN2B protein levels were investigated in the synaptic (left) and in the extrasynaptic (right) fractions. (c) GluN2A, (d) GluN2A/GluN2B ratio, (e) SAP102, (f) Rabphilin, (g) PSD95 and (i) *Arc/Arg3.1* protein levels were measured in the postsynaptic fraction. (h) Striatal *Arc/Arg3.1* mRNA levels. The (j) ratio pNL-1_{Thr739}/NL-1 was measured in the postsynaptic density and the (k) ratio $pCREB_{Ser133}$/CREB was investigated in the nuclear fraction of the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. (l) Representative immunoblots are shown for GluN1, GluN2B, GluN2A, pNL-1_{Thr739}, NL-1, PSD95, SAP102, Rabphilin, $pCREB_{Ser133}$, CREB and β-actin. Results are expressed as mean percentage ± mean standard error from five (for the protein levels) or eight (for mRNA levels) independent determinations for each experimental group. Unpaired Student's *t* test $^*P < 0.05$ versus $DAT^{+/+}$ rats.

acid (EDTA) to block DNase action. Expression levels of the genes of interest were analysed by TaqMan qRT-PCR 48 thermal cycler (CFX384 real-time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in triplicate in a 384-well plate. Thermal cycling was initiated with incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (TaqMan polymerase activation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10 s to enable the melting process and then for 30 s at 60°C for the annealing and extension reaction. Data were analysed with the comparative threshold cycle (ΔΔCt) using *36B4* as the internal standard.

RT-PCR analysis was performed to evaluate activity regulated cytoskeleton associated protein (*Arc/Arg3.1*) gene expression. Primers and probes for *Arc/Arg3.1 and 36B4* were purchased from Eurofins MWG-Operon. Their sequences are shown below:

- *Arc/Arg3.1*: forward primer 5′-GGTGGGTGGCTCTGAAGAAT-3′, reverse primer 5′-ACTCCACCCAGTTCTTCACC-3′, probe 5′-GATCCAGAACCACATGAATGGG-3′;
- *36B4*: forward primer 5′-TTCCCACTGGCTGAAAAGGT-3′, reverse primer 5′-CGCAGCCGCAAATGC-3′, probe 5′-AAGGCCT-TCCTGGCC GATCCATC-3′;

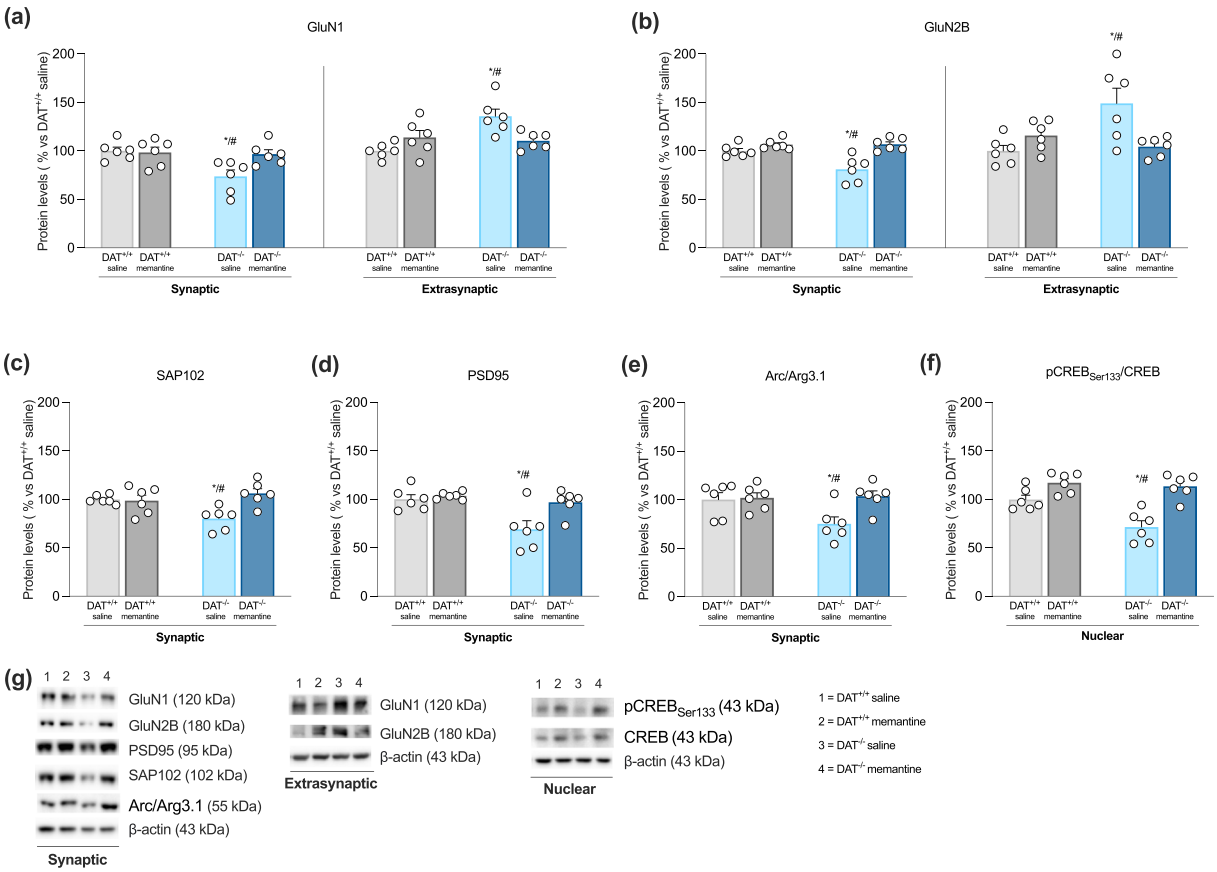

FIGURE 3 Effect of the memantine (30 mg kg^{-1}) treatment on the glutamatergic determinants in the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. Protein levels of the NMDA (a) subunit GluN1 and the (b) subunit GluN2B were measured in the synaptic (left) and in the extrasynaptic (right) fractions. The (c) anchoring proteins SAP102 as well as proteins involved in synaptic plasticity, (d) PSD95 and (e) Arc/Arg3.1, were investigated in the synaptic fraction. The ratio (f) $pCREB_{Ser133}$/CREB was investigated in the nuclear fraction. (g) Representative immunoblots are shown for GluN1, GluN2B, SAP102, PSD95, Arc/Arg3.1, $pCREB_{Ser133}$, CREB and β-actin. Results are expressed as mean percentage ± mean standard error from six independent determinations for each experimental group. Two-way ANOVA followed by Tukey's multiple comparisons test. $^{*}P < 0.05$ versus $DAT^{+/+}$ saline rats, $^{\#}P < 0.05$ versus $DAT^{-/-}$ memantine rats. 1 = $DAT^{+/+}$ saline; 2 = $DAT^{+/+}$ memantine; 3 = $DAT^{-/-}$ saline; 4 = $DAT^{-/-}$ memantine.

2.5 | Dendritic spine labelling and morphological classification

Neuronal labelling and morphological classification of dendritic spines in the whole striatum of $DAT^{+/+}$ ($n = 4$), $DAT^{-/-}$ ($n = 4$), $DAT^{+/+}$ treated with memantine ($n = 4$), $DAT^{-/-}$ treated with memantine ($n = 4$) rats were achieved using the lipophilic membrane tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DilC18[3]) (Life Technologies), as previously published (Caffino et al., 2023). Briefly, rats were deeply anesthetised and perfused with 0.1 M phopshate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Then brains were postfixed at 4°C in 4% PFA in PB for 40 min and stained with the dye. At least 20 neurons belonging to different dendritic segments, for each experimental group were used for quantification, and the neurons analysed belonged to eight hemispheres per group. The average dendritic length analysed is 50 µm and the length of the total dendrites analysed was at least 1500 µm for each experimental group. Analysis of dendritic spine morphology was performed with Fiji software (RRID: SCR_002285):- each protrusion was manually traced and specifically, spine length, head (Wh) and neck width (Wn) were measured. These parameters were used to classify and sort dendritic spines into three categories (thin, stubby and mushroom). The classification scheme is as follows: protrusions with a length greater than 3 µm were classified as filopodia, whereas those with a length below this threshold as dendritic spines. Among the dendritic spines, those with a Wh/Wn ratio greater than 1.7 were categorised as mushrooms, whereas those with a Wh/Wn ratio smaller than 1.7 were further subdivided into two groups:- stubby shorter than 1 µm and thin if longer than 1 µm (Gardoni et al., 2012). An operator who was 'blind' to the experimental conditions performed both image acquisition and quantification.

2.6 | Electrophysiology

Electrophysiology was performed as described (Villers & Ris, 2013) on $DAT^{+/+}$ and $DAT^{-/-}$ male rats ($DAT^{+/+}$ $n = 5$, $DAT^{-/-}$ $n = 7$, $DAT^{-/}$

$^{-}$ memantine $n = 5$) with minor modifications. Rats were anaesthetised with Nembutal IP (100 mg kg^{-1}) and then decapitated. Brains were placed in a 4°C artificial cerebrospinal fluid (aCSF) solution (124 mM NaCl, 4.4 mM KCl, 26 mM NaHCO3, 1 mM NaH2PO4, 2.5 mM CaCl2, 1.3 mM MgSO4, 10 mM D-glucose) and bubbled with a mixture of 95% O_2 and 5% CO_2. A thick section encompassing corticostriatal fibres was dissected, from which 400 µm slices were cut in aCSF with a vibratome (Leica VT1000S) (Hernandez et al., 2016). Slices were transferred to a recording chamber and allowed to recover for 1.5 h in interface mode. Recordings were made in an interface chamber (Tissue Slice Recording Chamber dual well 21051-00, FST, Canada) at 32°C, under constant perfusion with oxygenated aCSF (1 ml min^{-1}). Two bipolar twisted nickel–chrome stimulating electrodes, 50 µm in diameter, were placed in close proximity to the recording electrode, located close to the anterior commissure (Figure 5c). Extracellular field excitatory postsynaptic potentials (fEPSPs) were evoked using biphasic constant-voltage pulses (0.08 ms for each pulse) and recorded with low resistance (2–5 MΩ) glass microelectrode filled with aCSF. Baseline input/output (I/O) curves were determined by varying the stimulus intensity and determining the amplitude of the fEPSP. Paired-pulse response ratios were obtained by delivering two stimuli with an intensity to induce 40% of the maximal response at various intervals. For LTD recordings, stimuli were delivered every minute using a stimulus intensity to induce 40% of the maximal response for a total duration of 80 min. The initial slope of the fEPSP was measured on the average of four consecutive responses and a stable baseline was established for at least 20 min.

Theta burst stimulation was applied on one of the stimulating electrodes and consisted of 10 trains 15 s apart, each train consisting of 10 bursts at 200 Hz of 4 pulses at 50 Hz, using a twofold stimulation intensity. Control stimulation (eliciting 40% of maximal fEPSP) was then applied every minute until the end of the experiment. The second stimulating electrode was used as an internal control, with no LTD induction (control pathway). Data were normalised over the initial 20-min baseline and were excluded if recordings were unstable. Only experiments with LTD induction leading to initial (first 10 min after induction) fEPSP slope value lower than 70% of baseline value were considered. In memantine experiments, 10 µM of the substance was added to aCSF, starting during the recovery period and perfused continuously during the whole experiment.

Electrophysiological data acquisition was performed using WinLTP (Anderson et al., 2012), as previously described (Villers & Ris, 2013).

2.7 | Open field test

Two groups of male rats [$n = 12$ $DAT^{-/-}$, $n = 12$ wildtype ($DAT^{+/+}$)] were bred at the University of Mons (Mons, Belgium). The animals were tested for explorative and locomotor behaviour in the open field test (OFT), as previously described (Reinwald et al., 2018), at the age of 12 weeks on two consecutive days. In open field test, an individual rat was placed for 30 min in an unfamiliar illuminated arena and locomotor activity was recorded. The total distance travelled was measured and expressed in terms of centimetres travelled by the animal. Distance, central time and velocity were analysed using EthoVision® XT (Noldus, Wageningen, Netherlands, RRID: SCR_000441). Effects of memantine (10 µM; 30 $mg{\cdot}kg^{-1}$; i.p.) on locomotor activity in a novel environment were tested 30 min after injection. All solutions were made fresh daily.

2.8 | Statistical analysis

Data were collected in individual animals (independent determinations) and are presented as means ± standard errors. For each experiment, the normality of residuals was tested with the Kolmogorov–Smirnov test.

Molecular changes in protein levels (Experiment 1) or in mRNA levels produced by genotype with normal distribution were analysed by unpaired Student's *t*-test (*t*), using as control condition $DAT^{+/+}$ animals and as testing condition $DAT^{-/-}$. Data with a non-normal distribution were analysed by the Mann–Whitney test (*U*). Detailed statistics, such as *t* or *U* and *P* values of Student's *t*-test or Mann–Whitney test, are reported in Table S3.

After memantine treatment, molecular (Experiment 2) and morphological changes produced by genotype and memantine treatment were analysed by two-way (analysis of variance) ANOVA (Sample sizes were subjected to statistical analysis at least five animal per group ($n = 5$), where n = number of independent values.). When demanded by a relevant interaction ($P < 0.05$), Tukey's multiple comparisons test was performed to characterise differences among individual groups of rats. Detailed statistics, such as *F* and *P* values, of independent variables of two-way ANOVA, are reported in Supplementary Tables S4–S5.

For LTD statistical analysis, averaged fEPSP slopes recorded during the last 12 min of recording were used for statistical analysis among the experimental groups. Electrophysiological changes were analysed by a one-way ANOVA. When demanded by a relevant interaction, Tukey's multiple comparisons test was performed to characterise differences among individual groups of rats. Detailed statistics, such as *F* and *P* values of one-way ANOVA, are reported in Table S6.

For behavioural analysis, one-way ANOVA on velocity and distance travelled among groups was performed and if statistically significant, Tukey's multiple comparisons test was performed to characterise differences among individual groups of rats. Detailed statistics, such as *F* and *P* values of one-way ANOVA, are reported in Table S7.

Subjects were eliminated from the final dataset if their data deviated from the mean by 2 SDs. Prism 9 (GraphPad Software Prism v9, San Diego, CA, USA, RRID: SCR_002798) was used to analyse all data. Significance for all tests was assumed at $P < 0.05$.

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.9 | Materials

Memantine was purchased from Interchim (Montluçon, France), while the following were purchased from Sigma-Aldrich (Milan, Italy): phosphatase inhibitors, HEPES, $MgCl_2$, $NaHCO_3$, NaH_2PO_4, NaCl, KCl, $CaCl_2$, $MgSO_4$, D-glucose, peroxymonosulfate (PMS). Commercial cocktails of protease were purchased from Roche (Monza, Italy) and 1,1′-dioctadecyl- 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DilC18[3]) from Life Technologies (Segrate, Italy). Details of other materials and suppliers were provided in the specific sections.

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/2024 (Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Amarosi, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Annett, et al., 2023; Alexander, Mathie, Peters, Veale, Striessnig, Kelly, Armstrong, Faccenda, Harding, Davies, Aldrich, et al., 2023).

3 | RESULTS

3.1 | DAT deletion dysregulates the molecular composition of the glutamate synapse in the striatum

The **vesicular glutamate transporters type 1 (VGLUT1/SLC17A7)** and **type 2 (VGLUT2/SLC17A6)**) are responsible for storing glutamate in presynaptic vesicles and contribute to glutamate exocytic release (Fremeau et al., 2004). In the striatum of $DAT^{-/-}$ rats, VGLUT1 and VGLUT2 levels are significantly reduced (Figure 1a, b), suggesting reduced exocytotic glutamate release from presynaptic terminals.

Exocytosis involves the activation of intracellular signalling pathways such as, among others, the serine/threonine kinase**, calmodulin-dependent kinase II (αCaMKII)** (Hudmon & Schulman, 2002). It is known that, through the phosphorylation of several synaptic proteins, αCaMKII increases glutamate release (Nicholls, 1998). Among these, a critical role is played by αCaMKII-induced phosphorylation of Synapsin1 at serine residue 603 ($Synapsin1_{Ser603}$) (Leenders & Sheng, 2005). When phosphorylated at this residue, Synapsin1 contributes to mobilise an increased number of vesicles for release during presynaptic depolarisation. We found that the phosphorylation of both $\alpha CaMKII_{Thr286}$ (Figure 1c) and $Synapsin1_{Ser603}$ (Figure 1d) is significantly reduced in the striatum of $DAT^{-/-}$ rats, further corroborating the potential reduction of exocytotic glutamate release (Chang et al., 2021).

Extracellular glutamate concentration is primarily regulated by the action of the **glial glutamate transporter (excitatory amino acid transporter 2; GLT-1)**, which removes extracellular glutamate from the synaptic cleft (Murphy-Royal et al., 2017). However, striatal GLT-1 expression is unchanged in the striatum of $DAT^{-/-}$ rats (Figure 1e). The other glial mechanism regulates extracellular glutamate concentrations, the so-called glial cystine/glutamate antiporter X_c^-, which exchanges extracellular cystine for intracellular glutamate and, due to its glial localisation, delivers glutamate to the extrasynaptic milieu (LaCrosse et al., 2017). This mechanism is critical for glutamate homeostasis, especially in the striatum, where it supplies up to 60% of extracellular levels of glutamate (Massie et al., 2011). We found that xCT^-, the light chain component of the system X_c^-, expression is increased in the striatum of $DAT^{-/-}$ rats (Figure 1f), suggesting increased glutamate efflux at extrasynaptic sites (Baker et al., 2002).

Next, we investigated the expression of NMDA receptor subunits and their related anchoring protein, which are critical for glutamatergic neurotransmission. We found a reduction of **GluN1**, the main subunit, and **GluN2B**, the accessory subunit of NMDA receptor in the striatal synaptic fraction of $DAT^{-/-}$ rats. Further there was a concomitant increase of these subunits in the extrasynaptic fraction, suggesting that dopamine overactivity shapes the NMDA glutamate signal in the cleft (Figure 2a, b). No changes were observed in the expression of the accessory subunit **GluN2A** in the synaptic fraction, whereas in the extrasynaptic fraction its expression was not detected (Figure 2c), in line with the literature (Miwa et al., 2008). We also found an increase in the GluN2A/GluN2B ratio in the synaptic fraction (Figure 2d), which aligns with most of the GluN2B subunit being shifted toward the extrasynaptic sites. The scaffolding protein specific for the GluN2B subunit, that is SAP102, showed reduced expression, whereas the anchoring protein specific for the GluN2A subunit, that is Rabphilin, was unaltered (Figure 2e, f). We also investigated markers involved in controlling synaptic transmission and plasticity, as well as in regulating dendritic spine structure, that is PSD95 and the immediate early gene *Arc/Arg3.1* (Bramham, 2008; Fernández et al., 2017). Our findings found a decrease in the expression levels of PSD95. Similarly, both mRNA and protein levels of Arc/Arg3.1 were reduced, indicating an overall impairment of glutamatergic synaptic activity (Figure 2g–i).

Another critical determinant of glutamate homeostasis is represented by the trans-adhesion molecule **neuroligin 1 (NL-1)**, which glues together presynaptic and postsynaptic terminals for physiological glutamatergic neurotransmission (Haas et al., 2018). Because evidence exists that NL-1 activation is activity dependent, a mechanism primarily mediated by αCaMKII and NMDA receptors (Chubykin et al., 2007), and given that αCaMKII phosphorylates NL-1 to regulate excitatory synapses (Bemben et al., 2014), we measured the expression and phosphorylation of NL-1. We found that NL-1 phosphorylation in Thr739 was significantly reduced in the striatum of $DAT^{-/-}$ rats compared to wild types (Figure 2j), suggesting that presynaptic and postsynaptic glutamatergic terminals may not be tightly connected.

Searching for a mechanism to explain the increased extrasynaptic movement of NMDA receptors, we have investigated the phosphorylation of the transcription factor CREB. This information is critical

because inhibition of CREB phosphorylation is known to drive NMDA receptor extrasynaptic movement (Bemben et al., 2014). Notably, we found a significant reduction of CREB phosphorylation in Ser133 in the striatum of DAT$^{-/-}$ rats, suggesting a mechanism for NMDA extrasynaptic localisation (Figure 2k). Protein expression levels measured in the whole homogenate are reported in Table S1.

The observed changes in the striatum of DAT$^{-/-}$ rats indicate increased trafficking of GluN2B-containing NMDA receptors from synaptic towards extrasynaptic sites through a CREB-dependent mechanism (Hardingham et al., 2002).

3.2 | Memantine restores the molecular composition of the glutamate synapse in the striatum of DAT$^{-/-}$ rats

We wondered whether blocking NMDA receptors by memantine, an antagonist with a high affinity for extrasynaptic NMDA receptors, could, at least in part, restore the normal balance of NMDA receptors between synaptic and extrasynaptic sites in DAT$^{-/-}$ rats. Figure 3a–f illustrates that expression levels of the glutamatergic targets whose expression was altered in the striatum of DAT$^{-/-}$ rats were restored following a single injection of memantine.

3.3 | Memantine restores structural plasticity in the striatum of DAT$^{-/-}$ rats

Because the reduction of PSD95 and Arc/Arg3.1 levels in the striatum of DAT$^{-/-}$ rats could reflect spines less enriched in PSD95 or an overall loss of dendritic spines in this brain region, we performed morphological analyses in DAT$^{-/-}$ and DAT$^{+/+}$ rats treated with saline or memantine. We found that DA overactivity reduced dendritic spine density, which was restored by a single injection of memantine (Figure 4a, b). Evaluating the shape of all spines (mushroom, thin and stubby), DAT$^{-/-}$ rats showed a reduction in the percentages of mushroom-shaped spines, that is the active and mature spines (Figure 4c), and a concomitant increase in stubby-shaped spines, that is immature with a short lifetime (Figure 4d) (Arellano et al., 2007). Of note, treatment with the NMDA antagonist memantine modified the percentage of mushroom- and stubby-shaped spines, increasing and reducing their percentages, respectively (Figure 4c, d). No changes were instead observed in the percentages of thin-shaped spines and filopodia (Table 1) nor in the length and head-width size of spines analysed (Table S2).

3.4 | Memantine restores synaptic plasticity in the striatum of DAT$^{-/-}$ rats

The next step was to investigate whether the reorganisation of the glutamate synapse would lead to functional impairment. We analysed glutamate-dependent neurotransmission in DAT$^{-/-}$ rats. Electrophysiological experiments were conducted using extracellular recordings in ex vivo striatal slices from DAT$^{+/+}$ and DAT$^{-/-}$ rats. Specifically, we decided to evaluate striatal LTD expression because it is a strongly DA-dependent process (Calabresi et al., 1992; Centonze et al., 2001). In the striatum, theta-burst stimulation consistently produced a robust, long-lasting LTD induction in DAT$^{+/+}$ animals (Figure 5a, b). In contrast, LTD was impaired in DAT$^{-/-}$ rats, suggesting that this deficit is fine-tuned by the increased dopaminergic tone. To investigate whether the unphysiological activation of extrasynaptic NMDA receptors and/or the reduction of synaptic NMDA receptors might contribute to impaired LTD, we evaluated whether memantine exposure may induce changes in neuronal properties and restore LTD in DAT$^{-/-}$ rats. To this end, *ex vivo* recordings were performed in the ventral part of the striatum in the presence of the drug diluted in aCSF solution, and we observed that memantine-treated slices from DAT$^{-/-}$ rats showed a restored LTD induction (Figure 5a, b). Further, paired-pulse ratio measurements did not reveal any variations, indicating that the dynamic of synaptic release remained unchanged across all the experimental groups in the ventral part of the striatum (Figure S1).

3.5 | Memantine partially reduces hyperactivity in DAT$^{-/-}$ rats

We have previously shown that DAT$^{-/-}$ rats exhibit hyperactivity and impaired learning (Leo et al., 2018). We reasoned that if mis-localised NMDA receptor signalling is a crucial mechanism in triggering hyperactivity, then the suppression of NMDA receptor activity through memantine would reverse the behavioural and cognitive symptoms observed in DAT$^{-/-}$ rats. Indeed, memantine significantly reduced DAT$^{-/-}$ locomotor activity during the open-field test performed 30 min after drug administration, reducing both velocity and distance travelled by DAT$^{-/-}$ animals (Figure 6a, b). Moreover, acute memantine injection induced a change in locomotor patterns, as shown in Figure 6c. In addition, we found that the reduced locomotor activity is not because of stereotypies (data not shown), in line with previous data showing the lack of significant stereotypies at doses of memantine ranging from 20 to 60 mg kg^{-1} (Danysz et al., 1994).

4 | DISCUSSION

We here demonstrate that the hyperdopaminergic condition, caused by targeted inactivation of the DAT, impairs striatal glutamate homeostasis via alterations of NMDA receptor synaptic trafficking and spine morphology, leading to impaired LTD that ultimately compromises striatal functions. Such effects seem to be driven by extrasynaptic NMDA receptors because the administration of memantine, an NMDA receptor antagonist, restores molecular, structural and functional striatal glutamate homeostasis, and it reduces velocity and distance travelled by DAT$^{-/-}$ rats. These findings identify a previously unknown mechanism of dopamine-associated dysregulation of the glutamate synapse critical for disorders characterised by hyperdopaminergia.

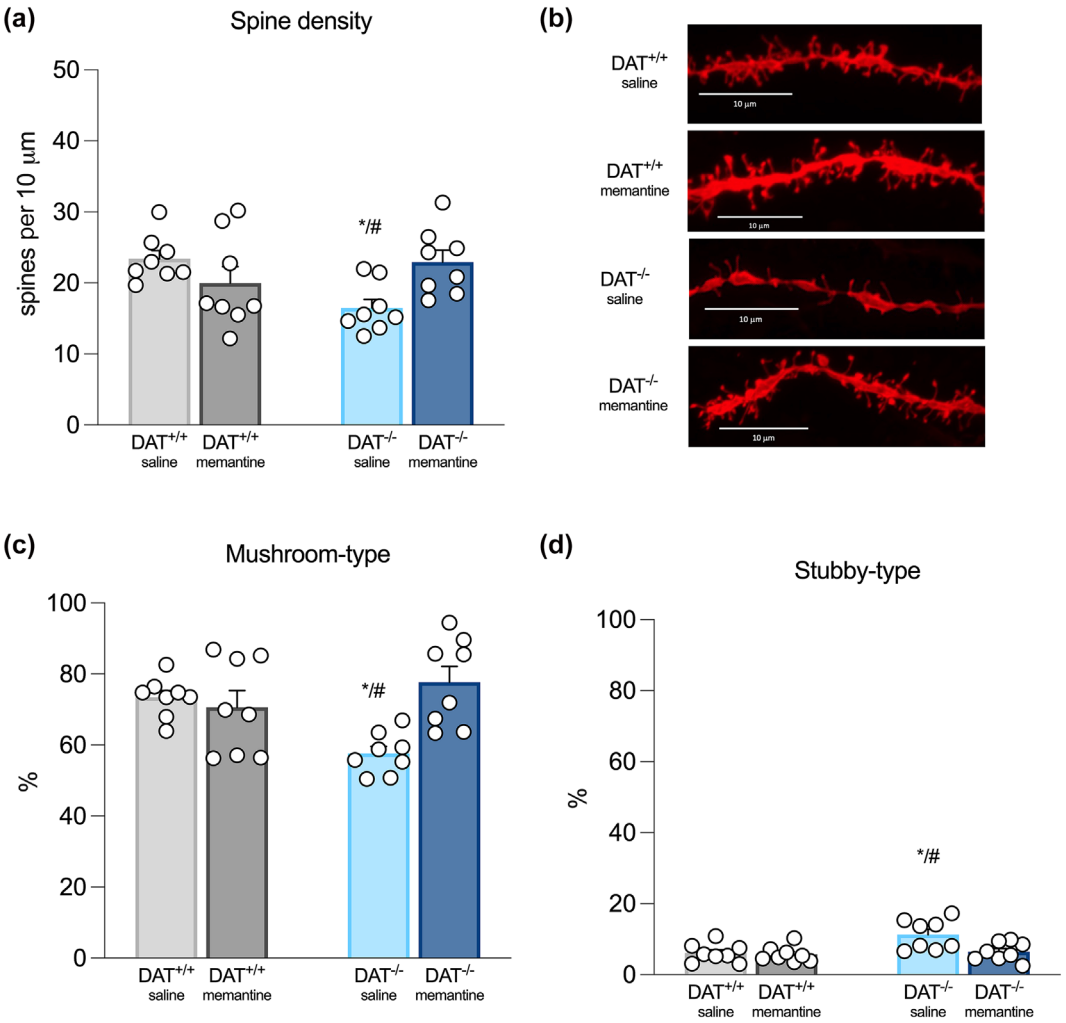

FIGURE 4 Effects of the memantine (30 mg kg^{-1}) treatment on dendritic spine density and morphology in the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats. Panel (a) shows the total spine density in the striatum, which is possible to appreciate in representative pictures from each (b) experimental group. The (c) percentage of mushroom- and (d) stubby-shaped spines are represented. $N > 2500$ spines from at least 20 different neurons for each group, neurons analysed belonged to eight hemispheres per group. Results are expressed as mean percentage ± mean standard error. Two-way ANOVA followed by Tukey's multiple comparisons test. $^{*}P < 0.05$ versus $DAT^{+/+}$ saline rats, $^{\#}P < 0.05$ versus $DAT^{-/-}$ memantine rats.

	$DAT^{+/+}$ Saline	$DAT^{+/+}$ memantine	$DAT^{-/-}$ Saline	$DAT^{-/-}$ memantine
% thin	10.37 ± 3.84	22.32 ± 6.75	12.64 ± 5.38	12.37 ± 4.37
% filopodia	11.77 ± 2.41	6.15 ± 1.25	14.88 ± 4.06	5.82 ± 1.51

TABLE 1 Effects of the memantine (30 mg kg^{-1}) treatment on the percentage of thin-shaped spines and filopodia in the striatum of $DAT^{+/+}$ and $DAT^{-/-}$ rats.

Note: Results are expressed as mean percentage ± mean standard error. Two-way ANOVA followed by Tukey's multiple comparisons test.

Our data indicate that dopamine overactivity causes striatal glutamate dyshomeostasis through glutamatergic changes occurring at both presynaptic and postsynaptic terminals. At the presynaptic level, our findings converge on a reduction of glutamate release as shown by reduced expression of VGLUT1 and VGLUT2 as well as reduced αCaMKII-induced $Synapsin1_{Ser603}$ phosphorylation. Further studies involving reliable approaches to measure glutamate release are necessary to explore this possibility. Interestingly, while the expression of the glial transporter GLT-1 is not altered, the glial antiporter xCT^{-}, which mediates the stoichiometric exchange of extracellular cystine for intracellular glutamate promoting the extrasynaptic release of this neurotransmitter (Bridges et al., 2012), is up-regulated, indicating increased non-vesicular glutamate release. Of note, activation of xCT^{-} causes reduced postsynaptic responses at cortico-accumbal synapses (Moran et al., 2005), in line with our evidence of impaired induction of LTD in the striatum and, together with presynaptic and postsynaptic terminals activity, it points to glial cells involvement in the mechanisms subserving glutamate dyshomeostasis in a condition of hyperdopaminergia.

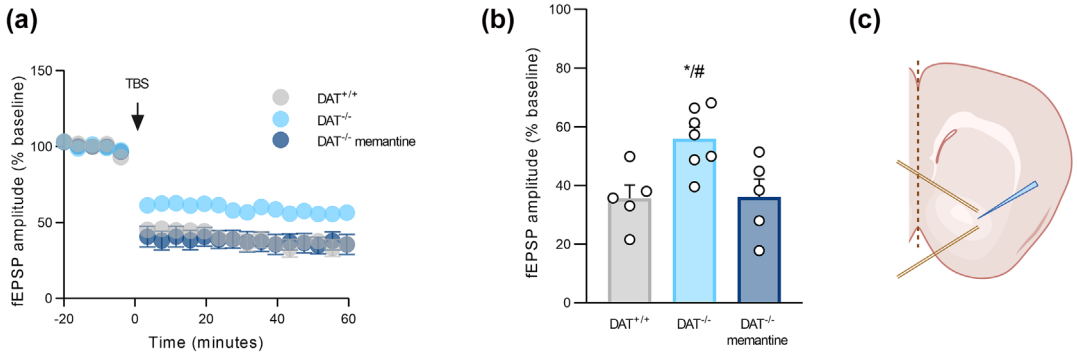

FIGURE 5 Field EPSPs (fEPSPs) were recorded from the ventral striatum. (a) Recordings performed on slices from $DAT^{+/+}$ saline-treated rats, $DAT^{-/-}$ saline-treated rats and $DAT^{-/-}$ rats treated with memantine (30 mg kg^{-1}). The ratio of the baseline over the normalised fEPSP slope (last 12 min) was used to quantify (b) LTD. (c) Representative picture where the recording and the stimulating electrodes were placed. Two-way ANOVA followed by Tukey's multiple comparisons test. *$P < 0.05$ versus $DAT^{+/+}$ saline rats, #$P < 0.05$ versus $DAT^{-/-}$ memantine rats.

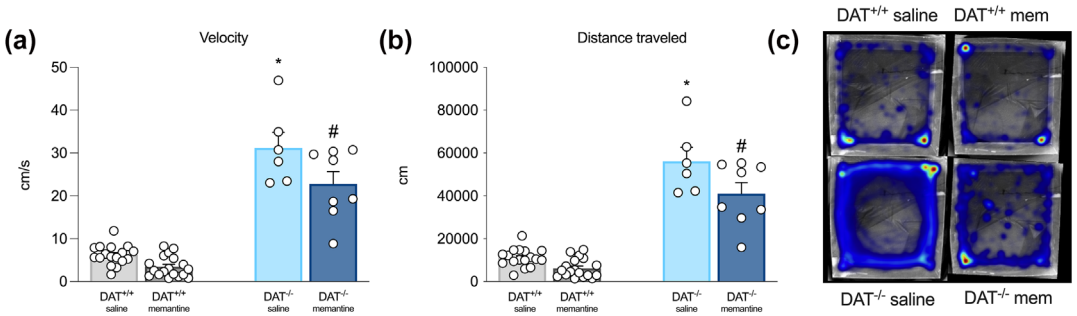

FIGURE 6 Effects of the memantine (30 mg kg^{-1}) treatment on (a) the velocity (cm/s) and (b) distance travelled (cm) during the open field test of $DAT^{+/+}$ and $DAT^{-/-}$ rats. (c) Representative images of the heat maps in the open field box. One-way ANOVA followed by Tukey's multiple comparisons test. *$P < 0.05$ versus $DAT^{+/+}$ saline-treated rats, #$P < 0.05$ versus $DAT^{-/-}$ memantine-treated rats. Supporting information is available on BJP's website.

Molecular changes at the presynaptic level might affect the postsynaptic response. We observed a hypofunctionality of the glutamate synapse, as shown by the reduced synaptic availability of NMDA receptors, which appear to move toward extrasynaptic sites. Such extrasynaptic localisation of NMDA receptors is also demonstrated by the reduced activation of striatal CREB, which is indicative of the extrasynaptic positioning of NMDA receptors (Hardingham et al., 2002). Moreover, such an effect is strengthened by a defective anchoring of these receptors at the postsynaptic membrane, as shown by the reduced expression of the main scaffolding proteins for NMDA receptors, indicating that these receptors are less retained at the postsynaptic membrane of the dendritic spine and, thereby, less stable. This evidence is further strengthened by the reduced expression and phosphorylation of the adhesion molecule NL-1, which glues together presynaptic and postsynaptic terminals in an activity-dependent manner and regulates the abundance of NMDA receptors at postsynaptic sites (Budreck et al., 2013). Because αCaMKII-induced phosphorylation of NL-1 regulates excitatory synapses (Bemben et al., 2014) and activity-dependent activation of NL-1 is a prerequisite to enhance synaptic functions in neurons (Chubykin et al., 2007), the herein-shown reduced activation of NL-1 may contribute to the reduced synaptic recruitment and retention of NMDA receptors at synaptic sites and to their mislocalisation. These postsynaptic changes driven by DA overactivity, together with the presynaptic reduction of VGLUTs and increased xCT^{-} expression, provide a mechanism to sustain the reduced postsynaptic responsiveness exhibited by $DAT^{-/-}$ rats, which results in the impairment of activity-dependent striatal plasticity.

Morphological analyses revealed reduced spine density in the striatum of $DAT^{-/-}$ rats. Previous work found a significant volume loss in the striatum of $DAT^{-/-}$ mice, with decreased spine density as a possible cause (Berlanga et al., 2011; Reinwald et al., 2022). Interestingly, we also found that PSD95 expression, a critical postsynaptic protein essential for stabilising glutamate receptors at excitatory synapses and for synaptic maturation and spine stabilisation (Ehrlich et al., 2007), was reduced in the striatum of $DAT^{-/-}$ rats, an effect previously observed also in $DAT^{-/-}$ mice (Yao et al., 2004), potentially contributing to the observed decrease in spine density. Moreover, these changes also result in a reshaping of spine morphology because we observed a reduction in mushroom-shaped spines, that is, the mature type, which are enriched in PSD95 (Cane et al., 2014). At the same time, we observed an increase in stubby-shaped spines, that

is, the immature type, which instead lack PSD95, probably because of spine shrinkage caused by the hyperdopaminergic condition (Ehrlich et al., 2007). The shift from mushroom- to stubby-shaped spines leads to less dynamic spines with a reduced turnover (Ehrlich et al., 2007), which may contribute to explain the impaired LTD herein shown and the stereotypical movements and cognitive rigidity we previously observed in DAT$^{-/-}$ rats (Leo et al., 2018).

Such molecular and structural rearrangements are likely to have functional relevance. In fact, dopamine overactivity modifies the composition of the NMDA receptors in the postsynaptic density, thus altering receptor functionality and, consequently, striatal synaptic strength. Interestingly, the increased GluN2A/GluN2B ratio induced by the hyperdopaminergic tone might affect the threshold for the frequency-dependent induction of LTD (Yashiro & Philpot, 2008). The NMDA receptor subunit switch toward GluN2A-containing receptors, limiting Ca^{2+} entry through NMDA and via reduced activation of CaMKII at the synapse, would require a stronger postsynaptic response to induce synaptic plasticity. *In vitro* electrophysiological studies have provided clear evidence that dopamine acts as a major modulator of the glutamatergic neurotransmission (Leo et al., 2018). Accordingly, we show that excessive DA tone impairs the induction of LTD in the striatum (Lovinger, 2010).

Striatal dopaminergic, cholinergic and glutamatergic transmission make an ensembled orchestra of multifaceted reciprocal interactions responsible for physiological and pathological behaviours (Cachope et al., 2012; Sulzer et al., 2016). Within this picture, glutamate is known to be co-released by the dopamine terminals (Chuhma et al., 2004; Steinkellner et al., 2022). The persistent hyperdopaminergia at the striatal terminals of DAT $^{-/-}$ rats induced a loss of LTD, suggesting that the impaired synaptic plasticity observed is linked to the constantly high level of dopamine signalling. Several *in vitro* studies showed that stimulation of dopamine receptors is a critical requirement to induce cortico-striatal LTD (Centonze et al., 2001) and this form of synaptic plasticity is absent in mice lacking **D_2 receptors** (Calabresi et al., 1997). Furthermore, LTD appears to be dependent upon *Arc/Arg3.1* translation, which we find to be reduced, further supporting the inability to induce LTD in the striatum of DAT $^{-/-}$ rats (Waung et al., 2008).

LTD is a common mechanism to reduce the synaptic weight of glutamatergic inputs in the basal ganglia network (Citri & Malenka, 2008; Ma et al., 2018). Under normal conditions, activation of both glutamate and dopamine receptors appears to be required to produce LTD (Kreitzer & Malenka, 2005; Mercuri et al., 2021). LTD of excitatory transmission can either occur through persistent presynaptic alterations in glutamate release or postsynaptic changes in glutamate receptor density. We can then speculate that the altered synaptic plasticity of DAT$^{-/-}$ rats in the ventral part of the striatum depends on the reduced **D_1 receptor** expression in combination with glutamate synaptic rearrangement, a possibility supported by the evidence that striatal *Arc/Arg3.1* expression, known to be strongly increased by D_1 agonists, is reduced (Fumagalli et al., 2006).

Our findings suggest that lateral movement of NMDA receptors toward extrasynaptic compartments might be responsible for the behavioural and electrophysiological alterations observed in DAT$^{-/-}$ rats. If this hypothesis were true, it would be possible to investigate whether a drug that blocks the extrasynaptic NMDA receptors could restore a physiological situation in the striatum of DAT$^{-/-}$ rats and, also, the molecular, structural and behavioural alterations. Among the various NMDA receptor blockers, memantine more potently inhibits extrasynaptic than synaptic NMDA receptors (Garcia-Munoz et al., 2015; Xia et al., 2010). Further, an injection of memantine restores not only the number of dendritic spines in DAT$^{-/-}$ rats back to control levels but also improves their functionality, because the drug increases the number of mature and active spines (mushroom) while reducing the immature spines (stubby) (Berry & Nedivi, 2017). We further found that memantine restores electrophysiological responses and induces a reduction in velocity and distance travelled by DAT$^{-/-}$ rats, exerting a robust calming effect. To sum up, memantine normalises the NMDA receptor trafficking, reverses dendritic spine changes and restores electrophysiological responses in the striatum. Whether such changes could be primarily due to a local redistribution of NMDA receptors or be secondary to the increased number of dendritic spines and mushroom-shaped spines is still to be demonstrated.

We are aware that this manuscript holds some limitations. We employed a single treatment with memantine because a growing body of evidence indicates that low, chronic doses of this drug have multiple side effects such as impaired memory in adult rats (Creeley et al., 2006), potentiated stress-induced reduction of cell proliferation (Babic et al., 2012) and significant anxiolytic effect in open field tests (Bagewadi et al., 2015). Moreover, repeated memantine exposure via an implanted cannula to minimise animal stress has proven to be difficult because of technical limitations. We are also aware that the dose of memantine herein used is high. However, we treated rats also with the lower dose of 5 mg kg^{-1}, which did not reduce locomotor activity (data not shown), and therefore molecular, structural and functional analyses were performed in the striatum of rats treated with the behaviourally effective dose of memantine (30 mg kg^{-1}).

It is conceivable that, with the dose herein employed of 30 mg kg^{-1}, receptors other than NMDA receptors were engaged because memantine also interacts with other receptors (**nACh**, **5-HT$_3$**, **A_{2A}**) and transporters (**SERT**, DAT) at physiological (and particularly supraphysiological) concentrations (Johnson & Kotermanski, 2006). Hence, the effects observed in this manuscript may not be unequivocally mediated by NDMA receptor inhibition but could have been influenced by off-target effects. Accordingly, it has been previously shown that serotonin can induce a 'paradoxical calming effect' in DAT$^{-/-}$ mice, which may also explain the small reduction seen here in the memantine-treated DAT$^{-/-}$ rats. However, Sontag and collaborators (1982) have shown reduced **5-hydroxtryptamine (5-HT; serotonin)** at the striatal level at the dose of 20 mg kg^{-1}, thus potentially ruling out a contributing effect of 5-HT.

We focussed on the LTD phenomenon because the induction of LTP in medium spiny neurons following high-frequency stimulation (HFS) is more difficult to elicit than LTD and it was shown to require the removal of Mg^{2+} ions from the perfusion solution to relieve the voltage-dependent block of NMDA receptors (Lovinger, 2010). In

addition, striatal LTP appears to be more capricious and protocol dependent to induce than LTD (Lovinger, 2010). Last, we limited our analysis to male rats and therefore we do not know whether our findings generalise to female rats.

In conclusion, we have here established a mechanism of dopamine-associated dysregulation of the glutamate synapse. In fact, dopamine overactivity, consequent to the elimination of DAT, has reorganised the striatal glutamate synapse in a way that NMDA receptors are de-recruited from active synaptic zones and moved outside the cleft to extrasynaptic sites, thus weakening glutamate neurotransmission, as confirmed by reduced spine density, resulting in LTD impairment. Of note, treatment with memantine normalises hyperlocomotion of $DAT^{-/-}$ rats, reverses spine alterations, rectifies NMDA receptor mislocalisation and restores a physiological LTD. Our findings delineate a conceptual foundation to better understand striatal glutamate homeostasis. We believe that these findings may shed light on the aetiology of different disorders that may possess dopaminergic origins providing a knowledge basis that can be exploited to develop improved treatments for disorders characterised by elevated dopaminergic activity.

AUTHOR CONTRIBUTIONS

Lucia Caffino conceived and planned the experiments, supervised the molecular analyses, wrote the original draft and supervised the project. Giorgia Targa performed morphological analyses, contributed to electrophysiological data, overviewed figures, managed literature research and contributed to the writing of the manuscript. Francesca Mottarlini conducted the Western blot experiments and analysed the results, managed literature research and statistical analyses and contributed to the writing of the manuscript. Sarah Thielens contributed to perform electrophysiological experiments. Beatrice Rizzi contributed to sample preparation, conducted the Western blot analyses and contributed to overview figures and literature research. Agnes Villers performed the electrophysiological experiments. Laurence Ris contributed to the writing of the manuscript. Raul R. Gainetdinov contributed to the interpretation of the results and to the writing of the manuscript. Damiana Leo conceived, planned and supervised the behavioural experiments and the electrophysiological analyses, contributed to the interpretation of the results and to the writing of the manuscript. Responsible for the funding acquisition. Fabio Fumagalli conceived and planned the experiments, supervised the molecular analyses, contributed to the interpretation of the results and contributed to the writing of the manuscript. Responsible for funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in relation to the work herein described.

DATA AVAILABILITY STATEMENT

Data available on request from the authors

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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