



Free D-aspartate modulates the expression of proteins linked to schizophrenia and autism spectrum disorder during early postnatal life

Francesco Errico^{1,2} · Rosita Russo³ · Federica Carrillo⁴ · Tommaso Nuzzo^{2,5} · Raffaella di Vito^{2,5} · Enza Canonico³ · Paolo Vincenzo Pedone³ · Ferdinando Di Cunto^{6,7} · Teresa Esposito^{4,8} · Alessandro Usiello^{2,5} · Angela Chambery³

Received: 5 May 2025 / Revised: 2 October 2025 / Accepted: 6 October 2025
© The Author(s) 2025

Abstract

D-aspartate is an endogenous agonist of NMDA and mGlu5 receptors, with a distinctive spatiotemporal expression profile that peaks in the prenatal and early postnatal brain. This suggests a critical role for D-aspartate metabolism in modulating neurodevelopmental processes linked to glutamatergic neurotransmission. However, the precise mechanisms through which D-aspartate exerts its effects remain unclear. To elucidate the molecular pathways orchestrated by early D-aspartate signalling, we employed a knock-in mouse model characterized by constitutive D-aspartate depletion due to the prenatal expression of its degradative enzyme, D-aspartate oxidase. Using an advanced quantitative proteomic approach based on Tandem Mass Tag isobaric labelling and nano-liquid chromatography coupled with high-resolution tandem mass spectrometry, we investigated the proteomic variations induced by D-aspartate depletion during postnatal brain development, comparing *Ddo* knock-in mice with their wild-type littermates. Our findings reveal that D-aspartate modulates the neonatal expression of proteins involved in glutamatergic neurotransmission, nervous system development, and cytoskeleton organization. Moreover, proteomic analysis identified a subset of D-aspartate-regulated proteins mapping molecular pathways associated with autism spectrum disorder and schizophrenia. These findings offer new perspectives on the complex protein networks influenced by D-aspartate metabolism in the developing brain and highlight its potential impact on cerebral function in health and psychiatric disorders.

Keywords High-resolution mass spectrometry · Tandem mass tag · D-aspartate oxidase · D-aspartate · Autism spectrum disorder · Schizophrenia

Francesco Errico and Rosita Russo are contributed equally to this work.

✉ Alessandro Usiello
usiello@ceinge.unina.it

✉ Angela Chambery
angela.chambery@unicampania.it

¹ Dipartimento di Agraria, Università degli Studi di Napoli “Federico II”, Portici, Italy

² Laboratory of Translational Neuroscience, CEINGE Biotechnologie Avanzate “Franco Salvatore”, Naples, Italy

³ Proteomic and mass spectrometry laboratory, Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania, “Luigi Vanvitelli”, Caserta, Italy

⁴ Institute of Genetics and Biophysics “Adriano Buzzati-Traverso”, National Research Council, Naples, Italy

⁵ Clinical Biochemistry Laboratory, Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania, “Luigi Vanvitelli”, Caserta, Italy

⁶ Department of Neuroscience, University of Turin, Turin, Italy

⁷ Neuroscience Institute “Cavalieri Ottolenghi”, Orbassano, Italy

⁸ IRCCS INM Neuromed, Pozzilli, Italy

Abbreviations

ANOVA	analysis of variance
ASD	autism spectrum disorder
BCA	bicinchoninic acid
BrdU	bromodeoxyuridine
CNS	central nervous system
D-Asp	D-aspartate
DDO	D-aspartate oxidase
Ddo-KI	Ddo knock-in
Ddo-WT	Ddo wild-type
DE	differentially expressed
WT	wild-type
GABA	gamma-aminobutyric acid
GO	gene ontology
LC-MS/MS	liquid chromatography tandem mass spectrometry
mGluR5	metabotropic Glu5 receptor
MRI	magnetic resonance imaging
NAC	N-acetyl-L-cysteine
NMDAR	NMDA receptor
OPA	o-phthalaldehyde
PCP	phencyclidine
PFC	prefrontal cortex
qRT-PCR	quantitative real-time polymerase chain reaction
TCA	trichloroacetic acid
TMT	tandem mass tag
SCZ	schizophrenia

Introduction

Free D-aspartate (D-Asp) is selectively present in high amounts in the mammalian brain during prenatal and early postnatal development [1–3]. The distinctive spatiotemporal profile of cerebral D-Asp occurrence is tightly regulated by the postnatal expression of D-aspartate oxidase (DDO or DASPO EC 1.4.3.1) [4–6], the enzyme that catalyzes its selective degradation [7, 8]. While the degrading mechanism of D-Asp is well-established, its biosynthesis in mammals remains unclear. However, studies in mice suggest that serine racemase (SR), the enzyme responsible for D-serine biosynthesis [9], may also function as a D-Asp synthase in the forebrain due to its ability to catalyze the chiral conversion of L-aspartate [10, 11].

Pharmacological studies support the idea that D-Asp acts as an endogenous agonist at the glutamate GluN2 site of ionotropic NMDA receptors (NMDARs) [2, 12–15] and at metabotropic Glu5 receptors (mGluR5) [16]. Microdialysis experiments in adult freely moving mice have further demonstrated that D-Asp crosses the blood-brain barrier and is present at nanomolar concentrations in the extracellular

space of the cortex [6, 17]. Moreover, pharmacological oral and intraperitoneal administration of D-Asp in adult mice triggers a significant *in vivo* release of L-glutamate in the prefrontal cortex (PFC) of freely moving mice [17], providing further evidence of its modulatory role on glutamatergic neurotransmission.

In line with its neuroactive role, studies in rodents and humans have shown that upregulation of D-Asp levels in *Ddo* knockout or D-Asp-treated mice improves cognitive functions at early adulthood [15, 18, 19] stimulates brain activity, synaptic transmission and plasticity [15, 18–23], and increases dendritic growth and spine density in the PFC and hippocampus [21, 24]. Specifically, in these brain regions, increased D-Asp levels appear to regulate both functional and structural synaptic plasticity through actin polymerization mechanisms [21, 24].

To further explore the role of this atypical amino acid in regulating the downstream events of NMDAR and mGluR5 activation during brain development, we generated a knock-in mouse model with prenatal and postnatal depletion of D-Asp, achieved through constitutive expression of the *Ddo* gene (*Ddo*-KI) [5]. Neuroanatomical investigations revealed that D-Asp depletion significantly perturbs brain morphology, with long-lasting effects into adulthood, as shown by the altered number of parvalbumin-positive GABAergic interneurons in the cortex of *Ddo*-KI mice [5] — an inhibitory neuron subclass strongly implicated in neurodevelopmental psychiatric disorders [25, 26]. Additionally, bromodeoxyuridine (BrdU) incorporation and immunohistochemical experiments showed a lower number of proliferating cells in the dorsal pallium during corticogenesis in these mice, which is reflected in a reduced grey matter volume in the adult cortex and striatum, as measured by structural magnetic resonance imaging (MRI) [27].

In line with the observed morphological brain abnormalities, early cerebral D-Asp depletion in *Ddo*-KI mice was associated with metabolomic alterations during development [28], and with memory and social behaviour deficits in juvenile and adult life [5, 27], resembling autism spectrum disorders (ASD)-like phenotypes described in other animal models [29]. Similarly, another study demonstrated that prenatal and early postnatal supplementation of D-Asp in fragile X syndrome (*Fmr1* KO) mice improved fear conditioning memory and motor coordination deficit [30]. Further studies have shown that non-physiological elevation of this D-amino acid in adult mice prevents schizophrenia (SCZ)-like phenotypes, including prepulse inhibition deficits, ataxia, and dysfunctional brain activation induced by the psychotomimetic NMDAR antagonists, dizocilpine (MK801) and phencyclidine (PCP) [20, 31, 32].

In addition to preclinical investigations, independent *post-mortem* studies have shown a significant reduction

in D-Asp levels in the PFC of SCZ patients compared to healthy controls [33, 34]. Supporting this evidence, a hypothesis-free machine learning algorithm identified a stable molecular cluster, including the D-Asp/total Asp ratio, in the *post-mortem* dorsolateral PFC, effectively distinguishing SCZ patients from non-psychiatric controls [35]. Consistently, altered D-Asp concentrations have also been detected in blood samples from living SCZ patients [36, 37]. Moreover, a chromosome 6 duplication encompassing the entire *DDO* gene was identified in a young patient with severe intellectual disability, thought disorders and behavioural abnormalities reminiscent of ASD and SCZ symptomatology [27]. In line with these clinical observations, dysregulated D-Asp levels have been reported in the idiopathic ASD mouse model BTBR [38], in environmental ASD rat models prenatally exposed to lipopolysaccharide or valproate [39], and in Duchenne muscular dystrophy mice [40]. Together, these findings reinforce the hypothesis that altered D-Asp metabolism contributes to neurodevelopmental disorders.

Although the data presented above underscores the role of D-Asp in modulating glutamatergic transmission and neurodevelopment, its specific influence in regulating protein expression downstream NMDAR and mGluR5 stimulation in the developing brain remains unknown. In the present study, we sought to investigate the consequences of D-Asp deprivation at the proteome level in the brain of *Ddo*-KI mice at early postnatal stages using an advanced quantitative proteomic approach.

Materials and methods

Animals

Ddo-KI and *Ddo*-WT mice were generated and genotyped by PCR according to the protocol previously reported [5]. Animals were housed in groups (4–5 per cage), at a constant temperature (22 ± 1 °C) on a 12 h light/dark cycle (lights on at 7 AM) with food and water ad libitum. All research involving animals was carried out following the directive of the Italian Ministry of Health governing animal welfare and protection (D.LGS 26/2014) and approved by “Direzione Generale della Sanità e dei Farmaci Veterinari (Ufficio 6)” (permission nr. 796/2018).

HPLC analysis

The levels of D-Asp, L-aspartate, L-glutamate, L-asparagine, D-serine, L-serine, glycine, L-threonine, L-arginine, taurine, L-alanine, and GABA were analyzed by HPLC in male and female mouse whole-brain tissue samples,

including the cerebellum. Brain samples were pulverized in liquid nitrogen and an aliquot of each (7–18 mg) was homogenized in 1:10 (w/v) 0.2 M trichloroacetic acid (TCA), sonicated (4 cycles, 10 s each), and centrifuged at $13,000 \times g$ for 20 min. TCA supernatants containing amino acids were then neutralized with NaOH and subjected to pre-column derivatization with o-phthalaldehyde (OPA)/N-acetyl-L-cysteine (NAC). Diastereoisomer derivatives were resolved on a UHPLC Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA, USA) using a ZORBAX Eclipse Plus C8, 4.6×150 mm, 5 μ m (Agilent Technologies, Santa Clara, CA, USA) under isocratic conditions (0.1 M sodium acetate buffer, pH 6.2, 1% tetrahydrofuran, and 1.5 mL/min flow rate). Each run was followed by a washing step with 0.1 M sodium acetate buffer, 3% tetrahydrofuran, and 47% acetonitrile. The precipitated protein pellets were solubilized in 1% SDS solution and quantified by bicinchoninic acid (BCA) assay method (Pierce™ BCA Protein Assay Kits, Thermofisher scientific, Rockford, IL, USA). Identification of amino acids was based on retention time, compared with the one associated with external standards. Each compound was quantified using 6–15 point calibration curve generated using external standards in the range of the predicted analyte concentration in the sample. The detected amino acid concentration in tissue homogenates was normalized by the total protein content and expressed as nmol/mg protein. If the amino acid concentration in the tissue analysed was below the limit of detection (i.e. D-Asp in *Ddo*-KI mice), the value was set at 0.

Quantitative RT-PCR

The expression profile of *Ddo* and 30 other genes, including *Gria3*, *Syncrin*, *Icam5*, *Tubb4a*, *Cntnap1*, *Ptk2b*, *Maob*, *Slc17a7*, *Pde1a*, *Macrod2*, *Homer1*, *Slc4a8*, *Mapt*, *Rasgrf2*, *Grin1*, *Igf2r*, *Gad1*, *Gria4*, *Cntnap4*, *Homer3*, *Iqsecl*, *Madd*, *Nav3*, *Nudt4*, *Oxr1*, *Srr*, *Syng1*, *Ppm1h*, *Igsf21*, *Fxyd6*, was evaluated on total RNAs of *Ddo*-KI and *Ddo*-WT ($n=5$ mice/genotype/age). RNA was isolated by using TRIzol RNA Isolation Reagents (Thermo Fisher Scientific, Waltham, MA, USA) protocol. Five μ g of total RNA were reverse transcribed with the Superscript III-First strand kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicate, using gene-specific primers (Suppl. Table 1) and ITaq Universal Sybr Green Supermix (Bio-Rad, Hercules, CA, USA) following the manufacturer's directions. Results were normalized versus the expression of the glyceraldehydes-3-phosphate dehydrogenase (*Gapdh*) gene. Data were analyzed with unpaired *t*-test to compare the gene expression between genotypes at each age. A value of $p < 0.05$ was considered to be statistically significant.

Sample preparation for proteomic analyses

Proteomic analyses were performed on pooled ($n = 3$) whole brains of male and female *Ddo*-KI and *Ddo*-WT mice and collected at different stages of postnatal development: P3, P7 and P14. Animals were sacrificed and the whole brains, including the cerebellum, were dissected within 20 s on an ice-cold surface. All tissue samples were pulverized in liquid nitrogen and stored at -80°C for subsequent processing. Tissues were then lysed in ice-cold lysis buffer (100 mM Triethylammonium bicarbonate TEAB, SDS 1%) and disrupted by two cycles of sonication at a 20% amplitude for 30 s on ice. Lysates were cleared by centrifugation at $16,000 \times g$ for 15 min at 4°C . Supernatants were transferred into new tubes, treated with 1 U of RQ1 Dnase (Promega) for 30 min at room temperature, and the protein concentration was determined by using the Pierce BCA Protein assay kit (Thermo Scientific, Rockford, IL). For each condition, equal amounts of proteins (50 μg in 100 μL of 100 mM TEAB) were reduced with 10 mM Tris-(2-carboxyethyl)-phosphine (TCEP) for 1 h at 55°C and alkylated with 18 mM iodoacetamide by incubating samples for 30 min at room temperature in the dark. Proteins were then precipitated overnight by adding six volumes of pre-chilled acetone. Following centrifugation at $8,000 \times g$ for 10 min at 4°C , protein pellets were resuspended in 100 μL of 100 mM TEAB and digested overnight with MS grade trypsin (Pierce) at an enzyme/substrate ratio of 1:40 at 37°C . The resulting peptide mixtures were chemically labelled with the TMT isobaric tags as previously reported [41, 42] by using the following isobaric tags: 131 P3 WT, 130 C P3 KI, 129 N P7 WT, 128 C P7 KI, 127 N P14 WT, 126 P14 KI. Briefly, 0.8 mg of Tandem Mass Tag (TMT) reagents in 41 μL of anhydrous acetonitrile were added to each sample. The reaction proceeded for 1 h and then was quenched for 15 min with hydroxylamine to a final concentration of 0.3%. Samples were then mixed in equal amounts and diluted in 0.1% TFA/2% CH_3CN to a final concentration of 0.5 $\mu\text{g}/\mu\text{L}$ for LC-MS analyses.

High-resolution nanoLC – Tandem mass spectrometry

Aliquots of TMT labelled samples (2.5 μg) were analyzed by high-resolution nanoLC-Tandem Mass Spectrometry using a Q-Exactive Orbitrap mass spectrometer equipped with an EASY-Spray nano-electrospray ion source (Thermo Fisher Scientific, Bremen, Germany) and coupled to a Thermo Scientific Dionex UltiMate 3000RSLC nano system (Thermo Fisher Scientific) [43]. The solvent composition was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Peptides were loaded on a trapping

PepMapTM100 $\mu\text{Cartridge}$ Column C18 (300 $\mu\text{m} \times 0.5 \text{ cm}$, 5 μm , 100 $\text{\AA}$) and desalted with solvent A for 3 min at a flow rate of 10 $\mu\text{L}/\text{min}$. After trapping, eluted peptides were separated on an EASY-Spray analytical column (50 $\text{cm} \times 75 \mu\text{m}$ ID PepMap RSLC C18, 3 μm , 100 Angstrom), heated to 35°C , at a flow rate of 300 nL/min applying the following gradient: 5% B for 3 min, from 5% to 27.5% B in 222 min, from 27.5% to 40% B in 10 min, from 40% to 95% B in 1 min. Washing (95% B for 4 min) and re-equilibration (5% B for 24 min) steps were always included at the end of the gradient. Eluting peptides were analysed on the Q-Exactive mass spectrometer operating in positive polarity mode with a capillary temperature of 280°C and a potential of 1.9 kV applied to the capillary probe [42, 44]. Full MS survey scan resolution was set to 70,000 with an automatic gain control (AGC) target value of 3×10^6 for a scan range of 375–1500 m/z and maximum ion injection time (IT) of 60 ms. The mass (m/z) 445.12003 was used as lock mass. A data-dependent top 12 method was operated during which high-energy collisional dissociation (HCD) spectra were obtained at 35,000 MS2 resolution with AGC target of 1×10^5 for a scan range of 200–2000 m/z , maximum IT of 120 ms, 1.6 m/z isolation width and normalized collisional energy (NCE) of 32. Precursor ions targeted for HCD were dynamically excluded for 30 s. Full scans and Orbitrap MS/MS scans were acquired in profile mode, whereas ion trap mass spectra were acquired in centroid mode. Charge state recognition was enabled by excluding unassigned and 1, 7, 8, >8 charged states. All data were acquired with Xcalibur 3.1 software (Thermo-Fisher Scientific).

Protein identification and quantitation

For data processing, the acquired raw files were analyzed with the Thermo Scientific Proteome Discoverer 2.4 software (Thermo Fisher Scientific) using the SEQUEST HT search engine. The HCD MS/MS spectra were searched against the *Mus musculus* database (UniProt release 2023_01, number of entries 17761 sequences) assuming trypsin (Full) as digestion enzyme and two allowed number of missed cleavage sites. Mass tolerances were set to 10 ppm and 0.02 Da for precursor and fragment ions, respectively. The oxidation of methionine (+15.995 Da) was set as a dynamic modification. Carbamidomethylation of cysteine (+57.021 Da) and the TMT label on lysines and the N-terminus (229.1629) were set as static modifications. False discovery rates (FDRs) for peptide spectral matches (PSMs) were calculated and filtered using the Percolator node in Proteome Discoverer that was run with the following settings: Maximum Delta Cn 0.05, a strict target FDR of 0.01, a relaxed target FDR of 0.05 and validation based on q-value. Protein identifications were accepted when the

protein FDR was below 1% and when present in at least two out of three replicate injections with at least two peptides.

Bioinformatic analyses

The clustered heatmap of the proteins that were differentially expressed with $\log_2$ fold change values $-0.6 \geq (\log_2 \text{fold change}) \geq 0.6$ in at least one out of three replicates was generated by hierarchical cluster analysis (Euclidean average distance) by using the Perseus software platform (version 2.0.11) after Z-score normalization of the protein dataset, and minimum and maximum intensity values were represented by default green and red colours, respectively. For bioinformatic analyses, proteins with $\log_2$ fold change values $-0.4 \geq (\log_2 \text{fold change}) \geq 0.4$ were considered as differentially expressed (DE). Molecular function enrichment analysis was performed by using the ClueGO plug-in of Cytoscape 3.6.0 to generate a functionally grouped GO/pathway term network of enriched molecular function categories for the identified proteins based on kappa statistics [45, 46]. For further data visualization, the results of this analysis were imported onto the Evenn platform to generate the Venn network of proteins mapped within enriched terms [47]. The enriched Gene Ontology (GO) terms for DE proteins were extracted by using a module integrating the clusterProfiler R package [48]. Enrichment plots and GOChord plots were generated and visualized by using the online platform for data analysis and visualization available at <https://www.bioinformatics.com.cn>. The networks of *Homo sapiens* proteins associated with autism spectrum disorders and schizophrenia were retrieved by using the String: disease query plug-in of Cytoscape using a confidence (score) cutoff of 0.4. The g: Orth tool of the g: Profiler web service platform was then used to retrieve mouse orthologous protein mappings of *Homo sapiens* entries based on the gene information from the Ensembl database [49]. This subset of disease-related proteins was subsequently matched against the list of D-Asp differentially expressed proteins to map modulated proteins associated with the selected neurodegenerative disorders.

The lists of human genes showing strong coexpression or anticorrelation with DDO was obtained from the dataset previously described [50], using as coexpression or anticorrelation cutoff the presence in top or bottom 1% ranks of the Pearson correlation coefficients, respectively, with reciprocity criterion. The GO enrichment analysis of these lists was performed using the DAVID analysis tool [51] and the statistical significance of intersections was assessed using the Fisher exact test.

Results

Constitutive overexpression of *Ddo* gene leads to stable and specific depletion of D-aspartate levels in the *Ddo* knock-in mouse brain

First, we confirmed *Ddo* gene overexpression in the whole brain of *Ddo*-KI mice at early postnatal stages, such as postnatal day 3 (P3) and P14, as well as at mature adult stage (around P300), compared to age-matched *Ddo* wild-type littermates (*Ddo*-WT). qRT-PCR showed a significant increase in *Ddo* mRNA expression at each age analyzed (*Ddo*-KI vs. *Ddo*-WT: P3, $p=0.0002$; P14, $p=0.0355$; P300, $p<0.001$; Student's *t*-test; Suppl. Figure 1).

Using the same cohort of mice, we then evaluated the impact of constitutive *Ddo* overexpression on cerebral levels of D-Asp, its precursor, L-aspartate, and other amino acids directly or indirectly involved in glutamatergic transmission, including L-glutamate, L-glutamine, D-serine, L-serine, glycine, gamma-aminobutyric acid (GABA), taurine, L-asparagine, L-arginine, L-alanine and L-threonine. Additionally, we analyzed key ratios, such as D-Asp/total Asp (%), D-serine/total serine (%), glycine/L-serine, L-glutamine/L-glutamate and GABA/L-glutamate to assess potential alterations in amino acid metabolic inter-conversion (Suppl. Data 1). Statistical analysis revealed that *Ddo* overexpression selectively alters D-Asp levels (two-way ANOVA: genotype, $F_{(1,28)}=407.1$, $p<0.0001$; genotype x age $F_{(2,28)}=49.28$, $p<0.0001$) and D-Asp/total Asp ratio (genotype, $F_{(1,28)}=427.0$, $p<0.0001$; genotype x age $F_{(2,28)}=40.13$, $p<0.0001$), resulting in an essentially complete D-Asp depletion across all analyzed ages under our experimental conditions (D-Asp and D-Asp/total Asp, $p<0.0001$, Fisher's *post-hoc* analysis) (Fig. 1a, c). In contrast, the levels of L-Asp and all other amino acids and metabolic ratios remained unchanged between genotypes (Fig. 1b, d-r).

A quantitative proteomics approach reveals that D-aspartate depletion affects proteome expression in early postnatal mouse brain

To investigate protein expression changes in *Ddo*-KI mice compared to the *Ddo*-WT littermates at different developmental stages, we applied a quantitative proteomic approach based on TMT isobaric labelling and nano-liquid chromatography coupled with high-resolution tandem mass spectrometry (LC-MS/MS) (Fig. 2a). Specifically, we analyzed whole brains from *Ddo*-KI and *Ddo*-WT mice at P3, P7 and P14. We used an isobaric labelling-based workflow combined with LC-MS/MS on a Q-Exactive mass spectrometer [43]. A high number of peptide groups (24,716) was

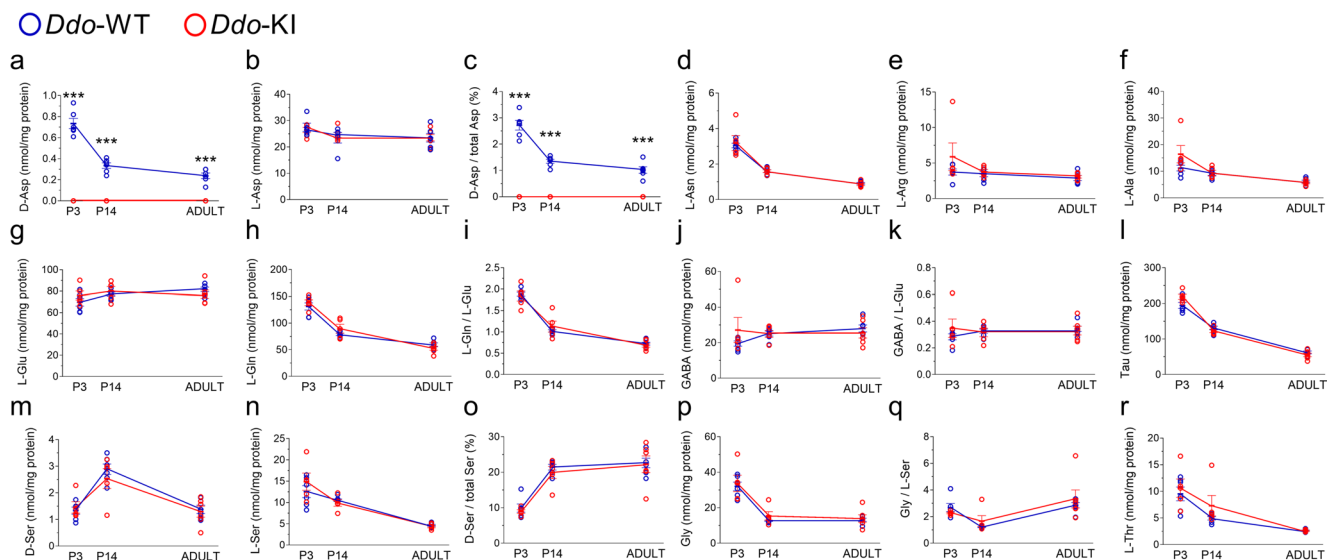


Fig. 1 Amino acid levels in the whole brain of *Ddo-KI* mice during lifetime. Analysis of (a) D-aspartate and (b) L-aspartate levels, (c) D-aspartate/total aspartate ratio, (d) L-asparagine, (e) L-arginine, (f) L-alanine, (g) L-glutamate and (h) L-glutamine levels, (i) L-glutamine/L-glutamate ratio, (j) GABA levels, (k) GABA/L-glutamate ratio, (l) taurine, (m) D-serine and (n) L-serine levels, (o) D-serine/total serine ratio, (p) glycine levels, (q) glycine/L-serine ratio, and (r) L-threonine levels in the whole brain of *Ddo-KI* and *Ddo-WT* mice at post-natal day 3 (P3), P14 and P300. The amino acid content was expressed as nmol/mg protein, while the ratios were expressed

as % (D-aspartate/total aspartate and D-serine/total serine) or absolute values (L-glutamine/L-glutamate, GABA/L-glutamate and glycine/L-serine). In *Ddo-KI* mice, D-aspartate levels were below the detection limit of the HPLC system; accordingly, the D-aspartate/total aspartate ratio could not be calculated, and both measures were set to 0. In each sample, free amino acids were detected in a single run. *** $p < 0.0001$, compared with age-matched *Ddo-WT* mice (Fisher's *post-hoc* comparison). Dots represent individual values; bars illustrate the median with interquartile range

used for protein identification, with more than 80% used as unique peptides for protein quantification, confirming the high efficiency of peptide labelling (data not shown).

MS/MS and database search identified and quantified 2,594 non-redundant proteins, each with more than one peptide in at least two out of three replicates (Suppl. Data 2). Approximately 60% of proteins were commonly identified, while only about 21% of proteins were found to be uniquely identified. We extracted subsets of differentially expressed proteins from this dataset, applying a 1.3-fold change threshold, in at least one of the three postnatal comparisons (Suppl. Data 3). In detail, most of the differentially expressed proteins (288 out of 295) were observed at P3 stage in *Ddo-KI* samples, as illustrated in the volcano plot reporting $-\text{Log}_{10}$ p-values versus Log_2 fold-change values of *Ddo-KI* samples, compared with *Ddo-WT* (Fig. 2b and Suppl. Data 3). Following data filtering, we identified a subset of twenty-one proteins whose relative expression levels changed 1.5-fold or more in at least one time-point (P3, P7, P14) compared to *Ddo-WT* samples (Table 1; Fig. 2c). Proteomic analysis indicated that in four out of six clusters (clusters 1–4), differentially expressed proteins were significantly downregulated at the P3 stage, while no significant alterations were revealed at later developmental stages (Fig. 2d).

D-aspartate depletion affects the expression of proteins involved in brain development, synaptic transmission and cytoskeletal organization

We next performed a GO enrichment analysis on differentially expressed proteins, revealing that D-Asp-modulated proteins were mainly associated with molecular functions related to nervous system development, chemical synaptic transmission and its modulation, cellular component and cell junction organization, and cytoskeleton-dependent transport (Fig. 3a). The network of proteins involved in the most enriched affected functions is shown in Fig. 3b. Consistently, the most significantly enriched biological processes included synapse organization, axogenesis, cytoskeleton-dependent transport, transport along microtubules, regulation of neurotransmitter receptor activity and regulation of axogenesis (Fig. 4a). Notably, the most enriched cellular component categories were exclusively linked to synaptic structures (Fig. 4b). To further dissect the pathways influenced by D-Asp, we conducted a pathway enrichment analysis identifying the “Glutamatergic synapse” and the “Alanine, aspartate and glutamate metabolism” as the most significantly impacted pathways (Fig. 4c). Specifically, we mapped nine downregulated proteins within the “Glutamatergic synapse” pathway: *Prkcg* ($\downarrow 0.43$ -fold), *Grk2* ($\downarrow 0.42$ -fold), *Gria4* ($\downarrow 0.48$ -fold), *Homer1* ($\downarrow 0.39$ -fold), *Gria3*

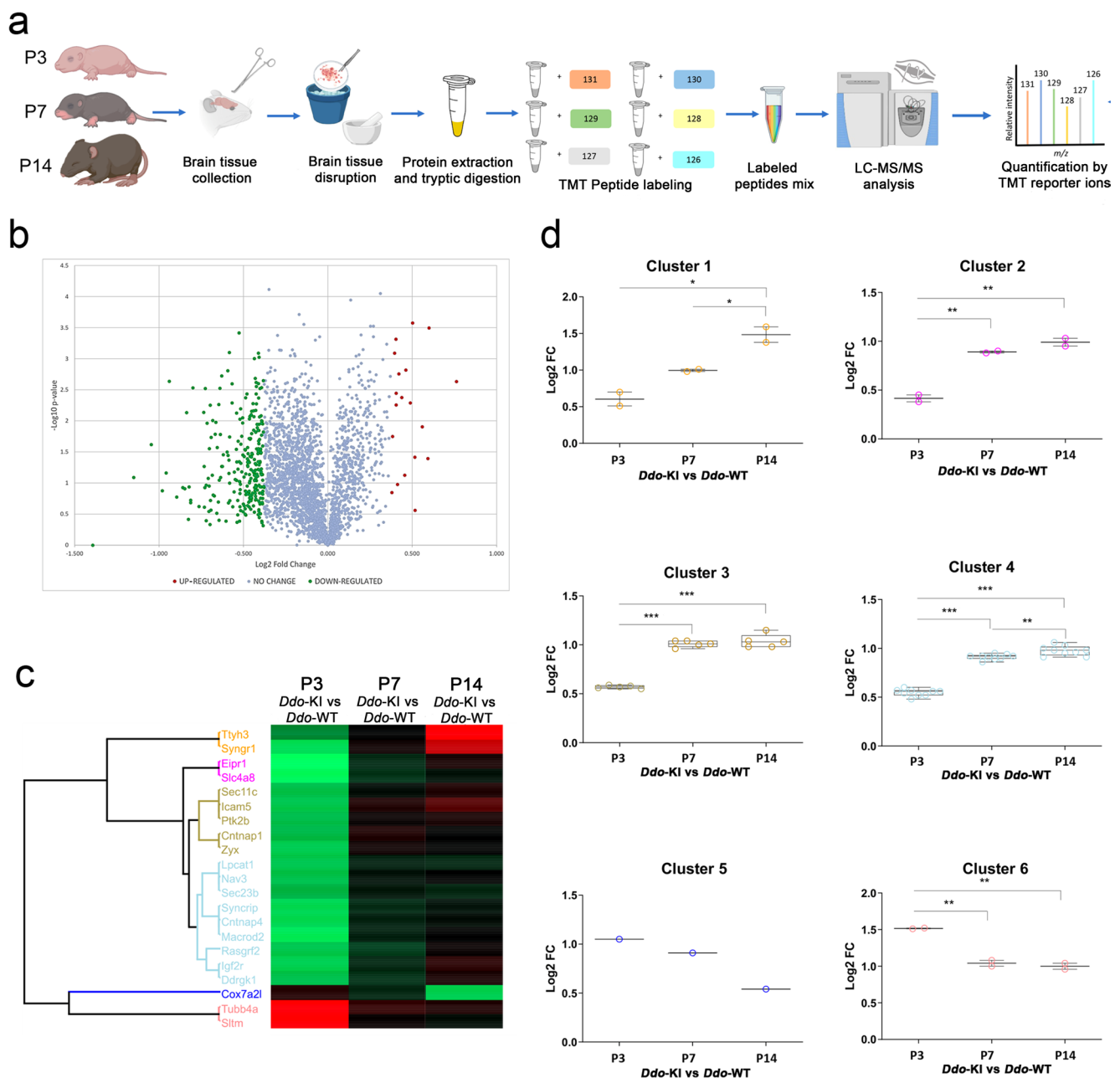


Fig. 2 Quantitative proteomic approach reveals differentially expressed proteins in the whole brain of post-natal day 3 *Ddo*-KI mice. **(a)** Experimental design and proteomic workflow for comprehensive quantitative analysis of whole brains from *Ddo*-KI and *Ddo*-WT mice collected at different post-natal developmental stages, including post-natal day 3 (P3), P7 and P14. For Tandem Mass Tag (TMT) isobaric labelling, proteins extracted from whole brains were digested into peptides and labelled with TMT isobaric stable isotope tags. After sample mixing, peptides were analysed by LC-ESI-MS/MS. In MS1, the peptides appear as a single precursor. When fragmented during MS2, in addition to the normal fragment ions, the reporter regions dissociated to produce ion signals, which provided accurate quantitative information regarding the relative amount of the peptide in the samples. Image created with BioRender.com (www.biorender.com). **(b)** Volcano plot

obtained from TMT-based quantitative proteomics analysis of P3 *Ddo*-KI versus age-matched *Ddo*-WT brain samples. Each point represents the difference in expression (Log2 fold-change) between the *Ddo*-KI and *Ddo*-WT plotted against the $-\log_{10}$ p-value. Identified proteins with no changes in their regulation level are reported in light blue. Up- and down-regulated proteins ($-0.4 \geq \log_2 \text{fold-change} \geq 0.4$) are indicated in red and green, respectively. **(c)** Hierarchical clustering (Euclidean average distance) of normalized protein intensities for differentially expressed proteins $-0.6 \leq (\log_2 \text{fold change}) \leq 0.6$ identified in whole brains from *Ddo*-KI and *Ddo*-WT mice collected at postnatal days P3, P7 and P14 by quantitative nanoLC-MS/MS. Down-regulated and up-regulated proteins in the heatmap are coloured in green and red, respectively. **(d)** Protein expression profiles of each cluster

Table 1 Differentially expressed proteins ($0.66 \geq FC \geq 1.50$) identified in the whole brain of *Ddo*-WT and *Ddo*-KI mice at postnatal day 3 (P3), P7, and P14 by quantitative nanoLC-MS/MS

Accession	Gene	Description	P3 Ddo- KI vs. Ddo-WT	P7 Ddo- KI vs. Ddo-WT	P14 Ddo- KI vs. Ddo-WT
Q6P5F7-1	Ttyh3	Protein tweety homolog 3	-0.51	-0.03	0.67
Q61387	Cox7a2l	Cytochrome c oxidase subunit 7 A-related protein, mitochondrial	0.07	-0.14	-0.89
Q9D6F9	Tubb4a	Tubulin beta-4 A chain	0.60	0.11	0.06
O54991	Cntnap1	Contactin-associated protein 1	-0.76	0.06	-0.03
Q60625	Icam5	Intercellular adhesion molecule 5	-0.84	0.06	0.20
O55100-1	Syngr1	Synaptogyrin-1	-0.97	0.01	0.46
Q62523	Zyx	Zyxin	-0.86	0.01	-0.03
Q9QVP9	Ptk2b	Protein-tyrosine kinase 2-beta	-0.79	0.00	0.04
Q8CH25-1	Sltm	SAFB-like transcription modulator	0.59	0.00	-0.06
Q9D8V7	Sec11c	Signal peptidase complex catalytic subunit SEC11C	-0.81	-0.06	0.06
Q80TN7	Nav3	Neuron navigator 3	-0.81	-0.07	-0.03
Q9D662	Sec23b	Protein transport protein Sec23B	-0.74	-0.09	-0.14
Q99P47	Cntnap4	Contactin-associated protein-like 4	-0.94	-0.12	-0.06
Q3TFD2-1	Lpcat1	Lysophosphatidylcholine acyltransferase 1	-0.84	-0.12	-0.14
Q3UYG8	MacroD2	O-acetyl-ADP-ribose deacetylase MACROD2	-1.06	-0.12	-0.03
Q07113	Igf2r	Cation-independent mannose-6-phosphate receptor	-0.89	-0.14	0.08
Q7TMK9-2	Syncrip	Isoform 2 of Heterogeneous nuclear ribonucleoprotein Q	-0.94	-0.15	-0.07
Q8K0G5	Eipr1	Protein TSSC1	-1.40	-0.15	0.04
Q80WW9	Ddrgk1	DDRGK domain-containing protein 1	-0.81	-0.17	0.04
Q8JZR6	Slc4a8	Electroneutral sodium bicarbonate exchanger 1	-1.15	-0.18	-0.07
P70392	Rasgrf2	Ras-specific guanine nucleotide-releasing factor 2	-0.84	-0.22	0.00

($\downarrow 0.41$ -fold), *Adcy1* ($\downarrow 0.70$ -fold), *Slc17a7* ($\downarrow 0.44$ -fold), *Grin1* ($\downarrow 0.42$ -fold) and *Homer3* ($\downarrow 0.72$ -fold). Additionally, three proteins involved in the “Alanine, aspartate and glutamate metabolism” pathway were significantly down-regulated: *Gad1* ($\downarrow 0.39$ -fold), *Aldh4a1* ($\downarrow 0.40$ -fold) and *Adsl* ($\downarrow 0.43$ -fold). Overall, these findings highlight a regulatory role for D-Asp in modulating the expression of key proteins involved in glutamatergic neurotransmission.

A subset of D-aspartate-modulated proteins in the early postnatal mouse brain is linked to autism spectrum disorder and schizophrenia

To gain insights into the potential involvement of D-Asp-modulated proteins in psychiatric disorders, we evaluated in the list of proteins affected by D-Asp depletion the prevalence of sequences previously related to ASD and SCZ. To this aim, we first extracted the subsets of *Homo sapiens* proteins associated with both psychiatric disorders by using the String: disease query plug-in of Cytoscape. Then, by using

the g: Orth tool of the g: Profiler platform, we retrieved for these subsets the corresponding mouse orthologous protein entries that were subsequently matched against the list of D-Asp differentially expressed proteins. By this approach, we found that 15 differentially expressed proteins at the P3 developmental stage in *Ddo*-KI mice were previously associated with ASD (Fig. 5a, subsets A and B). GO enrichment analysis revealed that these proteins were mainly involved in functions including the regulation of synapse structure/activity, modulation of chemical synaptic transmission, nervous system development, glutamate receptor signalling pathway, behaviour and regulation of neurotransmitter levels (Fig. 5b; Suppl. Figure 2a). Similarly, twenty-one differentially expressed proteins in *Ddo*-KI mice (20 at P3 and 1 at P14) were previously associated with SCZ (Fig. 5a, subsets A and C) and are involved in biological processes partially overlapping with those revealed for ASD (e.g. glutamate receptor signalling pathway and behaviour) as well as in the regulation of synaptic plasticity, glutamatergic synaptic transmission, postsynaptic chemical synaptic

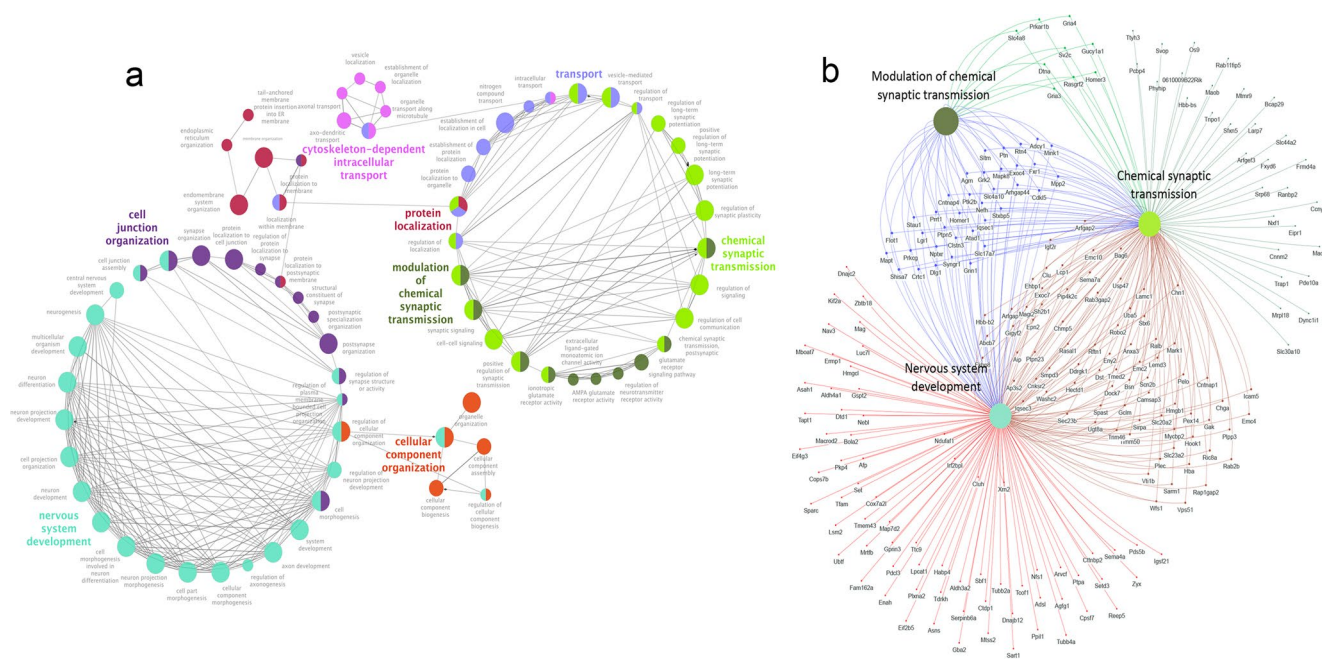


Fig. 3 D-aspartate depletion affects the expression of proteins involved in brain development, synaptic transmission and cytoskeletal organization during early postnatal life. **(a)** Functionally grouped network of enriched molecular function categories for differentially expressed proteins in the whole brain of *Ddo-KI* and *Ddo-WT* mice at postnatal

day (P) P3 and P14 generated using the ClueGO Cytoscape plug-in. The proportion of shared proteins between terms was evaluated using kappa statistics. GO terms are depicted as nodes whose size represents the term enrichment significance. **(b)** Venn network of proteins mapped within the top three enriched terms

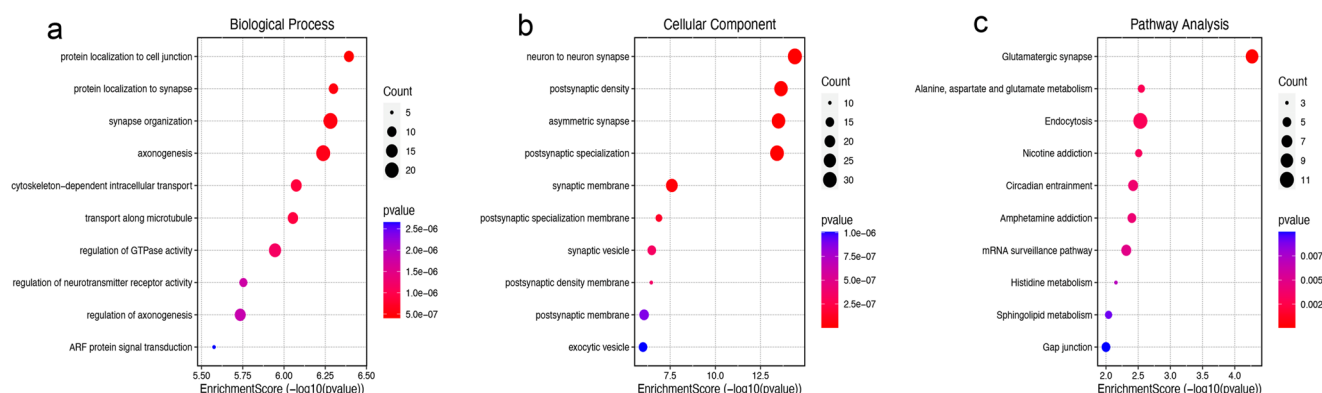


Fig. 4 Analysis of enriched gene ontology terms at postnatal day 3. Enriched gene ontology (GO) terms for **(a)** “Biological process”, **(b)** “Cellular component” and **(c)** “Pathway analysis” of differentially

expressed proteins in *Ddo-KI* and *Ddo-WT* mice at postnatal day 3. The colour and size of the plots represent the *p* value and the count of proteins, respectively

transmission, protein localization to synapse and regulation of axogenesis (Fig. 5c; Suppl. Figure 2b). Interestingly, a comparison of D-Asp-modulated proteins with both psychiatric disorders revealed that nine proteins, i.e. *Slc17a7* ($\downarrow 0.44$ -fold), *Gria3* ($\downarrow 0.41$ -fold), *Fxr1* ($\downarrow 0.39$ -fold), *Grin1* ($\downarrow 0.42$ -fold), *Mapt* ($\downarrow 0.46$ -fold), *Homer1* ($\downarrow 0.39$ -fold), *Maob* ($\downarrow 0.44$ -fold), *Cnnm2* ($\downarrow 0.62$ -fold), *Gad1* ($\downarrow 0.39$ -fold), represent a common signature of potential relevance to study the role of D-Asp in neurodevelopmental disorders (Fig. 5a, subset A).

Anticorrelation analysis of *DDO* in the human brain identifies genes overlapping with proteins downregulated in the *Ddo* knock-in mouse brain at postnatal day 3

The above results and the previous finding of *DDO* gene duplication in a patient affected by thought disorders and ASD symptomatology [27] suggest that *DDO* levels may influence the expression of genes critically implicated in

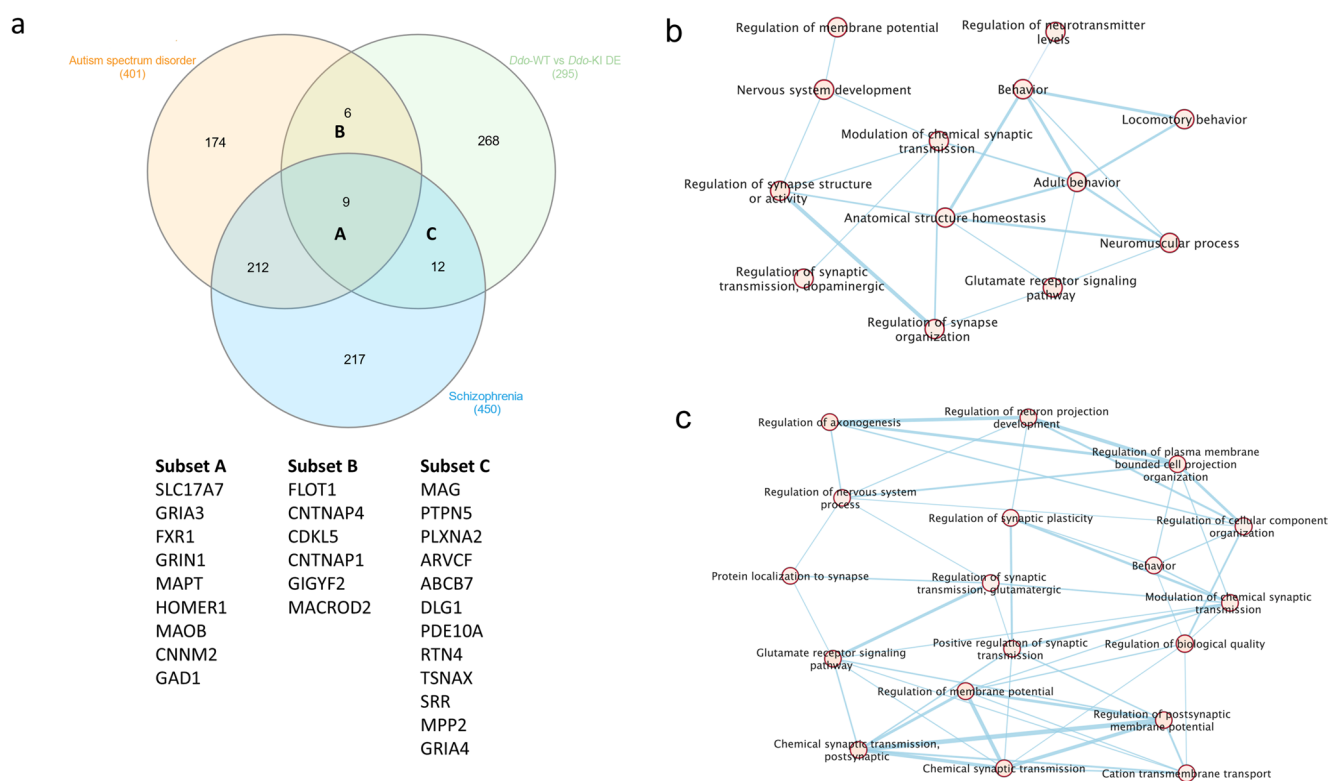


Fig. 5 A subset of D-aspartate-modulated proteins in the whole brain of post-natal day 3 *Ddo*-KI mice is linked to autism spectrum disorder and schizophrenia. **(a)** Venn diagram showing the overlapping of proteins differentially expressed (DE) by D-aspartate in *Ddo*-WT vs. *Ddo*-KI mice (*Ddo*-WT vs. *Ddo*-KI DE) and orthologous proteins

associated with autism spectrum disorder (ASD) and schizophrenia. **(b)** Enrichment map of biological processes involving proteins within the subsets A and B (*Ddo*-WT vs. *Ddo*-KI DE and ASD) or **(c)** within the subsets A and C (*Ddo*-WT vs. *Ddo*-KI DE and schizophrenia)

this disorder, even in the human brain. To explore this possibility, we systematically analyzed the strongest positive and negative correlations between *DDO* and other genes in the human CNS, using a published tissue-specific coexpression network derived from a dataset of 544 human microarray experiments [50]. Among the 62 genes coexpressed with *DDO* (Suppl. Data 4) only one (*Pde10a*) overlapped with mouse proteins modulated in *Ddo*-KI mice. In contrast, the list of 126 *DDO*-anticorrelated genes showed nine sequences in common with mouse proteins modulated in *Ddo*-KI brains (*Fxyd6*, *Igsf21*, *Iqsec1*, *Madd*, *Nudt4*, *Oxrl1*, *Pde1a*, *Ppm1h*, *Slc17a7*) (Suppl. Data 4), which represents a highly significant intersection ($p = 0.00007$, hypergeometric test). Notably, all the nine common proteins were downregulated in *Ddo*-KI transgenic brains at P3 (Suppl. Data 3). Interestingly, a GO enrichment analysis of *DDO*-anticorrelated genes in the human brain revealed a significant enrichment of terms associated with synaptic and cognitive functions.

D-aspartate metabolism regulates the expression of *Grin1*, *Homer3*, *Syngr1* and *Iqsec1* genes in the early postnatal brain of *Ddo* knock-in mice

We investigated whether the observed changes in protein expression may be a direct consequence of an altered gene expression caused by D-Asp depletion. To this aim, we performed qRT-PCR analysis on whole brain samples from *Ddo*-KI and *Ddo*-WT mice at P3 and P14. Specifically, we selected 30 genes encoding a subset of proteins identified in the mouse proteomic analysis, which are particularly relevant due to their role in glutamatergic transmission and/or brain development, as well as their known association with SCZ/ASD (Table 2; Suppl. Data 3). Some of these genes were also identified through in silico analysis based on anti-correlation/co-expression with *DDO* gene expression in the human CNS (Table 2; Suppl. Data 4). Consistent with proteomic data, statistical analysis revealed a significant reduction in *Grin1*, *Homer3*, *Syngr1* and *Iqsec1* mRNA expression in the brain of *Ddo*-KI mice at P3, compared with age-matched *Ddo*-WT control littermates (Table 2).

Table 2 Analysis of mRNA expression of specific D-aspartate-modulated proteins in the whole brain of post-natal day 3 (P3) and P14 *Ddo*-KI and *Ddo*-WT mice by qRT-PCR

P3				P14		
Gene	Ddo-WT Mean±SD	Ddo-KI Mean±SD	<i>p</i> value	Ddo-WT Mean±SD	Ddo-KI Mean±SD	<i>p</i> value
<i>Cntnap1</i>	0.004±0.001	0.004±0.001	0.9248	0.012±0.005	0.016±0.004	0.3075
<i>Cntnap4</i>	0.059±0.021	0.052±0.007	0.5211	0.023±0.0003	0.024±0.009	0.8540
<i>Fxyd6</i>	0.756±0.309	0.751±0.310	0.9835	0.237±0.152	0.140±0.112	0.2774
<i>Gad1</i>	0.066±0.024	0.066±0.020	0.9915	0.052±0.021	0.068±0.028	0.3258
<i>Gria3</i>	0.027±0.012	0.027±0.004	0.8942	0.033±0.011	0.052±0.021	0.1118
<i>Gria4</i>	0.176±0.079	0.165±0.022	0.7633	0.141±0.109	0.215±0.095	0.2853
<i>Grin1</i>	0.096±0.031	0.054±0.008	0.0344	0.108±0.034	0.116±0.064	0.8230
<i>Homer1</i>	0.047±0.015	0.035±0.006	0.1235	0.050±0.013	0.068±0.032	0.2821
<i>Homer3</i>	0.0041±0.0007	0.0031±0.0005	0.0424	0.015±0.005	0.009±0.005	0.1093
<i>Icam5</i>	0.0018±0.0007	0.0016±0.0004	0.4909	0.008±0.003	0.006±0.001	0.3019
<i>Igf2r</i>	0.157±0.066	0.143±0.020	0.6846	0.032±0.014	0.040±0.015	0.4422
<i>Igsf21</i>	0.068±0.037	0.072±0.032	0.8672	0.036±0.022	0.047±0.026	0.4782
<i>Iqsec1</i>	0.167±0.033	0.112±0.015	0.0105	0.123±0.028	0.137±0.082	0.7306
<i>Macrod2</i>	0.012±0.005	0.009±0.002	0.4857	0.003±0.002	0.003±0.001	0.5342
<i>Madd1</i>	0.107±0.023	0.093±0.036	0.4960	0.087±0.008	0.083±0.036	0.8137
<i>Maob</i>	0.012±0.003	0.010±0.004	0.5037	0.021±0.006	0.029±0.015	0.3077
<i>Mapt</i>	0.670±0.319	0.518±0.091	0.3346	0.141±0.008	0.174±0.091	0.4389
<i>Nav3</i>	0.049±0.035	0.041±0.019	0.6496	0.034±0.019	0.044±0.024	0.5027
<i>Nudt4</i>	0.053±0.021	0.037±0.008	0.1420	0.032±0.025	0.037±0.030	0.7951
<i>Oxr1</i>	0.013±0.005	0.010±0.003	0.3784	0.032±0.014	0.034±0.017	0.8017
<i>Pde1a</i>	0.007±0.004	0.008±0.002	0.7562	0.012±0.006	0.020±0.002	0.0344
<i>Ppm1h</i>	0.027±0.010	0.032±0.017	0.5638	0.037±0.0111	0.033±0.018	0.7218
<i>Ptk2b</i>	0.008±0.002	0.007±0.002	0.5357	0.019±0.003	0.020±0.005	0.8721
<i>Rasgrf2</i>	0.040±0.022	0.036±0.017	0.7283	0.012±0.004	0.024±0.016	0.1375
<i>Slc17a7</i>	0.045±0.0144	0.046±0.018	0.9148	0.0002±0.0815	0.0003±0.0342	0.2616
<i>Slc4a8</i>	0.014±0.003	0.011±0.001	0.1115	0.009±0.002	0.009±0.002	0.7101
<i>Srr</i>	0.064±0.035	0.058±0.017	0.7248	0.058±0.028	0.075±0.039	0.45
<i>Syncrip</i>	0.192±0.053	0.179±0.025	0.6407	0.0596±0.021	0.077±0.031	0.3207
<i>Syng1</i>	0.038±0.003	0.029±0.007	0.0296	0.035±0.012	0.063±0.054	0.3003
<i>Tubb4a</i>	0.167±0.032	0.127±0.018	0.0420	0.267±0.062	0.321±0.171	0.5242

Data are expressed as the difference in threshold cycle ($2^{-\Delta\Delta C_t}$) between each specific gene and the reference gene, *Gapdh* (arbitrary units), and represented as the mean±standard deviation (SD). Statistical analysis was performed by unpaired *t*-test. $p<0.05$ was considered statistically significant and reported in bold

Discussion

Glutamatergic neurotransmission plays a crucial role in CNS development, regulating key processes such as neuronal proliferation, apoptosis, differentiation, migration and refinement of synaptic connections [52–56]. Accordingly, alterations in glutamatergic receptor function during prenatal and early postnatal development can lead to permanent changes in brain structure and organization, contributing to the later emergence of neurodevelopmental disorders such as SCZ and ASD [57–60]. Despite the recognized influence of NMDAR and mGluR5 signaling in neurodevelopmental processes and related disorders, the specific contribution of endogenous ligands regulating the activity of these glutamatergic receptors remains unclear. In particular, whether the NMDAR and mGluR5 agonist D-Asp influences the

function of these receptor subclasses through selective changes in protein expression has not been clarified.

In this study, we found that metabolic depletion of D-Asp significantly alters the proteome profile of the developing mouse brain at an early postnatal stage. Notably, in *Ddo*-KI mice most differentially expressed proteins (cut-off level>1.3-fold) were detected at P3, suggesting that the regulatory effect of D-Asp on protein expression is confined to a narrow postnatal developmental window.

GO enrichment analysis revealed that proteins modulated by D-Asp are primarily associated with nervous system development, chemical synaptic transmission and its modulation, and cytoskeleton-mediated intracellular transport, confirming previous *in vivo* and *ex vivo* studies in animal models [5, 15, 16, 18–22, 24, 27, 30].

Among the proteins most significantly affected by D-Asp depletion (cut-off >1.3-fold), we identified key components of the glutamatergic synapse, including the essential NMDAR subunit, GluN1 (encoded by the *Grin1* gene), the AMPAR subunits, GluA3 (*Gria3*) and GluA4 (*Gria4*), and the post-synaptic density proteins, Homer1 and Homer3 [61]. Moreover, we identified GAD1, an enzyme central to GABA synthesis, and thus indirectly associated with glutamatergic neurotransmission [62]. In this regard, a previous study has revealed an altered number of parvalbumin-positive GABAergic interneurons in the cortex of *Ddo*-KI mice [5], supporting a potential implication of D-Asp in regulating inhibitory circuits.

Applying a more stringent protein expression cut-off (>1.5-fold), we further refined a subset of proteins involved in nervous system development and chemical synaptic transmission. Many of these proteins directly or indirectly interact with NMDARs and/or mGluR5, contributing to the modulation of glutamate-mediated synaptic activity. Notable examples include CNTNAP1, which regulates NMDAR-dependent synaptic plasticity [63] and mGluR5-dependent hippocampal memory formation [64], and ICAM5, which modulates dendritic outgrowth and spine maturation by competing with NMDARs [65–67]. Additionally, SYNGR1 is involved in synaptic vesicle function and significantly impacts NMDAR-dependent long-term potentiation (LTP) [68]. Moreover, mGluR5-mediated induction of PTK2B (also known as PYK2) promotes NMDAR phosphorylation, thereby contributing to intracellular Ca^{2+} homeostasis [69, 70]. Finally, RASGRF2 is essential for NMDAR-dependent hippocampal LTP and spine enlargement [71, 72], while SLC4A8 modulates the pH-dependent release of neuronal glutamate [73].

Among D-Asp-regulated proteins, several play a key role in processes critical for brain development, including synapse formation, synaptic plasticity and neurotransmission (SYNGR1, CNTNAP4, ICAM5, PTK2B, RASGRF2, GAD1) [68, 74–78], neuronal migration and axon guidance (CNTNAP1, TUBB4A, ICAM5, NAV3) [74, 79–81], myelination (TUBB4A, CNTNAP1) [81, 82], regulation of signaling pathways (IGF2R, SLC4A8) [83, 84] and gene expression (SYNCRIP) [85], cytoskeletal organization, dendrite morphogenesis and structural integrity (MAPT, TUBB4A, PTK2B) [79, 86, 87].

D-Asp has been shown to induce NMDAR-dependent late-phase LTP and dendritic spinogenesis in the rodent hippocampus by modulating actin polymerization mechanisms [21, 24]. Consistently, findings in the mouse testis indicate that D-Asp influences spermatogenesis by regulating actin- and tubulin-mediated cytoskeleton dynamics [88, 89]. The present results confirm the impact of D-Asp on cerebral protein levels implicated in cytoskeleton-dependent transport

and remodeling, including microtubule binding and stability (MAPT, TUBB4A) [79, 87], actin regulation (PTK2B, RASGRF2) [78, 87], cytoskeleton adhesion (CNTNAP1, ICAM5) [74, 81], and microtubule organization and axon guidance (NAV3) [80].

In line with the increasing evidence linking early cerebral D-Asp to neurodevelopmental psychiatric disorders, our proteomic analysis identified a subset of D-Asp-regulated proteins at P3 that are associated with ASD, SCZ or both disorders. Many of these proteins are known to modulate glutamatergic neurotransmission. Among them, we identified the aforementioned GluN1, GluA3, GluA4, Homer1, the synaptic vesicle-associated glutamate transporter, SLC17A7/VGLUT1 [90], the biosynthetic enzyme of glutamate-GABA conversion, GAD1 [25, 26, 62], as well as CNTNAP1 [63], FXR1 [91], FLOT1 [92], CDKL5 [93], PTPN5 [94], DLG1/SAP97 [95], RTN4/NogoA [96] and MPP2 [97].

To further support our proteomic findings, we analyzed publicly available human datasets for genes most strongly correlated with *DDO* expression. Among them, we identified nine anti-correlated human genes encoding orthologous proteins to those found in proteomic analysis. Beyond *Slc17a7*, which directly impacts glutamatergic transmission [90], the majority of these genes encode proteins that modulate glutamatergic functions, affecting synaptic transmission (*Iqsec1*, *Ppm1h*, *Pdel1a*) [98–100], neuronal excitability (*Fxyd6*, *Madd*) [101, 102], synapse organization (*Igsf21*) [103] or neuroprotection (*Oxr1*) [104]. Many of these proteins also play a critical role in brain development (*Slc17a7*, *Iqsec1*, *Ppm1h*, *Fxyd6*) [90, 98, 99, 101] and have been previously linked to neurodevelopmental psychiatric disorders (*Slc17a7*, *Iqsec1*, *Pdel1a*, *Madd*, *Fxyd6*) [105–109].

To investigate whether the modulatory effect of D-Asp on protein expression is somehow reflected at the mRNA level, we performed qRT-PCR analysis in the brain of *Ddo*-KI mice at P3 and P14. We focused on genes encoding a subset of differentially expressed proteins relevant to glutamatergic transmission, brain development, and/or neurodevelopmental psychiatric disorders. Some of these genes were also strongly anti-correlated with *DDO* expression in the human in silico database. Among the tested genes, we found that *Grin1*, *Homer3*, *Syng1* and *Iqsec1* were significantly downregulated in *Ddo*-KI mice compared to *Ddo*-WT controls at P3 but not at P14, in line with a selective, time-dependent decrease in the corresponding protein levels. Regardless of the precise molecular mechanism involved, our present data highlight that D-Asp metabolism physiologically influences the brain proteome at P3, affecting both transcriptional and translational processes.

Although further targeted studies are needed, we hypothesize that the selective impact of D-Asp metabolism on the

brain proteome during the early postnatal stage aligns with a critical role of NMDAR signaling in regulating neurodevelopmental processes relevant to psychiatric disorders [110, 111]. Specifically, the narrow time window during which D-Asp affects the brain proteome may coincide with the developmental shift from GluN2B to GluN2A subunit expression of NMDARs [112]. This transition is crucial for the maturation of synaptic signaling and cognitive functions, and alterations in its timing could influence neurodevelopmental pathways implicated in psychiatric conditions [112].

Taken together, our results not only suggest that fluctuations in the brain levels of D-Asp selectively affect the brain proteome but also lay the groundwork for several translational follow-up studies. One important direction will be to test whether temporal supplementation of D-Asp during the early postnatal period can rescue proteomic changes, as well as the metabolomic, morphological and social behavioral deficits previously observed in *Ddo*-KI mice [5, 27, 28]. Future analyses should also clarify whether, beyond the early postnatal onset of *Ddo* expression, maternal or developmental factors may influence D-Asp metabolism and thereby shape the brain proteome. In this regard, our recent study demonstrates that maternal environmental stressors linked to ASD risk — such as the infectious agent lipopolysaccharide or the mood stabilizer valproate — can alter cerebral D-Asp levels in ASD rat models [39], providing initial insight into its possible role in disease pathophysiology. Another relevant question will be whether peripheral D-Asp depletion at early postnatal stages contributes to the observed cerebral proteomic alterations. Since peripheral proteomic analyses and D-Asp measurements were not performed in the present study, this remains an important direction for future studies. Finally, given that the neurodevelopmental phenotypes observed in *Ddo*-KI mice arise from D-Asp depletion secondary to altered DDO expression/activity, future studies should clarify whether changes in SCZ and ASD patients involve not only D-Asp levels but also DDO itself, both centrally and peripherally. Such investigations could strengthen the mechanistic link with disease pathophysiology and help identify potential biomarker candidates for psychiatric neurodevelopmental disorders.

The present study also has some limitations. Proteomic analyses were conducted in both male and female *Ddo*-KI and *Ddo*-WT mice without sex stratification, preventing the assessment of sex-specific differences. In addition, the use of whole-brain samples limits the ability to determine whether specific regions — especially those most implicated in neurodevelopmental psychiatric disorders — are selectively affected by D-Asp dysmetabolism. Importantly, the cerebellum was included in the whole-brain dissections, and its contribution may have influenced the proteomic profile observed. This point is particularly relevant given accumulating

evidence for cerebellar involvement in neurodevelopmental psychiatric disorders, where structural abnormalities, disrupted cerebello-cortical connectivity (a circuit highly enriched in NMDARs), and altered glutamatergic signaling have been implicated in cognitive and affective deficits [113–118]. Future studies will be essential to establish whether the protein alterations and D-Asp changes identified at early postnatal stage are sex-dependent and region-specific.

In conclusion, the present findings highlight that D-Asp modulates the expression of key proteins implicated in NMDAR and mGluR5-dependent processes that are crucial for neurodevelopment and associated neuropsychiatric disorders. These observations emphasize the need for further research into the molecular mechanisms of D-Asp signaling and highlight its translational potential as a target for studies exploring pathophysiological mechanisms of neurodevelopmental disorders and their related therapeutic interventions.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00018-025-05938-9>.

Acknowledgements We kindly thank Mariangela Valletta for her support in mass spectrometry analyses and Arianna De Rosa for her support in mouse genotyping and critical discussion. We are grateful to Dr. E. Mautone for his valuable comments.

Author contributions F.E. supervised HPLC experiments, wrote and edited the paper. R.R. performed proteomic experiments, acquired data, prepared the figures and wrote the paper. F.C. performed qRT-PCR experiments and acquired data. T.N. supervised HPLC experiments. R.d.V. performed HPLC experiments, acquired data and prepared the figures. E.C. performed proteomic experiments. P.V.P. edited the paper. F.D.C. performed analysis on human in silico database and edited the paper. T.E. supervised qRT-PCR experiments and edited the paper. A.U. conceived and designed the study, and edited the paper. A.C. supervised proteomic experiments and edited the paper.

Funding A.U., T.N., and R.d.V. were supported by #NEXTGENERATIONEU (NGEU) funded by the Ministry of University and Research (Ministero dell'Università e della Ricerca, MUR), National Recovery and Resilience Plan (NRRP), project MNESYS (PE00000006) – A Multiscale integrated approach to the study of the nervous system in health and disease (DN. 1553 11.10.2022). A.U., F.E. and F.D.C. were supported by the Italian Ministry of University and Research (MUR) through PRIN 2017 - Project nr 2017M42834 (A.U. and F.E.) and PRIN PNRR 2022, financed by the European Union – Next Generation EU - Projects nr P2022ZEMZF (A.U. and F.E.) and 2022M75NN8 (F.D.C.). T.E. was supported by Ricerca Corrente - Italian Ministry of Health.

Data availability All data needed to evaluate the conclusions of the work are present in the paper and the supplementary materials.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. All research involving animals was carried out following the directive of the Italian Ministry of Health gov-

erning animal welfare and protection (D.LGS 26/2014) and approved by “Direzione Generale della Sanità e dei Farmaci Veterinari (Ufficio 6)” (permission nr. 796/2018).

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

References

- Hashimoto A, Oka T (1997) Free D-aspartate and D-serine in the mammalian brain and periphery. *Prog Neurobiol* 52(4):325–353
- Errico F, Cuomo M, Canu N, Caputo V, Usiello A (2020) New insights on the influence of free d-aspartate metabolism in the mammalian brain during prenatal and postnatal life. *Biochim Biophys Acta Proteins Proteom* 1868(10):140471. <https://doi.org/10.1016/j.bbapap.2020.140471>
- Hashimoto A, Kumashiro S, Nishikawa T, Oka T, Takahashi K, Mito T et al (1993) Embryonic development and postnatal changes in free D-aspartate and D-serine in the human prefrontal cortex. *J Neurochem* 61(1):348–351
- Errico F, Pirro MT, Affuso A, Spinelli P, De Felice M, D'Aniello A et al (2006) A physiological mechanism to regulate D-aspartic acid and NMDA levels in mammals revealed by D-aspartate oxidase deficient mice. *Gene* 374:50–57
- De Rosa A, Mastrostefano F, Di Maio A, Nuzzo T, Saitoh Y, Katane M et al (2020) Prenatal expression of D-aspartate oxidase causes early cerebral D-aspartate depletion and influences brain morphology and cognitive functions at adulthood. *Amino Acids* 52(4):597–617. <https://doi.org/10.1007/s00726-020-02839-y>
- Punzo D, Errico F, Cristino L, Sacchi S, Keller S, Belardo C et al (2016) Age-related changes in D-aspartate oxidase promoter methylation control extracellular D-aspartate levels and prevent precocious cell death during brain aging. *J Neurosci* 36(10):3064–3078. <https://doi.org/10.1523/JNEUROSCI.3881-15.2016>
- Katane M, Homma H (2010) D-aspartate oxidase: the sole catabolic enzyme acting on free D-aspartate in mammals. *Chem Biodivers* 7(6):1435–1449. <https://doi.org/10.1002/cbdv.200900250>
- Rabattoni V, Motta Z, Miceli M, Molla G, Fissore A, Adinolfi S et al (2023) On the regulation of human D-aspartate oxidase. *Protein Sci* 32(11):e4802. <https://doi.org/10.1002/pro.4802>
- Wolosker H, Blackshaw S, Snyder SH (1999) Serine racemase: a glial enzyme synthesizing d-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc Natl Acad Sci U S A* 96(23):13409–13414
- Horio M, Ishima T, Fujita Y, Inoue R, Mori H, Hashimoto K (2013) Decreased levels of free D-aspartic acid in the forebrain of Serine racemase (Srr) knock-out mice. *Neurochem Int* 62(6):843–847. <https://doi.org/10.1016/j.neuint.2013.02.015>
- Ito T, Hayashida M, Kobayashi S, Muto N, Hayashi A, Yoshimura T et al (2016) Serine racemase is involved in d-aspartate biosynthesis. *J Biochem*. <https://doi.org/10.1093/jb/mvw043>
- Olverman HJ, Jones AW, Mewett KN, Watkins JC (1988) Structure/activity relations of N-methyl-D-aspartate receptor ligands as studied by their inhibition of [3H]D-2-amino-5-phosphonopentanoic acid binding in rat brain membranes. *Neuroscience* 26(1):17–31
- Ransom RW, Stec NL (1988) Cooperative modulation of [3H] MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. *J Neurochem* 51(3):830–836. <https://doi.org/10.1111/j.1471-4159.1988.tb01818.x>
- Errico F, Bonito-Oliva A, Bagetta V, Vitucci D, Romano R, Zianni E et al (2011) Higher free D-aspartate and N-methyl-D-aspartate levels prevent striatal depotentiation and anticipate L-DOPA-induced dyskinesia. *Exp Neurol* 232(2):240–250. <https://doi.org/10.1016/j.expneurol.2011.09.013>
- Errico F, Nistico R, Napolitano F, Mazzola C, Astone D, Pisapia T et al (2011) Increased D-aspartate brain content rescues hippocampal age-related synaptic plasticity deterioration of mice. *Neurobiol Aging* 32(12):2229–2243. <https://doi.org/10.1016/j.neurobiolaging.2010.01.002>
- Molinaro G, Pietracupa S, Di Menna L, Pescatori L, Usiello A, Battaglia G et al (2010) D-aspartate activates mGlu receptors coupled to polyphosphoinositide hydrolysis in neonate rat brain slices. *Neurosci Lett* 478(3):128–130. <https://doi.org/10.1016/j.neulet.2010.04.077>
- Sacchi S, Novellis V, Paolone G, Nuzzo T, Iannotta M, Belardo C et al (2017) Olanzapine, but not clozapine, increases glutamate release in the prefrontal cortex of freely moving mice by inhibiting D-aspartate oxidase activity. *Sci Rep* 7:46288. <https://doi.org/10.1038/srep46288>
- Errico F, Nistico R, Napolitano F, Oliva AB, Romano R, Barbieri F et al (2011) Persistent increase of D-aspartate in D-aspartate oxidase mutant mice induces a precocious hippocampal age-dependent synaptic plasticity and spatial memory decay. *Neurobiol Aging* 32(11):2061–2074. <https://doi.org/10.1016/j.neurobiolaging.2009.12.007>
- Errico F, Nistico R, Palma G, Federici M, Affuso A, Brilli E et al (2008) Increased levels of d-aspartate in the hippocampus enhance LTP but do not facilitate cognitive flexibility. *Mol Cell Neurosci* 37(2):236–246. <https://doi.org/10.1016/j.mcn.2007.09.012>
- Errico F, Rossi S, Napolitano F, Catuogno V, Topo E, Fisone G et al (2008) D-aspartate prevents corticostriatal long-term depression and attenuates schizophrenia-like symptoms induced by amphetamine and MK-801. *J Neurosci* 28(41):10404–10414. <https://doi.org/10.1523/JNEUROSCI.1618-08.2008>
- Errico F, Nistico R, Di Giorgio A, Squillace M, Vitucci D, Galbusera A et al (2014) Free d-aspartate regulates neuronal dendritic morphology, synaptic plasticity, gray matter volume and brain activity in mammals. *Transl Psychiatry* 4:e417. <https://doi.org/10.1038/tp.2014.59>
- Krashia P, Ledonne A, Nobili A, Cordella A, Errico F, Usiello A et al (2016) Persistent elevation of D-aspartate enhances NMDA receptor-mediated responses in mouse substantia nigra pars compacta dopamine neurons. *Neuropharmacology* 103:69–78. <https://doi.org/10.1016/j.neuropharm.2015.12.013>
- Nicoletti CG, Monteleone F, Marfia GA, Usiello A, Buttari F, Centonze D et al (2020) Oral D-aspartate enhances synaptic plasticity reserve in progressive multiple sclerosis. *Mult Scler* 26(3):304–311. <https://doi.org/10.1177/1352458519828294>

24. Kitamura A, Hojo Y, Ikeda M, Karakawa S, Kuwahara T, Kim J et al (2019) Ingested d-aspartate facilitates the functional connectivity and modifies dendritic spine morphology in rat hippocampus. *Cereb Cortex* 29(6):2499–2508. <https://doi.org/10.1093/cercor/bhy120>
25. Gonzalez-Burgos G, Cho RY, Lewis DA (2015) Alterations in cortical network oscillations and parvalbumin neurons in schizophrenia. *Biol Psychiatry* 77(12):1031–1040. <https://doi.org/10.1016/j.biopsych.2015.03.010>
26. Lewis DA, Hashimoto T, Volk DW (2005) Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci* 6(4):312–324. <http://doi.org/10.1038/nrn1648>
27. Lombardo B, Pagani M, De Rosa A, Nunziato M, Migliarini S, Garofalo M et al (2022) D-aspartate oxidase gene duplication induces social recognition memory deficit in mice and intellectual disabilities in humans. *Transl Psychiatry* 12(1):305. <https://doi.org/10.1038/s41398-022-02088-5>
28. Grimaldi M, Marino C, Buonocore M, Santoro A, Sommella E, Merciai F et al (2021) Prenatal and early postnatal cerebral d-aspartate depletion influences l-amino acid pathways, bioenergetic processes, and developmental brain metabolism. *J Proteome Res* 20(1):727–739. <https://doi.org/10.1021/acs.jproteome.0c00622>
29. Crawley JN (2023) Twenty years of discoveries emerging from mouse models of autism. *Neurosci Biobehav Rev* 146:105053. <https://doi.org/10.1016/j.neubiorev.2023.105053>
30. Li YJ, Zhang K, Sun T, Guo YY, Yang Q, Liu SB et al (2023) Improvement of learning and memory by elevating brain D-aspartate in a mouse model of fragile X syndrome. *Mol Neurobiol* 60(11):6410–6423. <https://doi.org/10.1007/s12035-023-03438-0>
31. Errico F, D'Argenio V, Sforazzini F, Iasevoli F, Squillace M, Guerri G et al (2015) A role for D-aspartate oxidase in schizophrenia and in schizophrenia-related symptoms induced by phencyclidine in mice. *Transl Psychiatry* 5:e512. <https://doi.org/10.1038/tp.2015.2>
32. de Bartolomeis A, Errico F, Aceto G, Tomasetti C, Usiello A, Iasevoli F (2015) D-aspartate dysregulation in Ddo(-/-) mice modulates phencyclidine-induced gene expression changes of postsynaptic density molecules in cortex and striatum. *Prog Neuropsychopharmacol Biol Psychiatry* 62:35–43. <https://doi.org/10.1016/j.pnpbp.2015.05.003>
33. Errico F, Napolitano F, Squillace M, Vitucci D, Blasi G, de Bartolomeis A et al (2013) Decreased levels of D-aspartate and NMDA in the prefrontal cortex and striatum of patients with schizophrenia. *J Psychiatr Res* 47(10):1432–1437. <https://doi.org/10.1016/j.jpsychires.2013.06.013>
34. Nuzzo T, Sacchi S, Errico F, Keller S, Palumbo O, Florio E et al (2017) Decreased free d-aspartate levels are linked to enhanced d-aspartate oxidase activity in the dorsolateral prefrontal cortex of schizophrenia patients. *NPJ Schizophr* 3:16. <https://doi.org/10.1038/s41537-017-0015-7>
35. De Rosa A, Fontana A, Nuzzo T, Garofalo M, Di Maio A, Punzo D et al (2022) Machine learning algorithm unveils glutamatergic alterations in the post-mortem schizophrenia brain. *Schizophr* 8(1):8. <https://doi.org/10.1038/s41537-022-00231-1>
36. Garofalo M, De Simone G, Motta Z, Nuzzo T, De Grandis E, Bruno C et al (2024) Decreased free D-aspartate levels in the blood serum of patients with schizophrenia. *Front Psychiatry* 15:1408175. <https://doi.org/10.3389/fpsy.2024.1408175>
37. Rampino A, Garofalo M, Nuzzo T, Favia M, Saltarelli S, Masellis R et al (2024) Variations of blood D-serine and D-aspartate homeostasis track psychosis stages. *Schizophr* 10(1):115. <https://doi.org/10.1038/s41537-024-00537-2>
38. Nuzzo T, Sekine M, Punzo D, Miroballo M, Katane M, Saitoh Y et al (2020) Dysfunctional d-aspartate metabolism in BTBR mouse model of idiopathic autism. *Biochimica et Biophysica Acta (BBA)* 1868(12):140531. <https://doi.org/10.1016/j.bbapap.2020.140531>
39. Di Maio A, Yahyavi I, Buzzelli V, Motta Z, Ascone F, Putignano L et al (2025) Prenatal exposure to lipopolysaccharide or valproate leads to abnormal accumulation of the NMDA receptor agonist D-aspartate in the adolescent rat brain. *J Neurochem* 169(6):e70095. <https://doi.org/10.1111/jnc.70095>
40. Mastrostefano F, Garofalo M, Nuzzo T, Bruno C, Errico F, Usiello A et al (2025) Central neurophysiological alterations in dystrophic Mdx mice correlate with reduced hippocampal levels of the endogenous NMDA receptor ligand D-aspartate. *J Neurochem* 169(9):e70223. <https://doi.org/10.1111/jnc.70223>
41. Cimini D, Russo R, D'Ambrosio S, Dello Iacono I, Rega C, Carlinio E et al (2018) Physiological characterization and quantitative proteomic analyses of metabolically engineered *E. coli* K4 strains with improved pathways for capsular polysaccharide biosynthesis. *Biotechnol Bioeng* 115(7):1801–1814. <https://doi.org/10.1002/bit.26597>
42. Napolitano F, De Rosa A, Russo R, Di Maio A, Garofalo M, Federici M et al (2019) The striatal-enriched protein Rhes is a critical modulator of cocaine-induced molecular and behavioral responses. *Sci Rep* 9(1):15294. <https://doi.org/10.1038/s41598-019-51839-w>
43. Di Matteo F, Picicelli F, Kyrousi C, Tovecci I, Penna E, Crispino M et al (2020) Cystatin B is essential for proliferation and interneuron migration in individuals with EPM1 epilepsy. *Embo Mol Med* 12(6):ARTN e11419. doi: <https://doi.org/10.15252/emmm.201911419>
44. Citores L, Valletta M, Singh VP, Pedone PV, Iglesias R, Ferreras JM et al (2022) Deciphering molecular determinants underlying *Penicillium digitatum*'s response to biological and chemical antifungal agents by tandem mass tag (TMT)-based high-resolution LC-MS/MS. *Int J Mol Sci*. <https://doi.org/10.3390/ijms23020680>
45. Russo R, Russo V, Cecere F, Valletta M, Gentile MT, Colucci-D'Amato L et al (2021) ZBTB2 protein is a new partner of the nucleosome remodeling and deacetylase (NuRD) complex. *Int J Biol Macromol* 168:67–76. <https://doi.org/10.1016/j.ijbiomac.2020.12.029>
46. Russo R, Matrone N, Belli V, Ciardiello D, Valletta M, Esposito S et al (2019) Macrophage migration inhibitory factor is a molecular determinant of the anti-EGFR monoclonal antibody cetuximab resistance in human colorectal cancer cells. *Cancers*. <https://doi.org/10.3390/cancers11101430>
47. Chen T, Zhang HY, Liu Y, Liu YX, Huang LQ, EVenn (2021) Easy to create repeatable and editable Venn diagrams and Venn networks online. *J Genet Genomics* 48(9):863–866. <https://doi.org/10.1016/j.jgg.2021.07.007>
48. Yu GC, Wang LG, Han YY, He QY (2012) Clusterprofiler: an R package for comparing biological themes among gene clusters. *OMICS* 16(5):284–287. <https://doi.org/10.1089/omi.2011.0118>
49. Kolberg L, Raudvere U, Kuzmin I, Adler P, Vilo J, Peterson H (2023) g:Profiler-interoperable web service for functional enrichment analysis and gene identifier mapping (2023 update). *Nucleic Acids Res* 51(W1):W207–W12ARTN gkad347. <https://doi.org/10.1093/nar/gkad347>
50. Piro RM, Ala U, Molineris I, Grassi E, Bracco C, Perego GP et al (2011) An atlas of tissue-specific conserved coexpression for functional annotation and disease gene prediction. *Eur J Hum Genet* 19(11):1173–1180. <https://doi.org/10.1038/ejhg.2011.96>
51. Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, Lane HC et al (2022) DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acids Res* 50(W1):W216–W21. <https://doi.org/10.1093/nar/gkac194>

52. Olney JW (2002) New insights and new issues in developmental neurotoxicology. *Neurotoxicology* 23(6):659–668. [https://doi.org/10.1016/S0161-813X\(01\)00092-4](https://doi.org/10.1016/S0161-813X(01)00092-4)
53. Ikonomidou C, Bittigau P, Koch C, Genz K, Hoerster F, Felderhoff-Mueser U et al (2001) Neurotransmitters and apoptosis in the developing brain. *Biochem Pharmacol* 62(4):401–405
54. Contestabile A (2000) Roles of NMDA receptor activity and nitric oxide production in brain development. *Brain Res Brain Res Rev* 32(2–3):476–509. [https://doi.org/10.1016/S0165-0173\(00\)00018-7](https://doi.org/10.1016/S0165-0173(00)00018-7)
55. Jansson LC, Akerman KE (2014) The role of glutamate and its receptors in the proliferation, migration, differentiation and survival of neural progenitor cells. *J Neural Transm (Vienna)* 121(8):819–836. <https://doi.org/10.1007/s00702-014-1174-6>
56. Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. *Science* 260(5104):95–97. <https://doi.org/10.1126/science.8096653>
57. Uno Y, Coyle JT (2019) Glutamate hypothesis in schizophrenia. *Psychiatry Clin Neurosci* 73(5):204–215. <https://doi.org/10.1111/pcn.12823>
58. Olney JW, Newcomer JW, Farber NB (1999) NMDA receptor hypofunction model of schizophrenia. *J Psychiatr Res* 33(6):523–533. [https://doi.org/10.1016/S0022-3956\(99\)00029-1](https://doi.org/10.1016/S0022-3956(99)00029-1)
59. Moghaddam B, Javitt D (2012) From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment. *Neuropsychopharmacology* 37(1):4–15. <https://doi.org/10.1038/npp.2011.181>
60. Nisar S, Bhat AA, Masoodi T, Hashem S, Akhtar S, Ali TA et al (2022) Genetics of glutamate and its receptors in autism spectrum disorder. *Mol Psychiatry* 27(5):2380–2392. <https://doi.org/10.1038/s41380-022-01506-w>
61. de Bartolomeis A, Barone A, Buonaguro EF, Tomasetti C, Velucci L, Iasevoli F (2022) The Homer1 family of proteins at the crossroad of dopamine-glutamate signaling: an emerging molecular lego in the pathophysiology of psychiatric disorders. A systematic review and translational insight. *Neurosci Biobehav Rev* 136:104596. <https://doi.org/10.1016/j.neubiorev.2022.104596>
62. Soghomonian JJ, Martin DL (1998) Two isoforms of glutamate decarboxylase. Why? *Trends Pharmacol Sci* 19(12):500–505. [https://doi.org/10.1016/S0165-6147\(98\)01270-X](https://doi.org/10.1016/S0165-6147(98)01270-X)
63. Murai KK, Misner D, Ranscht B (2002) Contactin supports synaptic plasticity associated with hippocampal long-term depression but not potentiation. *Curr Biol* 12(3):181–190. [https://doi.org/10.1016/S0960-9822\(02\)00680-2](https://doi.org/10.1016/S0960-9822(02)00680-2)
64. Morato X, Lujan R, Goncalves N, Watanabe M, Altafaj X, Carvalho AL et al (2018) Metabotropic glutamate type 5 receptor requires contactin-associated protein 1 to control memory formation. *Hum Mol Genet* 27(20):3528–3541. <https://doi.org/10.1093/hmg/ddy264>
65. Conant K, Allen M, Lim ST (2015) Activity dependent CAM cleavage and neurotransmission. *Front Cell Neurosci* 9:305. <https://doi.org/10.3389/fncel.2015.00305>
66. Tian L, Stefanidakis M, Ning L, Van Lint P, Nyman-Huttunen H, Libert C et al (2007) Activation of NMDA receptors promotes dendritic spine development through MMP-mediated ICAM-5 cleavage. *J Cell Biol* 178(4):687–700. <https://doi.org/10.1083/jcb.200612097>
67. Ning L, Paetau S, Nyman-Huttunen H, Tian L, Gahmberg CG (2015) ICAM-5 affects spine maturation by regulation of NMDA receptor binding to alpha-actinin. *Biol Open* 4(2):125–136. <https://doi.org/10.1242/bio.201410439>
68. Janz R, Sudhof TC, Hammer RE, Unni V, Siegelbaum SA, Bolshakov VY (1999) Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I. *Neuron* 24(3):687–700. [https://doi.org/10.1016/S0896-6273\(00\)81122-8](https://doi.org/10.1016/S0896-6273(00)81122-8)
69. de Pins B, Mendes T, Giralt A, Girault JA (2021) The non-receptor tyrosine kinase Pyk2 in brain function and neurological and psychiatric diseases. *Front Synaptic Neurosci* 13:749001. <https://doi.org/10.3389/fnsyn.2021.749001>
70. Heidinger V, Manzerra P, Wang XQ, Strasser U, Yu SP, Choi DW et al (2002) Metabotropic glutamate receptor 1-induced upregulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. *J Neurosci* 22(13):5452–5461. <https://doi.org/10.1523/JNEUROSCI.22-13-05452.2002>
71. Schwechter B, Rosenmund C, Tolia KF (2013) RasGRF2 Rac-GEF activity couples NMDA receptor calcium flux to enhanced synaptic transmission. *Proc Natl Acad Sci U S A* 110(35):14462–14467. <https://doi.org/10.1073/pnas.1304340110>
72. Jin SX, Feig LA (2010) Long-term potentiation in the CA1 hippocampus induced by NR2A subunit-containing NMDA glutamate receptors is mediated by Ras-GRF2/Erk map kinase signaling. *PLoS ONE* 5(7):e11732. <https://doi.org/10.1371/journal.pone.0011732>
73. Sinning A, Liebmann L, Kougioumtzes A, Westermann M, Bruehl C, Hubner CA (2011) Synaptic glutamate release is modulated by the Na⁺-driven Cl⁻/HCO₃⁻ exchanger Slc4a8. *J Neurosci* 31(20):7300–7311. <https://doi.org/10.1523/JNEUROSCI.0269-11.2011>
74. Matsuno H, Okabe S, Mishina M, Yanagida T, Mori K, Yoshihara Y (2006) Telencephalin slows spine maturation. *J Neurosci* 26(6):1776–1786. <https://doi.org/10.1523/JNEUROSCI.2651-05.2006>
75. Girault JA, Costa A, Derkinderen P, Studler JM, Toutant M (1999) FAK and PYK2/CAKbeta in the nervous system: a link between neuronal activity, plasticity and survival? *Trends Neurosci* 22(6):257–263. [https://doi.org/10.1016/S0166-2236\(98\)01358-7](https://doi.org/10.1016/S0166-2236(98)01358-7)
76. Karayannis T, Au E, Patel JC, Kruglikov I, Markx S, Delorme R et al (2014) Cntnap4 differentially contributes to GABAergic and dopaminergic synaptic transmission. *Nature* 511(7508):236–240. <https://doi.org/10.1038/nature13248>
77. Burlingham SR, Wong NF, Peterkin L, Lubow L, Dos Santos Passos C, Benner O et al (2022) Induction of synapse formation by de novo neurotransmitter synthesis. *Nat Commun* 13(1):3060. <https://doi.org/10.1038/s41467-022-30756-z>
78. Ye X, Carew TJ (2010) Small G protein signaling in neuronal plasticity and memory formation: the specific role of Ras family proteins. *Neuron* 68(3):340–361. <https://doi.org/10.1016/j.neuron.2010.09.013>
79. Romaniello R, Arrigoni F, Fry AE, Bassi MT, Rees MI, Borgatti R et al (2018) Tubulin genes and malformations of cortical development. *Eur J Med Genet* 61(12):744–754. <https://doi.org/10.1016/j.ejmg.2018.07.012>
80. Powers RM, Hevner RF, Halpain S (2022) The neuron navigators: Structure, function, and evolutionary history. *Front Mol Neurosci* 15:1099554. <https://doi.org/10.3389/fnmol.2022.1099554>
81. Li W, Yang L, Tang C, Liu K, Lu Y, Wang H (2020) Mutations of CNTNAP1 led to defects in neuronal development. *JCI Insight*. <https://doi.org/10.1172/jci.insight.135697>
82. Curiel J, Rodriguez Bey G, Takanoashi A, Bugiani M, Fu X, Wolf NI et al (2017) *TUBB4A* mutations result in specific neuronal and oligodendrocytic defects that closely match clinically distinct phenotypes. *Hum Mol Genet* 26(22):4506–4518. <https://doi.org/10.1093/hmg/ddx338>
83. O'Kusky J, Ye P (2012) Neurodevelopmental effects of insulin-like growth factor signaling. *Front Neuroendocrinol* 33(3):230–251. <https://doi.org/10.1016/j.yfrme.2012.06.002>
84. Nordstrom T, Andersson LC, Akerman KEO (2019) Regulation of intracellular pH by electrogenic Na⁺/HCO₃⁻ co-transporters in

- embryonic neural stem cell-derived radial glia-like cells. *Biochim Biophys Acta Biomembr* 1861(6):1037–1048. <https://doi.org/10.1016/j.bbmem.2019.03.007>
85. Tilliole P, Fix S, Godin JD (2024) HnRNPs: roles in neurodevelopment and implication for brain disorders. *Front Mol Neurosci* 17:1411639. <https://doi.org/10.3389/fnmol.2024.1411639>
 86. Aiken J, Holzbaur ELF (2021) Cytoskeletal regulation guides neuronal trafficking to effectively supply the synapse. *Curr Biol* 31(10):R633–R50. <https://doi.org/10.1016/j.cub.2021.02.024>
 87. Fan L, Lu Y, Shen X, Shao H, Suo L, Wu Q (2018) Alpha protocadherins and Pyk2 kinase regulate cortical neuron migration and cytoskeletal dynamics via Rac1 GTPase and WAVE complex in mice. *Elife*. <https://doi.org/10.7554/eLife.35242>
 88. Usiello A, Di Fiore MM, De Rosa A, Falvo S, Errico F, Santillo A et al (2020) New evidence on the role of D-aspartate metabolism in regulating brain and endocrine system physiology: from preclinical observations to clinical applications. *Int J Mol Sci*. <https://doi.org/10.3390/ijms21228718>
 89. Santillo A, Falvo S, Venditti M, Di Maio A, Chieffi Baccari G, Errico F et al (2023) D-aspartate depletion perturbs steroidogenesis and spermatogenesis in mice. *Biomolecules*. <https://doi.org/10.3390/biom13040621>
 90. Freneau RT, Voglmaier S, Seal RP, Edwards RH (2004) VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci* 27(2):98–103. <https://doi.org/10.1016/j.tins.2003.11.005>
 91. Khlghatyan J, Beaulieu JM (2018) Are FXR family proteins integrators of dopamine signaling and glutamatergic neurotransmission in mental illnesses? *Front Synaptic Neurosci* 10:22. <https://doi.org/10.3389/fnsyn.2018.00022>
 92. Swanwick CC, Shapiro ME, Yi Z, Chang K, Wenthold RJ (2009) NMDA receptors interact with flotillin-1 and -2, lipid raft-associated proteins. *FEBS Lett* 583(8):1226–1230. <https://doi.org/10.1016/j.febslet.2009.03.017>
 93. Okuda K, Kobayashi S, Fukaya M, Watanabe A, Murakami T, Hagiwara M et al (2017) CDKL5 controls postsynaptic localization of GluN2B-containing NMDA receptors in the hippocampus and regulates seizure susceptibility. *Neurobiol Dis* 106:158–170. <https://doi.org/10.1016/j.nbd.2017.07.002>
 94. Won S, Roche KW (2021) Regulation of glutamate receptors by striatal-enriched tyrosine phosphatase 61 (STEP(61)). *J Physiol* 599(2):443–451. <https://doi.org/10.1113/JP278703>
 95. Howard MA, Elias GM, Elias LA, Swat W, Nicoll RA (2010) The role of SAP97 in synaptic glutamate receptor dynamics. *Proc Natl Acad Sci U S A* 107(8):3805–3810. <https://doi.org/10.1073/pnas.0914422107>
 96. Peng X, Kim J, Zhou Z, Fink DJ, Mata M (2011) Neuronal Nogo-A regulates glutamate receptor subunit expression in hippocampal neurons. *J Neurochem* 119(6):1183–1193. <https://doi.org/10.1111/j.1471-4159.2011.07520.x>
 97. Kim G, Lujan R, Schwenk J, Kelley MH, Aguado C, Watanabe M et al (2016) Membrane palmitoylated protein 2 is a synaptic scaffold protein required for synaptic SK2-containing channel function. *Elife*. <https://doi.org/10.7554/eLife.12637>
 98. Um JW (2017) Synaptic functions of the IQSEC family of ADP-ribosylation factor guanine nucleotide exchange factors. *Neurosci Res* 116:54–59. <https://doi.org/10.1016/j.neures.2016.06.007>
 99. Bliim N, Leshchynska I, Keable R, Chen BJ, Curry-Hyde A, Gray L et al (2019) Early transcriptome changes in response to chemical long-term potentiation induced via activation of synaptic NMDA receptors in mouse hippocampal neurons. *Genomics* 111(6):1676–1686. <https://doi.org/10.1016/j.ygeno.2018.11.019>
 100. Zhai S, Otsuka S, Xu J, Clarke VRJ, Tkatch T, Wokosin D et al (2024) Ca(2+)-dependent phosphodiesterase 1 regulates the plasticity of striatal spiny projection neuron glutamatergic synapses. *Cell Rep* 43(8):114540. <https://doi.org/10.1016/j.celrep.2024.114540>
 101. Garty H, Karlsh SJ (2006) Role of FXYD proteins in ion transport. *Annu Rev Physiol* 68:431–459. <https://doi.org/10.1146/annurev.physiol.68.040104.131852>
 102. Abdel-Salam GMH, Abdel-Hamid MS (2024) New insights into the clinical and molecular spectrum of the MADD-related neurodevelopmental disorder. *J Hum Genet* 69(6):263–270. <https://doi.org/10.1038/s10038-024-01236-7>
 103. Chofflet N, Naito Y, Pastore AJ, Padmanabhan N, Nguyen PT, Poitras C et al (2024) Structural and functional characterization of the IgSF21-neurexin2α complex and its related signaling pathways in the regulation of inhibitory synapse organization. *Front Mol Neurosci* 17:1371145. <https://doi.org/10.3389/fnmol.2024.1371145>
 104. Oliver PL, Finelli MJ, Edwards B, Bitoun E, Butts DL, Becker EB et al (2011) Oxrl1 is essential for protection against oxidative stress-induced neurodegeneration. *PLoS Genet* 7(10):e1002338. <https://doi.org/10.1371/journal.pgen.1002338>
 105. Ansar M, Chung HL, Al-Otaibi A, Elagabani MN, Ravenscroft TA, Paracha SA et al (2019) Bi-allelic variants in IQSEC1 cause intellectual disability, developmental delay, and short stature. *Am J Hum Genet* 105(5):907–920. <https://doi.org/10.1016/j.ajhg.2019.09.013>
 106. Hebb AL, Robertson HA (2007) Role of phosphodiesterases in neurological and psychiatric disease. *Curr Opin Pharmacol* 7(1):86–92. <https://doi.org/10.1016/j.coph.2006.08.014>
 107. Schneeberger PE, Kortum F, Korenke GC, Alawi M, Santer R, Woody M et al (2020) Biallelic MADD variants cause a phenotypic spectrum ranging from developmental delay to a multisystem disorder. *Brain* 143(8):2437–2453. <https://doi.org/10.1093/brain/awaa204>
 108. Zhong N, Zhang R, Qiu C, Yan H, Valenzuela RK, Zhang H et al (2011) A novel replicated association between FXYD6 gene and schizophrenia. *Biochem Biophys Res Commun* 405(1):118–121. <https://doi.org/10.1016/j.bbrc.2011.01.005>
 109. Shen YC, Liao DL, Chen JY, Wang YC, Lai IC, Liou YJ (2009) Resequencing and association study of vesicular glutamate transporter 1 gene (VGLUT1) with schizophrenia. *Schizophr Res* 115(2–3):254–260. <https://doi.org/10.1016/j.schres.2009.08.003>
 110. Belforte JE, Zsiros V, Sklar ER, Jiang Z, Yu G, Li Y et al (2010) Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci* 13(1):76–83. <https://doi.org/10.1038/nn.2447>
 111. Kruse AO, Bustillo JR (2022) Glutamatergic dysfunction in schizophrenia. *Transl Psychiatry* 12(1):500. <https://doi.org/10.1038/s41398-022-02253-w>
 112. Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 14(6):383–400. <https://doi.org/10.1038/nrn3504>
 113. Wei Y, Wang Z, Xue K, Niu X, Ma L, Han S et al (2025) Key regions aberrantly connected within cerebello-thalamo-cortical circuit and their genetic mechanism in schizophrenia: an fMRI meta-analysis and transcriptome study. *Schizophr* 11(1):8. <https://doi.org/10.1038/s41537-025-00558-5>
 114. Schmitt A, Koschel J, Zink M, Bauer M, Sommer C, Frank J et al (2010) Gene expression of NMDA receptor subunits in the cerebellum of elderly patients with schizophrenia. *Eur Arch Psychiatry Clin Neurosci* 260(2):101–111. <https://doi.org/10.1007/s00406-009-0017-1>
 115. Kang N, Chung S, Lee SH, Bang M (2024) Cerebro-cerebellar gray matter abnormalities associated with cognitive impairment in patients with recent-onset and chronic schizophrenia. *Schizophr* 10(1):11. <https://doi.org/10.1038/s41537-024-00434-8>

116. Yeganeh-Doost P, Gruber O, Falkai P, Schmitt A (2011) The role of the cerebellum in schizophrenia: from cognition to molecular pathways. *Clin (Sao Paulo)* 66(1):71–77. <https://doi.org/10.1590/s1807-59322011001300009>
117. Rodrigues BA, Silva JDN, Kawamoto EM (2025) Cerebellar alterations in autism spectrum disorder: a mini-review. *Cerebellum* 24(2):52. <https://doi.org/10.1007/s12311-025-01806-1>
118. Lee S, Moon H, Kim E (2025) NMDAR dysfunction in autism spectrum disorders: lessons learned from 10 years of study. *Curr Opin Neurobiol* 92:103023. <https://doi.org/10.1016/j.conb.2025.103023>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.