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Spermidine Treatment Improves GRIN2B Loss-Of-Function, A Primary Disorder of Glutamatergic Neurotransmission

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ABSTRACT

GRIN-related disorders (GRD) developmental and epileptic encephalopathies (DEEs) display a clinical spectrum including developmental delay, hypotonia, intellectual disability, epilepsy, and autistic traits. The presence of de novo pathogenic variants in the *GRIN* genes alters the *N*-methyl D-aspartate receptor (NMDAR) function, with a genotype-phenotype relationship. Despite recent advances to elucidate GRD pathophysiological mechanisms and to find treatments, to date, GRD therapeutic arms are still scarce and with limited efficacy. Herein, we investigated whether the natural polyamine spermine—positive allosteric modulators of GluN2B subunit-containing NMDARs—or its precursor spermidine might rescue NMDAR hypofunctionality. In heterologous cell systems, administration of spermine potentiated wild-type and loss-of-function (LoF) NMDAR-mediated currents and attenuated synaptic density deficits. Functionally, the putative therapeutic benefit of spermidine (spermine precursor) was assessed in constitutive *Grin2b*$^{+/-}$ heterozygous mice, a GRIN2B-LoF genetic murine model recapitulating GRD-like synaptic, motor, and cognitive alterations. Chronic spermidine administration in young adult *Grin2b*$^{+/-}$ mice partially rescued hippocampal long-term potentiation deficits in hippocampal slices of *Grin2b*$^{+/-}$ mice, supporting the cognitive improvement observed in behavioral phenotyping. Based on these preclinical findings, a case study was conducted in two pediatric patients harboring mild GRIN2B-LoF variants. Importantly, in line with preclinical findings, 18 months of spermidine treatment resulted in the

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amelioration of adaptive behavior (notably in the younger treated patient), with the absence of noticeable side effects. Overall, our findings provide both preclinical and clinical data supporting the benefit of spermidine for the treatment of GRD in individuals harboring GRIN2B-LoF variants.

1 | Introduction

Neurodevelopmental disorders (NDD) result from the disturbance of the nervous system's developmental and maturation programs. Consequently, the shaping of the developing nervous system is altered, influencing neurochemical, synaptic, and neurometabolic processes, ultimately disturbing brain function and behavior. The emergence of next-generation sequencing (NGS) methods strongly contributed to identifying monogenic causes of NDD. In particular, within the NDD-associated genes cluster, those encoding for proteins converging on the glutamatergic synapse are largely represented [1]. Among them, *de novo* pathogenic variants of *GRIN* genes have been recently associated with the so-called GRIN-related disorders (GRD), a group of rare developmental epileptic encephalopathies (prevalence 1:5000 births) [2] with an autosomal dominant inheritance pattern [3–5]. *GRIN* genes encode for the GluN subunits of the *N*-methyl D-Aspartate receptor (NMDAR), a family of ionotropic glutamate receptors involved in fast excitatory neurotransmission [6, 7]. Concomitant with the crucial role of the NMDAR in excitatory glutamatergic synapse physiology, GRD individuals exhibit neurological and behavioral symptoms including, but not limited to, developmental delay, intellectual disability, autistic traits, hypotonia, and epilepsy [8].

Genotype–phenotype studies are strongly suggestive that qualitative and quantitative GRD-associated symptoms are strongly dictated by gene variants, even if the penetrance is not complete and the environmental factors can also influence clinical severity [5]. Regarding the former, GRD clinical manifestations are dependent on the identity of the affected gene, the functional domain impacted, and the amino acid substitution and/or truncated domain [9]. Mechanistically, as for other channelopathies, GRD-associated *GRIN* variants are classified into the following categories: missense gain-of-function (GoF), missense loss-of-function (LoF), mixed/complex, unclear/indeterminate, and null variants [10]. The functional stratification of *GRIN* variants allowed the identification of *GRIN* genes vulnerability and their association with GRD, as well as their biophysical outcomes. Indeed, among disease-associated *GRIN* variants, *GRIN1*, *GRIN2A*, and *GRIN2B* are the most prevalent. *GRIN* variants are more frequently leading to a loss-of-function, especially for *GRIN2B* disease-associated variants (25.4% GoF vs. 74.6% LoF), according to the GRIN variants database, available at http://lmc.uab.es/grindb/home; updated June 2023) [9].

Since the identification of GRD genetic etiology and the functional correlates, several therapeutic strategies have been investigated, always considering the need to develop precision strategies. In the context of GRD, the efforts are focused on preclinically evaluating the therapeutic efficacy of pharmacological, nutraceutical, and, recently, genetic strategies. These approaches aim to generate a therapeutic toolbox toward personalized medicine required to compensate for the *GRIN* variants functional outcomes spectrum. Unfortunately, to date, both the number of GRD therapeutic compounds is limited, their preclinical efficacy validation is still ongoing, and the translation to the clinical practice of the therapeutic arms is still scarce and needs to be expanded [11–13]. Taking advantage of long-lasting studies on the NMDAR physiology [6], potential therapeutic strategies might be based on the use of NMDAR orthosteric and allosteric modulators which, in the context of GRIN-LoF variants, could be achieved by the supplementation of NMDAR coagonist/s or by the administration of NMDAR positive allosteric modulators (PAMs) [14]. Three decades ago, spermine [bis-*N*-(3-aminopropyl)-1,4-diaminobutane] and spermidine [*N*-(3-aminopropyl)-1,4-diaminobutane] were described as modulators of ionotropic receptors [15] and, particularly, as positive allosteric modulators of the NMDAR [16, 17]. Besides their metabolic and systemic physiological and beneficial functions (cardioprotection, increased life expectancy) [18], based on spermine's positive allosteric modulation on GluN2B subunit-containing NMDARs [14], several studies have investigated the potential therapeutic benefit of spermine (and its precursor, spermidine) administration to murine models of neurological disorders, showing a modulatory effect on learning and memory processes in murine models [15]. Moreover, spermidine supplementation was associated with a positive impact on memory performance in old adults with cognitive decline while maintaining a commendable safety and tolerability profile [19–21].

Overall, considering spermine positive allosteric modulatory effect on NMDARs [22], spermidine supplementation tolerability in humans, and previous studies showing the benefit of L-serine treatment (NMDAR coagonist) for the treatment of GRIN-LoF individuals [11, 12, 23], in the present study we aimed to explore (i) spermine in vitro impact on GRD-associated biophysical and cellular phenotypes, (ii) the effect of 3 mM spermidine supplementation in the drinking water of postweaning *Grin2b*$^{+/-}$ heterozygous model recapitulating GRD-LoF-like alterations, and (iii) the tolerability and potential clinical benefit of 18 months spermidine treatment (3 mg/day) in two GRIN2B-LoF individuals. In agreement with the preclinical findings showing a partial rescue of GRIN2B-LoF-mediated phenotypes, the proof-of-concept clinical study showed a clinical benefit, enlarging the therapeutic options for GRD associated with LoF *GRIN2B* variants.

2 | Subjects/Materials and Methods

2.1 | Mice

The *Grin2b*$^{+/-}$ heterozygous mouse model [24] was kindly provided by Dr. Ana Luisa Carvalho (Centro Neurocencia Coimbra, Portugal). The mouse colony maintenance and amplification were performed by breeding *Grin2b*$^{+/-}$ mice with

wild-type C57Bl6 mice. DNA was extracted from earsnip biopsies from the pups, and PCR genotyping was performed. Wild-type and haploinsufficient mice were co-housed by gender and labeled with earsnip combinatory code. All experiments were performed by experienced personnel blind to the animal's genotype/treatment, and direct or video-recording analysis were performed by trained personnel blind to the animal's class/treatment. Mice from all groups were housed in the same room of the Animal Facility to homogenize environmental conditions. For the establishment of primary neuronal cultures, pregnant CD1 females (Charles River Laboratories) were sacrificed when embryos reached embryonic day 18 (E18). All experimental procedures followed the European Union guidelines (Directive 2010/63/EU) and were approved by the Ethics Committee of the University of Barcelona (Procedure N. 84/20). Despite similar GRD prevalence in males and females, NMDARs are widely expressed in the brain and systemic organs that might be influenced by the endocrine system. Accordingly, the effect of sex was considered in mouse behavioral studies.

2.2 | Plasmids

The expression plasmids for rat GluN1 and GFP-GluN2B were kindly provided by Dr. Vicini [25]. Nucleotide changes for the generation of *GRIN2B* variants were achieved by oligonucleotide-directed mutagenesis using the QuickChange II XL site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene) and verified by Sanger sequencing.

2.3 | Cell Culture and Transfection

HEK-293T cell line was obtained from the American Type Culture Collection and maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and antibiotics (100 units/mL penicillin and 100 mg/mL streptomycin). Transient heterologous expression of NMDARs in HEK-293T cells was achieved by cotransfection of GluN1 and GluN2B subunits (1:2 ratio) using poly-ethylenimine (PEI)-based transfection methods. Cells were cultured in growing media supplemented with 0.5 mM D-2-amino-5-phosphonopentanoic acid (D-AP5, Abcam) to avoid excitotoxicity, and NMDAR-mediated currents were recorded 24 h after transfection.

2.4 | Primary Neuronal Cultures and Morphological Analysis of Dendritic Spines

Mouse hippocampal neuron cultures were established from mouse embryos (embryonic day E18) obtained from pregnant CD1 females. Briefly, the hippocampi were isolated and maintained in cold Hank's Balanced Salt Solution (HBSS, Gibco) supplemented with 0.45% glucose (HBSS-Glucose). After carefully removing the meninges, the hippocampi were digested with trypsin for 17 min at 37°C and dissociated. The cells were washed three times in HBSS and resuspended in Neurobasal medium supplemented with 2 mM Glutamax (Gibco) before filtering in 70 μm mesh filters (BD Falcon). The cells were plated onto glass coverslips (5×10^4 cells/cm^2) coated with 0.1 mg/mL poly-L-lysine (Sigma), and medium was substituted by complete growth medium (2 h after seeding) consisting of Neurobasal medium supplemented with 2% B27 (Invitrogen) and 2 mM Glutamax. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere, and half of the conditioned medium was replaced by fresh growth medium every 3–4 days. At day, in vitro 11 (DIV11) primary cultures were transfected with either GFP-GluN2Bwt or mutant GFP-GluN2B variants, using Lipofectamine™ 2000, for further morphological analysis of dendritic spines. This analysis was coupled to the induction of chemical (glycine, Gly) induced long term potentiation protocol (Gly-cLTP), previously described [12]. Briefly, at DIV14 primary hippocampal neurons were placed under basal conditions (non-stimulated), by incubating with Krebs-Ringer's solution supplemented with 1 mM Mg^{2+} and 1 μM TTX. For Gly-cLTP induction, cells were washed in 20 μM bicuculline (BIC) and 20 μM strychnine and incubated for 5 min in 20 μM BIC + 1 μM strychnine and 200 μM Gly (supplemented with 200 μM spermine, for the Gly + Spermine condition). The solutions were replaced by a medium supplemented with 20 μM BIC, 20 μM strychnine, and 1 mM Mg^{2+}. Following a 20 min incubation at 37°C, neurons were fixed with ice-cold 4% PFA and 4% sucrose, permeabilized-blocked, and incubated with rabbit anti-GFP (Life Technologies), then with Alexa 488-conjugated goat anti-rabbit Ab, washed, and mounted for confocal microscopy analysis, using a Nikon Eclipse 80i microscope (63×/1.4 N.A. immersion oil objective). GFP-GluN2B transfected neurons were immunolabeled and Z-stack images were acquired. Dendrites were manually traced with Neuron Studio software (http://research.mssm.edu/cnic/tools-ns.html) for further automatic classification of the spines into morphological categories (thin, mushroom, stubby).

2.5 | Electrophysiological Recordings of NMDAR-Mediated Whole-Cell Currents in HEK-293T Cells

Whole-cell patch-clamp experiments were performed 24 h after transfection, perfusing HEK-293T cells that were continuously perfused with extracellular physiological bath solution containing (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.42 with NaOH. Glutamate 1 mM (Glu, Sigma-Aldrich) and Gly 10 μM (Tocris) were co-applied for a 5 s period of time by piezoelectric translation (P-601.30; Physik Instrumente) of a theta-barrel application tool fabricated from borosilicate glass (1.5 mm o.d.; Sutter Instruments) and the activated currents were recorded in the whole-cell configuration at a holding potential of −60 mV, acquired at 5 kHz and filtered at 2 kHz by means of an Axopatch 200B amplifier, Digidata 1440A interface, and pClamp10 software (Molecular Devices Corporation). Electrodes were made from borosilicate glass (1.5 mm o.d., 0.86 mm i.d., Harvard Apparatus), pulled with a PC-10 vertical puller (Narishige) and displayed resistances of typically 2–4 MΩ when filled with intracellular pipette solution containing (in mM): 140 CsCl, 5 EGTA, 4 Na_2ATP, 0.1 Na_3GTP, and 10 HEPES, adjusted to pH 7.25 with CsOH. Glutamate plus glycine-evoked currents were expressed as current density (−pA/pF; maximum current divided by capacitance measured from the amplifier settings) to circumvent the putative differences derived from distinct cell sizes between

cells. Quantitative paired analysis of spermine or D-serine effects was performed, comparing the normalized peak amplitude of glutamate plus glycine supplemented with 200 μM spermine or 1–10 μM D-serine, respectively, and normalized to the response in the presence of glutamate plus glycine condition.

2.6 | Behavioral Studies

All behavioral tests were performed between 9 a.m. and 1 p.m. (light phase) and mice were habituated to the handling experimenter (5 min/day, five consecutive days) and to the testing room prior to testing. All tests were recorded and analyzed using a video-tracking system (Smart 3.0, Panlab). Spermidine supplementation was initiated after weaning (21-day-old mice) and maintained throughout the experiment. Spermidine was administered in drinking water, consisting of a 3 mM spermidine solution (Sigma-Aldrich) renewed every 2–3 days.

2.6.1 | Open Field Test

Open field arenas (40 cm × 40 cm × 40 cm) were made of gray methacrylate and dimly illuminated during testing (20 lx in the center). General locomotor activity was recorded for 10 min (distance traveled) and time spent in the central zone (20 cm × 20 cm) versus peripheral zone was measured.

2.6.2 | Accelerating Rotating Rod Test

In this task, the mice were placed on the top of a rotating rod. The rotating speed linearly increased from 4 to 40 rpm over 5 min. The trial consisted of three consecutive assays, and the best score (maximum speed reached) was considered as the mark for further analysis.

2.6.3 | Wire Suspension Test

In this task, mice were suspended from their forelimbs into the center of a 3 mm-diameter and 40 cm-long rod. The trial lasted 1 min, with mice left on the rod and the number of falls and times reaching the edge counted. Once the mouse fell or reached the edge, it was repositioned into the center of the rod. The number of falls was counted and subtracted from 10 (i.e., perfect performance = 10), and the score of "reaching" directly indicated the number of times the mouse reached the edges of the rod.

2.6.4 | Novel Object Recognition Test

The mice were habituated to the empty open field arenas (50 cm × 50 cm × 38 cm) for 10 min 1 day before testing. During the acquisition trial, mice were allowed to explore two identical copies of an object for 10 min. The objects were either Lego towers or T25 cell culture flasks filled with wood bedding (5 cm height × 3 cm large × 1 cm width) and were placed 5 cm apart from the arena walls in two adjacent corners. Twenty-four hours after acquisition, the mice were placed in the arena containing one familiar and one novel object in the trained object locations. The position and identity of the novel object were randomly counterbalanced among animals. Object exploration was manually scored during a 10 min trial, considering the exploration time/events only when the mouse's nose or front paws were in direct contact with the object. The preference for the novel object was determined as the ratio between time exploration of the novel object and total exploration time (discrimination index). Mice that did not explore the objects (cut-off: 20 s exploration/10 min trial) were excluded from statistical analysis.

2.7 | Ex Vivo Electrophysiological Recordings

Electrophysiological studies were performed in hippocampal acute slices of 3–4-month-old wildtype and *Grin2b*$^{+/-}$ mice (standard or 3 mM Spermidine-supplemented water), as described in [26]. Briefly, local field excitatory postsynaptic potentials (fEPSP) were measured in the *stratum radiatum* in the CA1 region of hippocampal coronal acute slices ($N = 7$ acute slices/mouse, 5–6 mice/group) after Schaffer collateral stimulation. The baseline was obtained after 30 min of stimulation at 0.03 Hz. Long-term potentiation (LTP) was induced by 10 theta bursts of 100 Hz (four pulses) with an interstimulus interval of 200 ms repeated seven times at 0.03 Hz, followed by fEPSPs recording for 60 min at 0.03 Hz. Paired-pulse fEPSPs were measured before baseline recordings with an interstimulus interval of 50 ms. All recordings were amplified and stored using an AxoClamp 2B amplifier and analyzed using Axon pClamp software and GraphPad statistical package.

2.8 | Patients

In this case study, two patients with de novo *GRIN2B* LoF variants (identified by whole exome sequencing and functionally annotated) [27] were referred to the Hospital Sant Joan de Déu (Barcelona, Spain) for clinical evaluation and management. All legal guardians provided informed written consent for functional testing of *GRIN* variants and for investigational clinical evaluation of spermidine treatment conducted along this proof-of-concept clinical study. All studies have been performed in accordance with the Declaration of Helsinki, and the protocol was approved by the local Ethics Committee (PIC-158-21). Prior to spermidine treatment, both patients received—among other treatments—L-serine supplementation (500 mg/kg/day) for 18 months, which was discontinued 1 month before spermidine treatment.

2.9 | Procedures

Study duration per participant was 18 months total. Spermidine was orally administered at a daily dose of 3 mg, divided into two doses of spermidine powder (1.5 mg after breakfast and 1.5 mg after the afternoon snack, around 5 p.m.) and mixed with food and/or drinks. Spermidine was provided through Spermidine-rich wheat germ extract. The spermidine-rich wheat germ extract was authorized as a novel food by the European Union's (EU's) Novel Foods Regulation (EC). The proposed dosage was based on previous studies, below the calculated no observed adverse effects level (Spermidine NOAEL for humans: 13.5 mg/kg bw/day) [28] and following EU regulatory concerning this supplement (https://eur-lex.

europa.eu/legal-content/ES/TXT/?uri=uriserv:OJ.L_.2020.092.01.0007.01.SPA&toc=OJ:L:2020:092:TOC).

2.10 | Follow-Up Protocol: Outcome Measures and Evaluations

Outcome measures included safety and tolerability of oral spermidine treatment, as well as efficacy.

2.10.1 | Tolerability

A first objective of the clinical investigational study was to evaluate the potential side effects of spermidine treatment in children. Despite being a metabolic product and EMA-approved food supply in adults, spermidine treatment in the paediatric population has not been reported yet. Therefore, a major goal of the clinical investigational study was to assess the safety and tolerability of spermidine treatment along the 18-month treatment protocol. The surveillance was performed by the referred neuropaediatrician, applying follow-up procedures consisting of physical examinations, including anthropometric parameters and a complete neurological examination before (V_0) and after 3 (V_1) and 18 months (V_2) spermidine treatment. Furthermore, safety and tolerability were assessed based on adverse events (AEs) and serious AEs (SAEs) collection.

2.10.2 | Efficacy

The evaluation of efficacy was conducted using the Vineland Adaptive Behavior Scale—second edition (VABS-II) in patient 1 and patient 2. VABS-II semi-structured interviews [29] were conducted by a trained neuropsychologist before (V_0, baseline, prior to spermidine treatment initiation), and following 3 and 18 months (V_1 and V_2, respectively) after initiating spermidine treatment. Standardized scores (SS) for VABS-II domains and for the Adaptive Behavior Composite (ABC) (mean = 100 and standard deviation [SD] = 15; higher scores indicating better adaptive function), as well as V-Scale Scores for each subdomain (mean = 15, SD = 3) were summarized and assessed for changes occurring from baseline to 18 months after starting spermidine treatment.

2.11 | Statistical Analysis

Comparison between experimental groups was evaluated using Prism9 (GraphPad Software Inc.), applying a two-tailed Student's *t* test or One-Way Analysis of Variance (ANOVA) test followed by Dunnett's post hoc tests on data with normal distribution. The statistical analysis of data with no normal distribution was performed using the nonparametric two-tailed Mann–Whitney test.

3 | Results

3.1 | Spermine Potentiates Currents of Hypofunctional NMDA Receptors

Spermine has been reported as a positive allosteric modulator (PAM) of GluN2B subunit-containing NMDAR [30]. Accordingly, spermine could potentially represent a new therapeutic arm for GRIN-LoF hypofunctionality, functionally mimicking and/or increasing D-serine-mediated potentiation of the NMDARs, the current treatment for GRIN-LoF [11, 12, 23, 31]. To address this hypothesis, NMDAR-mediated currents were assessed in HEK-293T cells heterologously expressing wildtype GluN1-GluN2B diheteromeric receptors or the well-reported (GluN1wt/GluN2B-P553T) mutant diheteromer, a paradigmatic *GRIN* missense variant with a dominant negative effect provoking a decrease in NMDAR activity [12]. Although the pathophysiological mechanisms of this missense *GRIN2B* LoF variant are different from those of *GRIN2B* null variants (truncating variants GRIN2B p.(Arg926Ter)(NM_000834.3:c.2776C>T) and GRIN2B p.(Lys670ValfsTer5)(NM_000834.3:c.2010 + 1G>A), harbored in the patients enrolled in this study) or *GRIN2B* haploinsufficiency (e.g., *grin2b*$^{+/-}$ heterozygous mice employed in this study), they all result in a decrease in NMDAR-mediated currents and were therefore used to test spermine-mediated potentiation of hypofunctional NMDARs along in vitro studies. Whole cell patch-clamp experiments were performed, simultaneously applying the coagonists (1 mM glutamate and 1 μM glycine) in the absence or in the presence of either D-serine (1–10 μM; $EC_{50} \approx 0.65$ μM) or spermine (200 μM; $EC_{50} \approx 150$ μM). Paired analysis of the normalized current density showed a significantly stronger potentiation of NMDAR-mediated currents in the presence of 200 μM spermine, compared to the application of saturating (10 μM) D-serine, both in wildtype- and in LoF-NMDAR-expressing HEK-293T cells (Figure 1A,B).

The boosting effect of spermine on the NMDAR was also consistently detected in GluN2B-LoF subunit-containing NMDARs. To this end, the spermine effect was assessed in HEK-293T cells expressing different patient-associated *de novo GRIN2B* LoF genetic variant subtypes (missense, nonsense), scattered along the different domains of the NMDAR. Similarly to the observed effects in GluN2B-P553T-containing NMDARs, co-administration of 1 mM glutamate and 10 μM glycine with 200 μM spermine resulted in the potentiation of NMDAR-mediated currents in HEK-293T cells expressing different reported GRD *de novo* missense GRIN2B and GRIN1 dominant negative variants. These experiments were conducted under heterozygous (monoallelic) conditions, mimicking the *GRIN* allelic scenario in GRD individuals harboring dominant negative *GRIN* missense mutations (Figure S1). In summary, we found that 200 μM spermine enhances NMDAR-mediated currents to a greater extent than L-Serine, with a potentiation observed in cell lines expressing either wildtype or different hypofunctional GluN2B or GluN1-subunit-containing NMDARs.

3.2 | Spermine Administration Ameliorates Synaptic Density Deficits in GRIN2B-LoF Neurons

After demonstrating spermine's ability to potentiate NMDAR currents in cell lines, its putative ability to rescue the dendritic spine disturbance in neurons expressing the GRIN2B loss-of-function genetic variant GluN2B-P553T was assessed. Thus, hippocampal primary neurons transfected with either GFP-GluN2B wildtype or GFP-GluN2B-P553T (LoF variant) [12] were acutely stimulated with the administration of 200 μM glycine (glycine-induced chemical long-term potentiation paradigm, Gly-cLTP), in the absence or in combination with 200 μM spermine. Based on previous findings showing the presence of abnormal spine

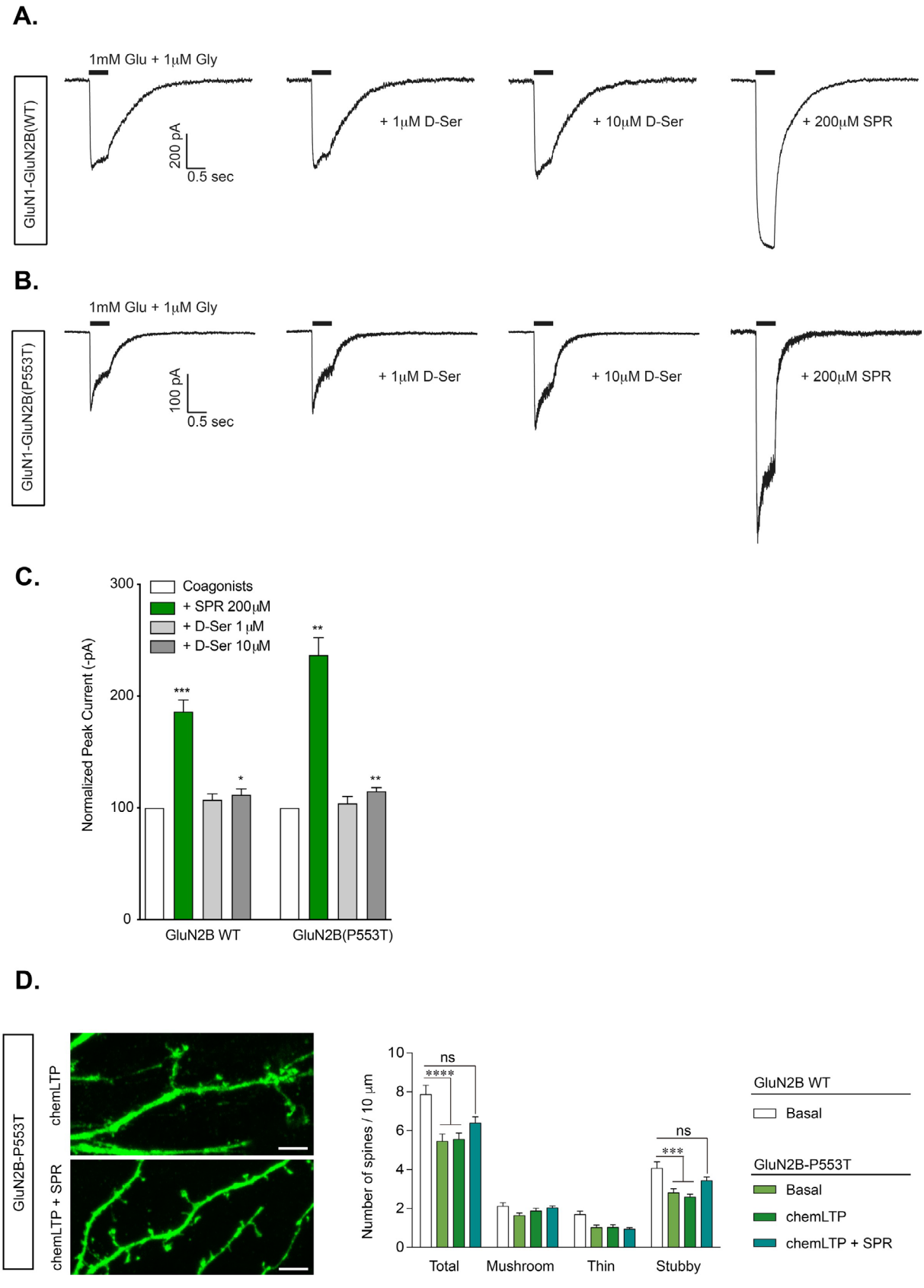

FIGURE 1 | Legend on next page.

density in primary neurons expressing the GRIN2B-LoF variant (i.e., GRIN2B-P553T) [12], immunofluorescent analysis was conducted in GFP-GluN2B (wt- or LoF-GRIN2B) transfected primary neurons.

Immunolabeling of GFP–GluN2B transfected neurons was used to visualize spines in secondary and tertiary dendrites (Figure 1D) and to quantify spine density and morphology (bar graph). Under basal conditions, compared with GFP–GluN2Bwt expressing neurons, the expression of GluN2B-P553T resulted in the reduction of dendritic spine density, mostly associated with a significant reduction of stubby spines, while mushroom and thin-type spines were unaffected. Further, cLTP induction did not elicit a significant increase in spine density in GRIN2B-LoF expressing neurons. Importantly, the lack of responsiveness to cLTP induction was rescued by co-application of 200 μM spermine in the presence of glycine, indicating the boosting effect of spermine administration in a neuronal context, enabling the increase of spine density (Figure 1C). Similar findings were observed in primary neurons transiently transfected with patient-associated GRIN2B-LoF variants GRIN2B p.(K1293del) and GRIN2B p.(R519Ter) (data not shown). Overall, in vitro findings supported the use of spermine toward the potentiation of GRIN2B-LoF and suggested that this strategy could mitigate the clinical symptoms of GRIN2B-LoF patients. Indeed, previous studies showed the safety, biodistribution, and metabolization of spermidine into spermine. Therefore, we hypothesized that spermidine treatment of spermidine will result in its metabolization, increasing spermine levels ultimately enhancing the NMDAR function in preclinical GRD models (*Grin2b*$^{+/-}$ mouse model of GRIN2B-LoF) and, more importantly, in GRIN2B-LoF patients.

3.3 | Spermidine Diet Partially Rescues Phenotypic Alterations in Grin2b$^{+/-}$ Mice

To investigate the putative spermidine-mediated potentiation of hypofunctional NMDAR in vivo, the *Grin2b*$^{+/-}$ mouse model [24] was selected. This constitutive *Grin2b*$^{+/-}$ haploinsufficient mouse model was initially generated to elucidate GluN2B subunit (and NMDAR) roles in synaptic transmission and plasticity mechanisms. The initial behavioral characterization showed an enhanced pre-pulse inhibition and an altered startle response indicative of sensorimotor gating deficits [32, 33]. In the present study, young adult *Grin2b*$^{+/-}$ mice (female and male) were submitted to a behavioral phenotyping battery (e.g., motor, anxiety, social and cognitive dimensions) toward the identification of putative GRD-like endophenotypes. Since no sex differences have been described in GRD individuals, and to identify robust sex-independent GRD-like endophenotypes, exclusively phenotypic alterations exhibited both in male and female *Grin2b*$^{+/-}$ mice were selected as behavioral readouts for further evaluation of putative spermidine treatment efficacy. Methodologically, after weaning, mice were separated by sex, genotyped, and housed in a genotype-independent manner (e.g., wildtype and heterozygous mice co-housed, $N = 4$ mice/cage). Mice were split into two drinking water regimens, consisting of either standard or 3 mM spermidine-supplemented water (a dosage based on previous reports showing tolerability and lack of side effects) [18, 34, 35] starting 6 weeks prior to behavioral and electrophysiological phenotyping. Water consumption was monitored along treatment, showing no significant differences (standard vs. spermidine-supplemented water).

Behavioral studies showed that spontaneous motor activity in the open field test was significantly reduced in female *Grin2b*$^{+/-}$ mice (35.6 ± 1.4 m in wildtype vs. 24.6 ± 6.2 m in *Grin2b*$^{+/-}$ mice; $p < 0.0001$, Student's *t* test) and decreased in male *Grin2b*$^{+/-}$(30.2 ± 1.4 m in wildtype vs. 27.6 ± 1.7 m in *Grin2b*$^{+/-}$ mice; $p = 0.25$, Student's *t* test). Motor hypoactivity detected in *Grin2b*$^{+/-}$ mice was accompanied by motor coordination impairment, as observed in the wire suspension test and in the accelerating rotarod task performance. In the wire suspension test, *Grin2b*$^{+/-}$ mice exhibited a reduced ability to hold on to the thin wire during the 60-s trial (holding score: 9.2 ± 0.3 in wildtype vs. 7.0 ± 0.5 in *Grin2b*$^{+/-}$ mice; $p = 0.002$, Mann–Whitney test; Figure 2A). In the accelerating rotarod test, heterozygous mice fell at slower rotating speeds (15.3 ± 1.0 rpm in wildtype vs. 12.0 ± 0.7 rpm in *Grin2b*$^{+/-}$ mice; $p = 0.0004$, Student's *t* test, Figure 2B). Together with the motor phenotypic abnormalities, a mild cognitive phenotype was detected in the novel object recognition test, with a reduction of the preference to visit the novel object (discrimination index, DI = 0.68 ± 0.02 in wildtype vs. 0.55 ± 0.02 in *Grin2b*$^{+/-}$ mice; $p < 0.0001$, Student's *t* test, Figure 2C). The phenotypic defects observed in *Grin2b*$^{+/-}$ mice were rescued by 6 weeks of spermidine treatment, with no changes observed in treated wildtype mice. More precisely, the motor coordination alterations were abrogated in treated *Grin2b*$^{+/-}$ mice, as shown in the wire suspension test performance (holding score: 9.2 ± 0.3 vs. 8.6 ± 0.3 in wildtype and *Grin2b*$^{+/-}$ treated mice, respectively; $p = 0.13$, Mann–Whitney test; Figure 2A) and in the speed at which they fell in the accelerating rotarod test (15.3 ± 1.0 rpm in wildtype vs. 14.0 ± 0.7 rpm in *Grin2b*$^{+/-}$ mice; $F_{(3,92)} = 3.608$; $p = 0.016$; Tukey's *post hoc* analysis indicated no significant differences ($p < 0.05$) between wildtype and Grin2b$^{+/-}$ treated mice; Figure 2B).

FIGURE 1 | Functional characterization of spermine potentiation of GluN2B subunit-containing NMDARs. (A) Representative whole-cell currents evoked by rapid application (0.5 s duration; −60 mV) of 1 mM glutamate plus 1 μM glycine ±1–10 μM D-serine or ± 200 μM spermine in HEK-293T cells expressing wildtype GluN1-GluN2B (top) or GluN1-GluN2B(P5533) LoF variant (bottom). (B) Bar graph representing Spermine- and D-serine-mediated potentiation of NMDAR currents (% with respect to current density upon coagonists application) ± SEM in HEK-293T cells expressing wildtype GluN1-GluN2B or GluN1-GluN2B(P5533) LoF variant (*, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001; two-way ANOVA + Bonferroni post hoc test). (C) *Left*, Representative images of immunofluorescence analysis of murine primary hippocampal neurons transfected with GFP-GluN2B (P553T) construct. Scale bar = 3 μm. *Right*, Bar graphs representing the quantification of spine density and morphology in neurons at basal conditions and after chemical LTP induction alone and with additional 200 μM spermine (green bars, GluN2B variant) comparing with the wild-type (empty bars) (> 20 dendrites per condition; ns, *p*-value > 0.05; *, *p*-value < 0.05; **, *p*-value < 0.01; ***, *p*-value < 0.001; ****, *p*-value < 0.0001; two-way ANOVA + Bonferroni post hoc test).

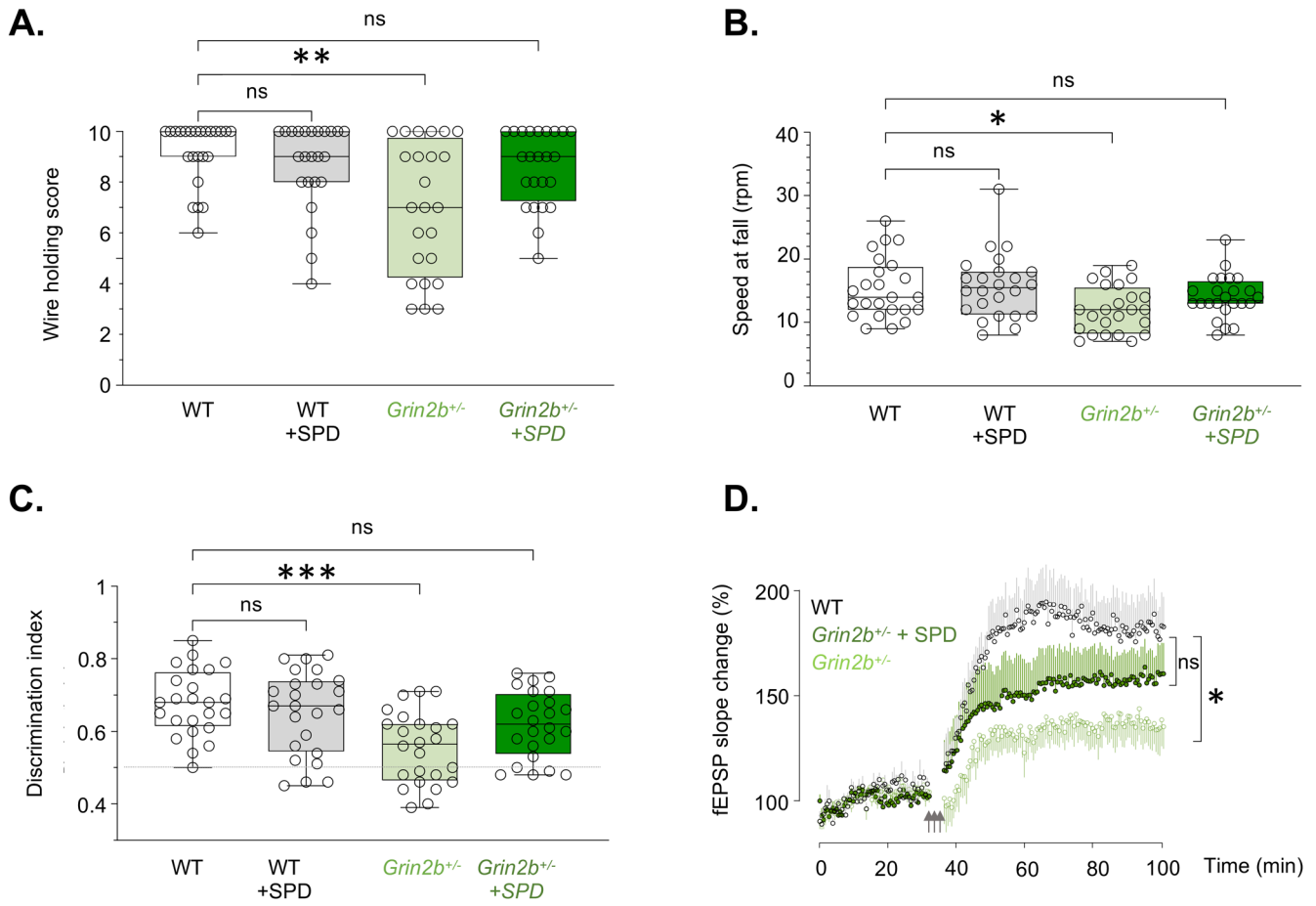

FIGURE 2 | Assessment of spermidine-mediated attenuation of *Grin2b*$^{+/-}$-mouse model phenotypic alterations. (A) Box and whiskers plot of the score in the wire suspension test performance (discrete values represented for each group: WT, empty boxes; WT + SPD, gray boxes; *Grin2b*$^{+/-}$, light green boxes; *Grin2b*$^{+/-}$ + SPD, dark green boxes; $N = 12$ male +12 female mice/group). (B) Box and whiskers plot of the speed at fall (rpm) of mice placed in the accelerating rotarod test, starting at 4 rpm and with a linearly accelerating speed reaching 40 rpm maximum rotating speed. (C) Box and whiskers plot of the discrimination index values for mice in the novel object recognition task. Each point represents the ratio (preference) between the time exploring the novel object and the total exploration time. (D) Time-course LTP after theta-burst stimulation, showing fEPSP slope changes in acute hippocampal slices of 3–5 months-old wildtype (black), *Grin2b*$^{+/-}$ (light green); and *Grin2b*$^{+/-}$ + 3 mM spermidine (dark green) treated mice ($N = 4$–5 mice/group, 6–7 LTP recordings). Black arrow shows theta-burst stimulation. Statistical analysis of LTP induction differences between groups was performed by measuring the change in fEPSP slope values in the stable phase ($t = 60$–100 min time-frame); (ns, p-value > 0.05; ****, p-value < 0.0001).

Importantly, the cognitive deficits associated with *Grin2b*$^{+/-}$ haploinsufficiency were also rescued in heterozygous mice that received chronic spermidine treatment, as observed in the novel object recognition task performance (DI = 0.68 ± 0.02 in wildtype vs. 0.62 ± 0.02 in *Grin2b*$^{+/-}$ treated mice; $F_{(3,92)} = 7.089$; $p = 0.0002$; Dunnet's *post hoc* analysis indicated no significant differences ($p = 0.099$) between wildtype and G*rin2b*$^{+/-}$ treated mice; Figure 2C).

To correlate the hippocampal-dependent behavioral task improvement with synaptic plasticity processes, electrophysiological studies were conducted in acute hippocampal slices dissected from the same mice cohort (1–4 weeks after performing the battery of behavioral phenotyping tests) maintained under vehicle or spermidine supplementation. Field potential recording of Schaffer collateral connections in ex vivo hippocampal slices showed that the LTP deficits observed in non-treated *Grin2b*$^{+/-}$ mice (176.3 ± 14.6 for wildtype mice vs. 140.4 ± 8.3 for *Grin2b*$^{+/-}$ mice with vehicle; $p < 0.0001$, Student's t test, Figure 2D) were rescued in *Grin2b*$^{+/-}$ mice chronically treated with spermidine (176.3 ± 14.6 for wildtype mice vs. 156.1 ± 18.5 for *Grin2b*$^{+/-}$ mice with spermidine; $p = 0.29$, Mann–Whitney test Figure 2D). Overall, the phenotypic assessment of *Grin2b*$^{+/-}$ mice showed the beneficial impact of spermidine treatment for in vivo rescue in heterozygous *Grin2b*$^{+/-}$ mice, supporting further use of this intervention in GRIN2B-LoF individuals.

3.4 | Clinical Studies

3.4.1 | Patients Description

The proof-of-concept clinical study consisted of 3 mg spermidine daily supplementation in two *GRIN2B* patients (girls) aged 15.3 and 5.7 years old. The patients included in the study were functionally stratified as GRIN2B-LoF patients since they are harboring GRIN2B p.(Arg926Ter)(NM_000834.3:c.2776C>T) and GRIN2B p.(Lys670ValfsTer5) (NM_000834.3:c.2010 + 1G>A) protein

TABLE 1 | Clinical assessment of GRIN2B-LoF patients participating in the proof-of-concept clinical study.

	Patient 1	Patient 2
Age (years)	16.2	6.7
Sex	Female	Female
Nucleotide variant	GRIN2B (NM_000834.3:c.2776C>T)	GRIN2B (NM_000834.3:c.2010 + 1G>A)
Protein variant	p.Arg926Ter	p.Lys670ValfsTer5
Age at diagnosis (years)	13.6	3.4
Developmental milestones acquisition (months, m)	Sitting at 13 m, walking at 30 m, first words at 24 m	Sitting at 24 m, walking at 30 m, first words at 36 m
Intellectual disability	Mild	Moderate
Expressive language deficit	Mild impairment	Limited output
Epilepsy	No	No clinical seizures, only EEG abnormalities (multifocal epileptiform discharges)
Motor function	Hypotonia first years of life. Gross motor abilities: GMFM-88 score = 100%; normal fine motor abilities	Mild hypotonia. Gross motor abilities: Mild limitations (balance and coordination impairment). Fine motor abilities: limited performance of manual tasks requiring speed and accuracy
Movement disorders	No	Yes. Mild stereotypic movements
Neurobehavioral disorders	Yes, autistic traits, impulsivity, attention deficits and mild hyperactivity	Yes, autistic traits, impulsivity, attention deficits and mild hyperactivity

Note: Clinical assessment of GRIN2B-LoF patients participating in the proof-of-concept clinical study. The table summarizes the neurological symptoms associated with the presence of *de novo GRIN2B* variants, prior to spermidine treatment.
Abbreviations: EEG, electroencephalogram; GMFM-88, gross motor function measure-88.

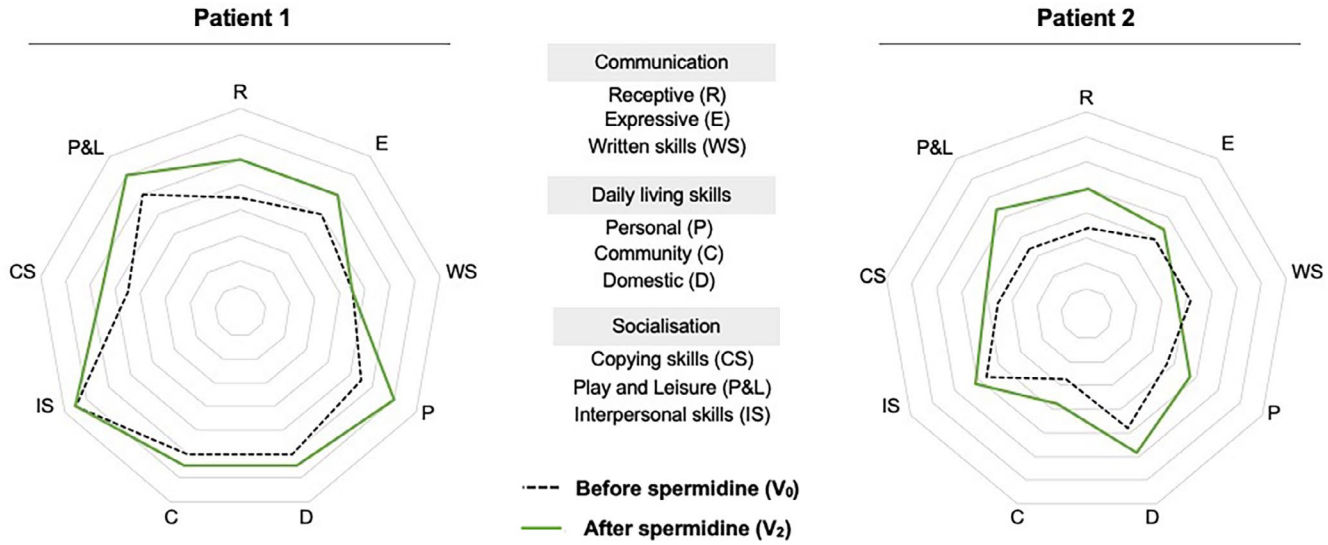

FIGURE 3 | Neuropsychological assessment of GRIN2B-LoF children before and after 18-month spermidine treatment. Spider graphs representing VABS-II subdomains before (baseline, V_0) and after 1.5 years of spermidine treatment (V_2). *Left*, Patient 1 subdomains V-Scale Scores, before treatment (red dotted lines) and after 18 months of spermidine treatment (red solid lines). *Right*, Patient 2 subdomains V-Scale Scores before treatment (green dotted lines) and after 18 months of spermidine treatment (green solid lines). Concentric radial increase corresponds to 2 score units of V-Scale (outer limit = 16).

truncating variants which, according to previous studies, invariably trigger GRIN2B-LoF [27]. The functional stratification was in accordance with the exhibited clinical symptoms, comprising mild–moderate intellectual disability, expressive language deficit, and behavioral disorders including autistic traits, impulsivity, inattention, and hyperactivity. The clinical history of both patients was

absent of seizure episodes, although Patient 2 presented an abnormal electroencephalogram pattern. The demographic, genetic, and clinical data of both patients are presented in Table 1.

3.4.2 | Assessment of Spermidine Safety, Tolerability, and Clinical Efficacy

Guardians and caregivers were compliant with the two times daily Spermidine administration protocol. Physical examination findings were considered AEs if, among others, they were associated with accompanying symptoms, requiring additional diagnostic testing or medical/surgical intervention, leading to a change in study nutraceutical dosing or discontinuation from the study and/or to be considered as an AE by the investigator. The follow-up showed the absence of side effects or serious adverse events along the 18-month treatment.

Adaptive behavior assessments by means of the VABS-II test showed that, despite inter-individual variability, both patients improved following 18-month spermidine treatment in the ABC, as shown by an increase in the standard scores (SS), as well as in several domains and subdomains (Figure 3, Supp. Table 1). The increase in ABC standard scores was observed for both patients, with an increase from 73 to 80 for patient 1 (P1) and from 53 to 66 for patient 2 (P2). The improvement was observed in both the Communication Domain, with an SS increase from 68 to 74 for patient 1 and from 54 to 70 for patient 2, and in the Daily Living Skills domain, where there was an SS increase from 79 to 91 for P1 (accompanied by increased socialization, from 80 to 87) and from 48 to 68 for P2. Upon analyzing the subdomains, V-scale Scores increased in almost all the subdomains for both patients. Data for all VABS-II subdomains, domains, and composite SS are reported in Figure 3 and Table S1. Besides the neuropsychological evaluation, the families of the two patients reported behavioral improvement. Parents reported an increase in children's attention to the environment, an improvement in social communicative interaction, and a more pronounced sense of humor and laughing in funny situations. Further, for P1, an important improvement was noticed in daily domestic autonomy (i.e., starting to go alone to school by train).

4 | Discussion

This study aimed to evaluate the potential therapeutic benefit of spermidine for the treatment of GRD caused by the presence of pathogenic GRIN2B-LoF variants. In cellular models, spermine administration showed a strong potentiating effect, boosting wild-type and hypofunctional GluN1/GluN2B-mediated currents and mitigating the associated synaptic alterations. Further, chronic administration of spermidine (spermine precursor) in the constitutive *Grin2b*$^{+/-}$ haploinsufficient (LoF) adult mouse model of GRD revealed spermidine-mediated attenuation of motor and cognitive GRD-like endophenotypes. Importantly, these findings were translated into a clinical investigation in two pediatric patients with GRIN2B-LoF variants. In these patients, chronic spermidine treatment showed tolerability and remarkable efficacy in improving communication, socialization, and daily living skills.

Despite being a therapeutic target convergent from a plethora of neurological disorders (Alzheimer's disease, schizophrenia, depression, epilepsy), to date, therapies for GRD are unfortunately very scarce. Current strategies, still under clinical investigation, are facing the difficulty of treating patients with a clinical spectrum of heterogeneous genetic (*GRIN* affected gene) and functional alterations (LoF vs. GoF). Accordingly, precision medicine is compulsory in the frame of GRD, and current pharmacological strategies are pursuing the identification of compounds modulating (enhancing or attenuating) overall NMDAR activity. In the context of *GRIN* variants producing a LoF of the NMDAR (caused by null or missense variants), the use of treatments potentiating NMDAR activity (wildtype and/or mutant) has been proposed. Importantly, a growing number of clinical studies have shown the therapeutic benefit of L-serine (metabolic precursor of D-serine, the natural NMDAR coagonist) for GRIN-LoF individuals [11, 12, 23, 31]. Despite its efficacy, additional therapeutic agents with higher potency, bioavailability, limited cost, and specificity are still required. The use of NMDAR positive allosteric modulators (PAM) is of special interest since, rather than activating per se the signalling process, their effects are theoretically restrained to strictly potentiate neurotransmitter-activated receptors. Spermine has been widely reported as an NMDAR PAM [30].

In our study, we have shown that, beyond the previously reported positive modulatory effect of spermine on wildtype NMDARs, in cells co-expressing hypofunctional and wildtype NMDARs, the PAM effect of spermine was preserved. This result suggests that, in the context of hypofunctional NMDARs (frequently exhibiting residual NMDAR-mediated currents, compared to NMDAR wildtype-driven currents), most of the PAM and coagonist efficacy must be ascribed to a potentiating effect on the predominantly active NMDAR that contains the wildtype subunits. Corollary, the observation of PAM effects exclusively on hypofunctional NMDAR is informative of PAM-NMDAR interaction [14], but in terms of therapeutic efficacy, should be placed in the heterozygous context to avoid missing the PAM overall net effect on NMDAR sub-populations. The study has been conducted on discrete preclinical and clinical GRIN2B-LoF variants, together with the evaluation of spermine efficacy in mutant *GRIN1* variants using cellular models. Despite the results showing the efficacy of spermine-mediated potentiation on all the hypofunctional NMDARs evaluated, the potential use of spermidine as a universal potentiator for the ensemble of GRIN2B LoF variants and the potential benefit for variants affecting other *GRIN* genes remains unsolved. Regarding the former, it is worth considering the autosomal dominant inheritance pattern, resulting in the presence of both wildtype and mutant GluN2B subunits. Therefore, independently of spermine effect on mutant GluN2B-LoF containing NMDARs, the pool of NMDARs containing the GluN2B wildtype subunit will be potentiated by spermine, as shown in the present work and, previously, by other teams [17, 30]. Regarding the potential interference of missense variants with spermine-binding to mutant NMDARs, structural studies showed that the spermine binding site is located within the amino terminal domain of the NMDAR. Genetic studies showed that this extracellular domain is largely resilient to genetic variability [9], and thus *GRIN2B* disease-associated variants are not likely to interfere with spermine-binding. Together with *GRIN2B*, *GRIN1* and *GRIN2A* genes are the most prevalently mutated genes

associated with GRD [9], and whether they might benefit from spermidine treatment remains unsolved. In vitro studies have described spermine specificity for GluN2B subunit-containing NMDARs, although at low glycine and/or Mg^{2+} concentrations the subunit-specificity is mitigated and the potentiation effect can be exacerbated or blocked [17, 30].

Along this work, the phenotypic assessment of the *Grin2b*$^{+/-}$ mouse model of GRIN2B-LoF has been performed, revealing the presence of GRD-like endophenotypes (e.g., motor defects, learning and memory alterations) that can be used as readouts to assess the benefit of potential GRD therapies. Currently, in the context of GRD research, extensive efforts are focused on generating and characterizing novel knockin (KI) mouse models harboring missense *GRIN* variants. Besides the relevant KI models, revisiting and deeply characterizing conventional *Grin1*, *Grin2a*, and *Grin2b* knockdown (KD) mouse models created three decades ago [24, 32, 36] can provide important insights to unveil GRD pathophysiology associated with LoF and to evaluate the efficacy of therapeutic strategies. In this line, a pioneer recent study described the presence of GRD-like phenotypes in the Grin1-knockdown mouse model and, importantly, a partial rescue throughout *Grin1* gene dosage restoration in adult *Grin1* knockdown mice [37]. In relation to the *Grin2b*$^{+/-}$ mouse model, its previous characterization showed diminished NMDAR-mediated excitatory postsynaptic currents (EPSCs) and long-term potentiation (LTP) alterations, together with sensorimotor gating alterations [32, 33]. In this study, we have identified additional behavioral GRD-like phenotypes, establishing this genetic GRIN2B-LoF model for future preclinical efforts to comprehensively study GRIN2B-LoF pathophysiology and to evaluate the potential efficacy of novel treatments. In relation to spermidine-mediated effects in vivo, several aspects need to be weighed. In addition to spermine biodistribution and concentration (resulting from metabolic activity, blood brain barrier permeability, stability and kinetics), the spatiotemporal expression pattern of *GRIN* genes [7] and also the intracellular effects of polyamines (i.e., AMPA receptors, autophagy processes) [38] might have a contribution to the overall improvement, and further studies need to be performed. Despite this wide range of pharmacological scenarios that cannot be addressed yet, the physiological findings obtained in the *Grin2b*$^{+/-}$ mouse model and in the clinical study indicate that the overall effect of spermidine is beneficial for GRIN2B-LoF treatment at different developmental stages. Further efforts in animal models (e.g., constitutive *Grin1* knockdown, *Grin2a*$^{+/-}$ models) following similar experimental approaches might help to elucidate the therapeutic interest of spermidine in GRIN1-LoF and/or GRIN2A-LoF individuals.

Regarding GRD therapeutic window, it is worth mentioning that GRD are developmental encephalopathies, and thus early therapeutic interventions should ideally be established, while the therapeutic window remains poorly explored. Genetic testing has been tremendously accelerated, and early intervention is becoming a reality for this and other neurogenetic disorders. Nevertheless, GRD therapeutic strategies are still scarce, and the age range of diagnosed and reported GRD is wide, raising the question about the efficacy of treatments at different developmental stages. Previous findings obtained in GRD genetic adult mouse models ([37], this study) support the possibility of treating adult mouse models of GRD with an improvement of phenotypic alterations.

Despite the reduced sample size of our clinical study, the ameliorations observed after 18-month spermidine treatment in developing children of different ages suggest that the therapeutic window for GRD is wide. Importantly, both patients showed spermidine tolerance and the lack of side effects along the treatment, together with a clinical improvement in both patients, still continuing spermidine treatment. The interindividual differences in the improved domains and subdomains might be explained by different factors, including genetic determinants, developmental stages, and neuroplasticity. In relation to the genetic factor, the patients harbor different GRIN2B-LoF variants differently affecting the NMDAR biophysical properties, dictating the severity of the clinical symptoms and the responsiveness to spermidine treatment. Regarding the developmental dimension, the pharmacological response (spermine bioavailability in the brain and pharmacodynamics) might differ between patients, while it is important to consider the spatiotemporal expression pattern of *GRIN* genes. Indeed, spermine is preferentially acting as a positive allosteric modulator of GluN2B subunit-containing NMDARs [30], which are predominant in the early developmental stages of neurotypical individuals (patient 2) [7]. With the GluN2B to GluN2A switch occurring during development, the concomitant decreasing GluN2B:GluN2A ratio results in a change in the NMDAR pools composition (reducing GluN2B relative expression) [39], which might attenuate the spermine effect in later developmental stages (patient 2). Also, since GRD are neurodevelopmental disorders, the therapeutic intervention at early developmental stages (as for P2, compared to P1) can improve treatment efficacy. In that sense, an early genetic diagnosis and functional stratification will likely be crucial to improve the efficacy of the current and future treatments. Finally, besides the potential differences between patients due to pharmacodynamics modulation by age, it must be noted that the spermidine dose was identical for both patients (not adjusted to weight), likely resulting in a higher spermine concentration in the younger patient (P2). Finally, the identification of the therapeutic benefit of spermidine treatment in GRIN2B LoF individuals, and the previously reported benefit of L-serine supplementation opens the possibility to explore a putative synergistic effect of both compounds in future studies.

In summary, despite a more extensive study including a large cohort of GRD patients being necessary, this work presented spermidine treatment as a new promising therapeutic arm for GRIN2B-LoF patients. Further studies should evaluate whether spermidine indications could be enlarged to GRIN-LoF and, beyond GRD, expanded to patients with NDD associated with glutamatergic synapse hypofunctionality.

Author Contributions

M.M., M.O., À.G.-C., and X.A. conceived and designed the study. A.S.-G., N.J.-P., A.R.-B., R.M.-V., F.M.-C., and D.S. participated in the acquisition and analysis of data. D.S., M.M., M.O., À.G.-C., N.J.-P., and X.A. participated in drafting a significant portion of the manuscript or figures. X.A. is the guarantor of the article.

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Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000, and the protocol was approved by the local Ethics Committee (PIC-158-21). Informed consent was obtained from all patients for being included in the study. All legal guardians provided informed written consent for functional testing of *GRIN* variants and for investigational clinical evaluation of spermidine treatment conducted along this proof-of-concept clinical study. All institutional and national guidelines for the care and use of laboratory animals were followed. All experimental procedures with laboratory animals followed the European Union guidelines (Directive 2010/63/EU) and were approved by the Ethics Committee of the University of Barcelona (Procedure No. 84/20).

Conflicts of Interest

À.G.-C. has received honoraria for research support and lectures from PTC Therapeutics, honoraria for lectures from Biomarin, Immedica, and Recordati Rare Diseases Foundation, and is a co-founder of the Hospital Sant Joan de Déu start-up 'Neuroprotect Life Sciences'.

Data Availability Statement

The data presented in this study are available to other investigators for the purpose of replication and re-use.

References

1. L. Volk, S.-L. Chiu, K. Sharma, and R. L. Huganir, "Glutamate Synapses in Human Cognitive Disorders," *Annual Review of Neuroscience* 38 (2015): 127–149.

2. J. R. Lemke, "Predicting Incidences of Neurodevelopmental Disorders," *Brain* 143 (2020): 1046–1048.

3. J. R. Lemke, K. Geider, K. L. Helbig, et al., "Delineating the GRIN1 Phenotypic Spectrum: A Distinct Genetic NMDA Receptor Encephalopathy," *Neurology* 86 (2016): 2171–2178.

4. K. Platzer, H. Yuan, H. Schütz, et al., "GRIN2B Encephalopathy: Novel Findings on Phenotype, Variant Clustering, Functional Consequences and Treatment Aspects," *Journal of Medical Genetics* 54 (2017): 460–470.

5. V. Strehlow, H. O. Heyne, D. R. M. Vlaskamp, et al., "GRIN2A-Related Disorders: Genotype and Functional Consequence Predict Phenotype," *Brain* 142 (2019): 80–92.

6. K. B. Hansen, L. P. Wollmuth, D. Bowie, et al., "Structure, Function, and Pharmacology of Glutamate Receptor Ion Channels," *Pharmacological Reviews* 73 (2021): 298–487.

7. P. Paoletti, C. Bellone, and Q. Zhou, "NMDA Receptor Subunit Diversity: Impact on Receptor Properties, Synaptic Plasticity and Disease," *Nature Reviews. Neuroscience* 14 (2013): 383–400.

8. T. A. Benke, K. Park, I. Krey, et al., "Clinical and Therapeutic Significance of Genetic Variation in the GRIN Gene Family Encoding NMDARs," *Neuropharmacology* 199 (2021): 108805, https://doi.org/10.1016/j.neuropharm.2021.108805.

9. A. García Recio, A. Santos-Gómez, D. Soto, et al., "GRIN Database: A Unified and Manually Curated Repertoire of GRIN Variants," *Human Mutation* 42 (2020): 8–18.

10. A. Santos-Gómez, F. Miguez-Cabello, N. Juliá-Palacios, et al., "Paradigmatic De Novo GRIN1 Variants Recapitulate Pathophysiological Mechanisms Underlying GRIN1-Related Disorder Clinical Spectrum," *International Journal of Molecular Sciences* 22 (2021): 12656, https://doi.org/10.3390/ijms222312656.

11. B. den Hollander, A. R. J. Veenvliet, M. Rothuizen-Lindenschot, et al., "Evidence for Effect of l-Serine, a Novel Therapy for GRIN2B-Related Neurodevelopmental Disorder," *Molecular Genetics and Metabolism* 138 (2023): 107523.

12. D. Soto, M. Olivella, C. Grau, et al., "L-Serine Dietary Supplementation Is Associated With Clinical Improvement of Loss-Of-Function GRIN2B-Related Pediatric Encephalopathy," *Science Signaling* 12 (2019): eaaw0936.

13. W. Tang, D. Liu, S. F. Traynelis, and H. Yuan, "Positive Allosteric Modulators That Target NMDA Receptors Rectify Loss-Of-Function GRIN Variants Associated With Neurological and Neuropsychiatric Disorders," *Neuropharmacology* 177 (2020): 108247.

14. C. Geoffroy, P. Paoletti, and L. Mony, "Positive Allosteric Modulation of NMDA Receptors: Mechanisms, Physiological Impact and Therapeutic Potential," *Journal of Physiology* 600 (2022): 233–259.

15. G. P. Guerra, M. A. Rubin, and C. F. Mello, "Modulation of Learning and Memory by Natural Polyamines," *Pharmacological Research* 112 (2016): 99–118.

16. C. Romano, K. Williams, S. DePriest, et al., "Effects of Mono-, Di-, and Triamines on the N-Methyl-D-Aspartate Receptor Complex: A Model of the Polyamine Recognition Site," *Molecular Pharmacology* 41 (1992): 785–792.

17. K. Williams, "Modulation and Block of Ion Channels: A New Biology of Polyamines," *Cellular Signalling* 9 (1997): 1–13.

18. T. Eisenberg, M. Abdellatif, S. Schroeder, et al., "Cardioprotection and Lifespan Extension by the Natural Polyamine Spermidine," *Nature Medicine* 22 (2016): 1428–1438.

19. C. Schwarz, S. Stekovic, M. Wirth, et al., "Safety and Tolerability of Spermidine Supplementation in Mice and Older Adults With Subjective Cognitive Decline," *Aging* 10 (2018): 19–33.

20. C. Schwarz, G. S. Benson, N. Horn, et al., "Effects of Spermidine Supplementation on Cognition and Biomarkers in Older Adults With Subjective Cognitive Decline: A Randomized Clinical Trial," *JAMA Network Open* 5 (2022): e2213875.

21. M. Wirth, G. Benson, C. Schwarz, et al., "The Effect of Spermidine on Memory Performance in Older Adults at Risk for Dementia: A Randomized Controlled Trial," *Cortex* 109 (2018): 181–188.

22. A. E. Pegg, "The Function of Spermine," *IUBMB Life* 66 (2014): 8–18.

23. I. Krey, S. von Spiczak, K. M. Johannesen, et al., "L-Serine Treatment Is Associated With Improvements in Behavior, EEG, and Seizure Frequency in Individuals With GRIN-Related Disorders due to Null Variants," *Neurotherapeutics* 19 (2022): 334–341.

24. T. Kutsuwada, K. Sakimura, T. Manabe, et al., "Impairment of Suckling Response, Trigeminal Neuronal Pattern Formation, and Hippocampal LTD in NMDA Receptor Epsilon 2 Subunit Mutant Mice," *Neuron* 16 (1996): 333–344.

25. J. H. Luo, Z.-Y. Fu, G. Losi, et al., "Functional Expression of Distinct NMDA Channel Subunits Tagged With Green Fluorescent Protein in Hippocampal Neurons in Culture," *Neuropharmacology* 42 (2002): 306–318.

26. F. Mannara, M. Radosevic, J. Planagumà, et al., "Allosteric Modulation of NMDA Receptors Prevents the Antibody Effects of Patients With Anti-NMDAR Encephalitis," *Brain* 143 (2020): 2709–2720.

27. A. Santos-Gómez, F. Miguez-Cabello, A. García-Recio, et al., "Disease-Associated GRIN Protein Truncating Variants Trigger NMDA Receptor Loss-Of-Function," *Human Molecular Genetics* 29 (2021a): 3859–3871.

28. M. Wirth, C. Schwarz, G. Benson, et al., "Effects of Spermidine Supplementation on Cognition and Biomarkers in Older Adults With Subjective Cognitive Decline (SmartAge)-Study Protocol for a Randomized Controlled Trial," *Alzheimer's Research & Therapy* 11 (2019): 36.

29. S. S. Sparrow and D. V. Cicchetti, "Diagnostic Uses of the Vineland Adaptive Behavior Scales," *Journal of Pediatric Psychology* 10 (1985): 215–225.

30. L. Mony, S. Zhu, S. E. P. Carvalho, and P. Paoletti, "Molecular Basis of Positive Allosteric Modulation of GluN2B NMDA Receptors by Polyamines," *EMBO Journal* 30 (2011): 3134–3146.

31. N. Juliá-Palacios, M. Olivella, M. Sigatullina Bondarenko, et al., "L-Serine Treatment in Patients With GRIN-Related Encephalopathy: A Phase 2A, Non-randomized Study," *Brain* 147 (2024): 1653–1666.

32. I. Ito, K. Futai, H. Katagiri, et al., "Synapse-Selective Impairment of NMDA Receptor Functions in Mice Lacking NMDA Receptor Epsilon 1 or Epsilon 2 Subunit," *Journal of Physiology* 500 (1997): 401–408.

33. T. Takeuchi, Y. Kiyama, K. Nakamura, et al., "Roles of the Glutamate Receptor epsilon2 and delta2 Subunits in the Potentiation and Prepulse Inhibition of the Acoustic Startle Reflex," *European Journal of Neuroscience* 14 (2001): 153–160.

34. M. Chrisam, M. Pirozzi, S. Castagnaro, et al., "Reactivation of Autophagy by Spermidine Ameliorates the Myopathic Defects of Collagen VI-Null Mice," *Autophagy* 11 (2015): 2142–2152.

35. A. Wirth, B. Wolf, C.-K. Huang, et al., "Novel Aspects of Age-Protection by Spermidine Supplementation Are Associated With Preserved Telomere Length," *Geroscience* 43 (2021): 673–690.

36. A. R. Mohn, R. R. Gainetdinov, M. G. Caron, and B. H. Koller, "Mice With Reduced NMDA Receptor Expression Display Behaviors Related to Schizophrenia," *Cell* 98 (1999): 427–436.

37. C. A. Mielnik, M. A. Binko, Y. Chen, et al., "Consequences of NMDA Receptor Deficiency Can Be Rescued in the Adult Brain," *Molecular Psychiatry* 26, no. 7 (2020): 2929–2942, https://doi.org/10.1038/s41380-020-00859-4.

38. F. Madeo, T. Eisenberg, F. Pietrocola, and G. Kroemer, "Spermidine in Health and Disease," *Science* 359 (2018): eaan2788, https://doi.org/10.1126/science.aan2788.

39. O. Bar-Shira, R. Maor, and G. Chechik, "Gene Expression Switching of Receptor Subunits in Human Brain Development," *PLoS Computational Biology* 11 (2015): e1004559.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.