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Synaptic NMDA receptor activation induces ubiquitination and degradation of STEP₆₁

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Abstract

NMDA receptor signaling is critical for the development of synaptic plasticity, learning and memory, and dysregulation of NMDAR signaling is implicated in a number of neurological disorders including schizophrenia (SZ). Previous work has demonstrated that the STriatal-Enriched protein tyrosine Phosphatase 61 kDa (STEP₆₁) is elevated in human SZ postmortem cortical samples and after administration of psychotomimetics to cultures or mice. Here we report that activation of synaptic NMDAR by bicuculline or D-serine results in the ubiquitination and proteasomal degradation of STEP₆₁, and increased surface localization of GluN1/GluN2B receptors. Moreover, bicuculline or D-serine treatments rescue the motor and cognitive deficits in MK-801-treated mice and reduce STEP₆₁ in mouse frontal cortex. These results suggest that STEP₆₁ may contribute to the therapeutic effects of D-serine.

Keywords

STEP₆₁; NMDA receptor; ubiquitination; schizophrenia; D-serine; cognitive function

Introduction

Schizophrenia (SZ) is a debilitating disorder that affects approximately 1% of the world's population and is characterized by positive and negative symptoms as well as cognitive deficits. Although the etiology remains unclear, SZ has been linked to activation of dopaminergic, glutamatergic and GABAergic pathways [1–7]. Neuroleptics have been used for decades for the treatment of SZ, although they are effective primarily against positive symptoms [8–11]. Psychotomimetic inhibition of glutamate receptors is used to create mouse models of SZ and have suggested hypofunction of glutamate signaling, consistent

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with the glutamate hypothesis of SZ [12–14]. More recently, deficits in GABAergic interneuron function have been proposed to contribute to the pathophysiology of SZ [6,15,16].

Recent studies have demonstrated that expression levels of STriatal-Enriched protein tyrosine Phosphatase 61 kDa (STEP₆₁) are increased in human and animal models of SZ [17–19]. STEP₆₁ levels are elevated in cortex of SZ patients, in human induced pluripotent stem cells derived from SZ patients and differentiated into glutamatergic neurons [20], as well as after administration of psychotomimetics to mice (e.g., PCP and MK-801). STEP₆₁ is a membrane-associated isoform that is targeted, in part, to the endoplasmic reticulum within postsynaptic terminals, while STEP₄₆ is a cytosolic protein [21]. STEP normally opposes synaptic strengthening by dephosphorylation of regulatory tyrosine residues in Fyn, Pyk2, and ERK1/2, thereby inactivating them [22–26]. STEP₆₁ also dephosphorylates subunits of ionotropic glutamate receptors, resulting in the internalization of NMDARs (GluN1/GluN2B) and AMPARs (GluA1/GluA2) [27,28]. Elevated levels of STEP₆₁ disrupt synaptic function and has been proposed to contribute to the cognitive deficits often present in SZ [17–19].

Neuroleptic medications exert their efficacy, at least in part, through the phosphorylation and inactivation of STEP₆₁, resulting in trafficking of NMDARs to synaptic membranes [17]. Neuroleptics, however, have a high incidence of side-effects [29,30]. NMDAR modulators represent a new class of neuroleptics that are currently under clinical investigation [31]. Co-agonists such as glycine and D-serine have been proposed to potentiate NMDAR signaling without inducing excitotoxicity [32]. Consistent with this, D-serine improves behavioral and cognitive deficits in psychotomimetic-induced animal models of SZ [33,34]. Furthermore, combining D-serine with neuroleptics medication improves neuroleptic efficacy in patients, although negative studies also exist [35,36].

The mechanisms by which D-serine exerts its effects are largely unknown. In this study, we test the hypothesis that STEP₆₁ expression is down-regulated by bicuculline or D-serine through NMDAR signaling. We demonstrate that bicuculline and D-serine both suppress STEP₆₁ expression and function and this is associated with the ability of these drugs to reverse motor and cognitive deficits in an MK-801 mouse model of SZ.

Materials and Methods

Antibodies and reagents

All antibodies used in this study are listed in the Supplementary Table 1. Bicuculline, MG-132, LY294002 calpeptin, tetrodotoxin and glycine were obtained from Calbiochem (San Diego, CA). (+)-MK-801, CNQX, LY 367385, MPEP, L-689560, D-serine and picrotoxin were purchased from Tocris Biosciences (Ellisville, MO). 2-Bromopalmitate (2-BP) was purchased from Sigma-Aldrich (St. Louis, MO). PSD-95 shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary neuronal cultures and treatments

The Yale University Institutional Animal Care and Use Committee approved all procedures. Primary cortical neurons were isolated from rat E18 embryos as previously described [37]. Cells were plated on poly-D-lysine-coated plates (1×10^6 cells/well) in Neurobasal Media supplemented with 2% B27 (Invitrogen, San Diego, CA) and grown for 14–18 days. Neurons were treated with bicuculline (10 μ M for 15–60 min), picrotoxin (50 μ M for 15–60 min) or D-serine (100 μ M for 30–120 min). In some experiments, drugs were pre-incubated for 30–60 min followed by bicuculline treatment. To test the priming effect of glycine, neurons were rinsed with the extracellular solution (ECS, pH 7.35 and osmolarity 330 mosM, consisting of the following: 140 mM NaCl, 1.3 mM CaCl_2 , 5 mM KCl, 25 mM HEPES, 33 mM glucose and 500 nM tetrodotoxin) 3 times and incubated in ECS 1 h prior to treatments. To inhibit the palmitoylation of PSD-95, neurons were treated with 2-BP (50 μ M) for 8 h, followed by bicuculline treatment.

Immunoprecipitation of STEP

Neurons were lysed in 1 $\times$ RIPA buffer (Pierce Biotechnology, Rockford, IL) with complete phosphatase and protease inhibitors (Roche, Indianapolis, IN) and spun at 1,000 $\times$ g for 10 min to obtain supernatants. Protein concentrations were measured using BCA protein assay kit (Pierce). For immunoprecipitations, supernatants (300 μ g) were first precleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and mixed with anti-STEP antibody (4 μ g) overnight at 4°C as described [37]. On the second day, protein A/G-agarose was added to the antibody-bound complex for 4 h at 4°C and precipitates were washed 3 times with 1 $\times$ RIPA buffer and analyzed by SDS-PAGE and immunoblotting.

Short hairpin RNA (shRNA) transduction

PSD-95 shRNA lentiviral particles (Santa Cruz Biotechnology) or control shRNA lentiviral particles [19] was added to primary cortical neurons at days *in vitro* (DIV) 7. After 7 days of growth in the presence of the shRNAs, neurons were treated with bicuculline or vehicle and harvested in RIPA buffer with phosphatase and protease inhibitors.

Immunofluorescence imaging and analysis

Cortical neurons were seeded at 40,000 cells/cm² on coverslips in Neurobasal medium with 2% B27. After bicuculline treatment at DIV 14–18, neurons were rinsed with 1 $\times$ PBS and fixed in 4% paraformaldehyde with 4% sucrose. For STEP and MAP2 double labeling, cells were permeabilized in PBS + 0.2% Triton X-100 for 20 min after fixation and blocked with 10% NGS + 1% BSA for 1 h at RT. Cultures were incubated with mouse anti-STEP (23E5) and rabbit anti-MAP2 antibodies overnight at 4°C. For surface receptor staining, cells were blocked with 10% NGS + 1% BSA for 1 h at RT and incubated with mouse anti-GluN1 (clone 54.1) or mouse anti-GluN2B (clone N59/20) antibodies overnight at 4°C. On the second day, coverslips were washed in PBS and incubated with goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594 secondary antibodies, respectively (Molecular Probes, Eugene, OR). For receptor double labeling, cells were permeabilized in PBS + 0.1% Triton X-100 for 20 min, washed with PBS and incubated with rabbit anti-synapsin I antibody for 2 h at RT, followed by goat anti-rabbit Alexa Fluor 594 secondary antibody.

Microscopy was performed with a Zeiss Axiovert 2000 microscope with an apotome (Applied Scientific Instruments, Eugene, OR) using a 100 × objective lens. All analyses were performed blind to the stimulation conditions of the culture. Cells were selected under phase contrast imaging to avoid bias on the fluorescence intensity. Twenty μm of each process (starting from one soma diameter away from the soma) was selected for analyses. To measure STEP level along dendrites, STEP and MAP2 co-localization was counted using the NIH ImageJ based Fiji (<https://imagej.net/Fiji>) with the Coloc2 plug-in as described [38]. To measure surface receptors (GluN1 and GluN2B), the correlation between GluN1 or GluN2B puncta with synapsin puncta was calculated by the intensity correlation analysis (ICA) using the ImageJ ICA plug-in (Wright Cell Imaging Facility: http://www.uhnresearch.ca/facilities/wcif/imagej/colour_analysis.htm). The product of the relative differences from the mean (PDM) was quantitated using ImageJ as described [38]. All fluorescence intensity was normalized to control levels and data were expressed as means ± SEM. Statistical significance ($P < 0.05$; $n = 31$ –50 processes analysed) was determined by one-way ANOVA with post hoc Tukey test or Student's t-test when appropriate.

Surface biotinylation

The amounts of NMDA receptors on plasma membranes were measured by biotinylation as described [39,17]. Briefly, neurons were rinsed twice with ice-cold 1×PBS, pH 7.4 (Sigma) after various treatments and incubated in 1 mg/ml EZ link sulfo-NHS-SS-biotin (Pierce) in PBS for 20 min with gentle shaking at 4 °C. After labeling, cells were washed three times with quenching buffer (1×PBS+ 100 mM glycine, pH 7.4) to scavenge the unreacted biotin. Cells were lysed in 1×RIPA buffer with brief sonication for 10 s. Insoluble cell debris was removed by centrifugation at 1,000×g for 10 min. Same amounts of supernatants were incubated with NeutrAvidin-agarose (Pierce) overnight at 4 °C and the resultant beads were washed three times in 1×RIPA buffer. The proteins bound to NeutrAvidin-agarose beads (membrane fractions) and supernatants inputs (total proteins) were subjected to SDS-PAGE and immunoblotting.

Animals and treatments

All experimental procedures were approved by the Yale University Institutional Animal Care and Use Committee and in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals. Male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, <http://jaxmice.jax.org/strain/013636.html>). All mice (3–6 months) were maintained on a 12 h light/dark cycle.

For drug administration, MK-801 and bicuculline were first dissolved in DMSO at 100 mM, further diluted with saline (0.9 % sodium chloride) and administered at 0.15 mg/kg and 1 mg/kg, respectively. The dosage of bicuculline (0.5, 1 or 2 mg/kg) was determined in a series of pilot studies. Vehicle (saline with same final concentration of DMSO) was used as controls. D-serine was dissolved in saline and used at 600 mg/kg. All drugs were administrated acutely with a single dose via intraperitoneal (i.p.) route. Naïve mice were used for each behavioral tests to avoid possible sensitization to drugs.

Behavioral tests

Locomotor activity—Mice were placed in an open-field activity chamber (43×43×30 cm, Med Associates Inc., St Albans, VT) and allowed to explore freely for 30 min prior to MK-801 or vehicle administration to get the baseline as previously described [19,40]. After drug administration mice were immediately put back to the activity chamber and monitored for 1 h. The activity monitor program (version 5.10, Med Associates) was used to record ambulatory distance and stereotypic counts.

Spontaneous alternation in Y-maze—Spontaneous alternation performance was tested using a symmetrical Y-maze (42×4.8×20 cm) as described previously [39]. Briefly, each mouse was placed in the center of the Y-maze and allowed to explore freely through the maze during a 5 min session. The sequence and total number of arms entered were recorded using ANY-maze (Stoelting, Wood Dale, IL). The percentage of spontaneous alternation was calculated by dividing the number of alternations by the total of arm entries completed during the trial. The percentage of alternations was defined according to the following equation: % alternation = [(number of alternations)/(total arm entries - 2)] × 100. An actual alternation was defined as entries into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB)

Novel object recognition—The task was carried out in a testing arena (60 cm × 60 cm) with opaque white walls as describe [39,17]. Briefly, mice were allowed to explore two identical objects for a total of 30 sec during the acquisition trial. Memory retention was evaluated at 24 h post training. During the retention trial, mice were exposed to a familiar object from the acquisition trial and a novel object (different objects were used for short-term or long-term memory tests). Objects (different shapes, texture, and color) and location of an object were randomly assigned to avoid the bias. The exploration time (sec) of each object in each trial was recorded using ANY-maze program.

Immunoblotting

Primary cortical cultures were lysed in 1 ×RIPA buffer (Pierce biotechnology, Rockford, IL) with complete protease and phosphatase inhibitors (Roche, Indianapolis, IN), lysates were spun at 1000×g for 10 min and supernatants (referred to as RIPA extracts herein) were saved for further analyses. Brain tissues (frontal cortices) were homogenized in TEVP+ 320 mM sucrose to obtain total homogenates and crude synaptic membrane fractions (P2) as described [37,17]. Samples were resolved on 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA) and incubated with specific primary antibodies (as listed in Supplementary Table 1) overnight at 4 °C. On the second day, membranes were incubated with peroxidase-conjugated secondary antibodies (Pierce, 1:5,000) for 2 h at RT. Immunoreactivity was developed using a Chemiluminescent Substrate kit (Pierce) and visualized by a G:BOX with the GeneSnap software (Syngene, Cambridge, UK). All densitometric bands were quantified using the Genetools program (Syngene).

Data analysis

All experiments were repeated at least three times. For immunoblotting quantification, phosphorylated protein levels were normalized to total protein levels, then to β -actin as loading control, except when phospho-GluN2B was evaluated in synaptosomal fractions (P2). Regulation of GluN2B phosphorylation at Y¹⁴⁷² by STEP₆₁ leads to a concomitant change of total GluN2B in P2 fractions [37,41]. In this case, pGluN2B levels were normalized to β -actin directly. Data were expressed as means $\pm$ SEM. Statistical significance ($P < 0.05$) was determined by one-way ANOVA with post hoc Tukey test. Student's t-tests were used for some experiments when appropriate. For locomotor activity, data were analyzed with two-way ANOVA with repeated measures, with post hoc Bonferroni or paired t-test.

Results

Activation of synaptic NMDAR leads to ubiquitination and degradation of STEP₆₁ in primary cortical neurons

Bicuculline treatment was used to potentiate synaptic NMDAR signaling [42,37,43]. Consistent with previous findings [37], bicuculline led to a rapid decrease in STEP₆₁ expression and a concomitant increase in the tyrosine phosphorylation of STEP substrates including GluN2B, Pyk2 and ERK1/2 (compared with Ctl at 15 min: STEP₆₁: 0.47 ± 0.07 , $P < 0.01$; pGluN2B: 1.42 ± 0.06 , $P < 0.05$; pPyk2: 1.61 ± 0.08 , $P < 0.01$; pERK1/2: 1.85 ± 0.08 , $P < 0.01$, $n = 4$, one-way ANOVA, Fig. 1a). To visualize the loss of STEP₆₁ along dendrites, we double-labeled STEP₆₁ (the prominent isoform in cortical neurons) and MAP2 using well-established antibodies. STEP₆₁ fluorescence density along dendrites was counted using the ImageJ plug-in Coloc2 as described [38]. In agreement with the biochemistry data, we found a significant reduction of STEP₆₁ intensity upon bicuculline treatment (0.60 ± 0.04 of Ctl, $P < 0.001$, $n = 49$ for Ctl, $n = 50$ for Bic, Student's t-test, Fig. 1b).

To determine whether the degradation of STEP₆₁ occurred through the ubiquitination pathway [41], we repeated the bicuculline treatment of cortical cultures in the presence of a proteasome inhibitor (MG-132). Under these conditions, bicuculline administration led to the accumulation of high molecular weight ubiquitinated STEP species (Fig. 2a). STEP₆₁ is known to undergo calpain-mediated proteolysis under certain conditions [37,44]. To test whether cleavage of STEP₆₁ may result in the loss of full-length STEP₆₁, we probed for STEP₃₃, the calpain-cleaved STEP product after bicuculline treatment. No change in STEP₃₃ or fodrin (a well-established calpain substrate) was found (Supplementary Fig. 1), suggesting that calpain did not account for the loss of STEP₆₁ induced by bicuculline.

Mono-ubiquitination or poly-ubiquitination have different effects on target proteins [45]. For instance, poly-ubiquitination of the glutamate receptor interacting protein 1 (GRIP1) results in its degradation, which leads to the loss of surface GluA2 receptors [46]. Mono-ubiquitination of another scaffolding molecule protein interacting with C-kinase 1 (PICK1) regulates the activity of an acid-sensing ion channel, without change in expression levels [47]. We used specific ubiquitin antibodies to examine whether STEP₆₁ was poly- or mono-ubiquitinated after bicuculline treatment. Ubiquitin FK1 antibody recognizes poly-

ubiquitinated proteins, while ubiquitin FK2 antibody recognizes both mono- and poly-ubiquitinated proteins [48]. Cultures were pretreated with MG-132 (10 μ M) for 30 min to prevent rapid degradation, STEP species were immunoprecipitated using anti-STEP antibody (23E5) in RIPA extracts, followed by immunoblotting with anti-ubiquitin FK1 and FK2 antibodies. Both antibodies labeled STEP₆₁. Inhibition of the UPS by MG-132 alone induced the accumulation of ubiquitinated-STEP₆₁, and there was a further increase in the poly-ubiquitination of STEP₆₁ after bicuculline treatment (Fig. 2b). However, as a mono-ubiquitin-specific antibody is not available, we cannot exclude the possibility that mono-ubiquitination of STEP₆₁ also occurs with bicuculline treatment.

The ubiquitination of different side chains within ubiquitin also determines the fate of target proteins, including degradation, activation of signaling pathways, or subcellular localization [49]. Generally, ubiquitination on Lys⁴⁸ leads to protein degradation whereas ubiquitination on Lys⁶³ results in modification of protein function [49]. To determine the modification of the poly-ubiquitin chain on STEP₆₁, we pretreated cultures with MG-132, followed by immunoprecipitation of STEP after bicuculline treatment. We found that STEP₆₁ was ubiquitinated at Lys⁴⁸-linked but not Lys⁶³-linked chains using selective antibodies against these sites (Fig. 2c). Inhibition of the UPS by MG-132 treatment alone was sufficient to induce ubiquitination of STEP₆₁ at Lys⁴⁸, whereas bicuculline treatment resulted in a further increase of this modification. These results are consistent with a rapid degradation of STEP₆₁ upon bicuculline treatment (Fig. 1a).

We also tested another GABA_A receptor antagonist (picrotoxin; PTX, 50 μ M) that enhances neuronal activity in primary cortical neurons [50,51]. There was a rapid reduction of STEP₆₁ and increased tyrosine phosphorylation of its substrates upon picrotoxin treatment for 15–60 min (Supplementary Fig. 2a). Similar to the bicuculline treatment (Fig. 2b), there was an increase in the ubiquitination of STEP₆₁ (Supplementary Fig. 2b).

To investigate the possible involvement of specific glutamate receptors in STEP₆₁ ubiquitination and degradation, we blocked these channels with selective antagonists. The NMDAR antagonist MK-801 blocked bicuculline-induced STEP₆₁ degradation (0.94 ± 0.08 , $P > 0.05$ compared with Ctl, $n = 6$), while blockade of AMPAR or mGluRs (mGluR1 and mGluR5) had no effect (CNQX: 0.54 ± 0.07 ; LY367385: 0.58 ± 0.06 ; MPEP: 0.65 ± 0.06 , $P < 0.05$, $n = 6$) (Fig. 3a). These antagonists did not alter STEP₆₁ levels in the absence of bicuculline treatment. As expected, degradation of STEP₆₁ was associated with an increase in the tyrosine phosphorylation of STEP substrates (pY¹⁴⁷² GluN2B, pY⁴⁰² Pyk2 and pY^{204/187} ERK1/2). In contrast, MK-801 significantly attenuated the effects of bicuculline on STEP substrates.

Full activation of the NMDAR requires the binding both glutamate and glycine as a co-agonist [52,53]. In the experiments described so far, we carried out the bicuculline treatment by addition to the widely-used Neurobasal, which contains 400 μ M glycine. To investigate whether glycine has priming effect on bicuculline-induced STEP₆₁ degradation, we repeat the treatment in the absence of glycine in the extracellular solution (ECS) as describe [52]. Bicuculline alone failed to induce STEP₆₁ degradation, whereas co-application of bicuculline and glycine (100 μ M) replicated the findings using Neurobasal medium, i.e.

degradation of STEP₆₁ (0.41 ± 0.13 , $P < 0.05$, $n = 4$) and an increase in the tyrosine phosphorylation of STEP substrates (pGluN2B: 1.53 ± 0.08 , $P < 0.05$; pPyk2: 1.34 ± 0.07 , $P < 0.05$; pERK1/2: 2.09 ± 0.19 , $P < 0.05$, $n = 4$, one-way ANOVA, Fig. 3b). As expected, the priming effect of glycine was blocked by the glycine site antagonist L-689560 (Fig. 3b). These results suggest that activation of the NMDAR by glycine is necessary for bicuculline-induced degradation of STEP₆₁.

Inhibition of PSD-95 function does not block bicuculline-induced STEP₆₁ degradation

A recent study showed that PSD-95 regulates STEP₆₁ stability through the UPS. Loss of membrane targeting of a PSD-95 cysteine mutant that can not be palmitoylated or knockdown of PSD-95 levels in cortical neurons led to accumulation of STEP₆₁ in the PSD fraction as well as in RIPA extracts [54]. To investigate whether PSD-95 is required for bicuculline-induced STEP₆₁ degradation, firstly we treated cortical neurons with 2-bromopalmitate (2-BP), commonly used to inhibit palmitoylation of PSD-95 [54,55]. Pilot study confirmed that 2-BP treatment resulted in increased PSD-95 in cytosol (Supplementary Fig. 3) as described [55], whereas another synaptic scaffolding protein SAP102 showed no difference in these compartments. In agreement with the previous study [54], we also found a significant increase of STEP₆₁ when PSD-95 palmitoylation was inhibited by 2-BP (RIPA extracts: 1.65 ± 0.11 ; pellet: 2.53 ± 0.22 , $P < 0.05$, $n = 4$, Student's t-test). Next, we repeated bicuculline treatment in 2-BP treated neurons. Bicuculline resulted in STEP₆₁ degradation in both vehicle-treated and 2-BP treated cultures, with an increase in the tyrosine phosphorylation of STEP substrates (Fig. 4a), suggesting loss of PSD-95 membrane targeting by 2-BP does not affect bicuculline-induced STEP₆₁ degradation.

Secondly, we examined the involvement of PSD-95 by reducing PSD-95 levels using shRNA-mediated knockdown. We achieved about 50% knockdown of PSD-95 levels in RIPA extracts after 7 days transduction (Fig. 4b). Consistent with the previous study [54], reduced PSD-95 led to increased STEP₆₁ in RIPA extracts. However, PSD-95 knockdown did not block bicuculline-induced degradation of STEP₆₁ (Fig. 4b). Since in both approaches (2-BP and shPSD-95 treatments) remaining membrane bound PSD-95 may be sufficient to initiate signaling pathways, we cannot completely exclude the possibility that PSD-95 contributes to the effects by bicuculline treatment.

Bicuculline-induced degradation of STEP₆₁ results in increased surface expression of GluN1/GluN2B

One mechanism by which STEP opposes synaptic strengthening is by promoting the internalization of NMDARs [27,39]. We performed surface biotinylation experiments to determine whether bicuculline-induced degradation of STEP₆₁ increased trafficking of NMDARs to surface membranes. Bicuculline treatment increased the surface expression of GluN1 and GluN2B (GluN1: 2.12 ± 0.12 ; GluN2B: 2.02 ± 0.15 , $P < 0.05$, $n = 4$, Fig. 5a) without changing total levels of these proteins in RIPA extracts. Treatment with bicuculline also led to increased tyrosine phosphorylation of GluN2B in the membrane surface and in RIPA extracts (Fig. 5a). Treatment with bicuculline was not associated with the alteration of surface levels of the control receptors GABA_Aβ(2/3) (Fig. 5a). In addition, MK-801 prevented the enhanced surface expression of GluN1/GluN2B (GluN1: 1.22 ± 0.20 ;

GluN2B: 0.89 ± 0.10 , $P > 0.05$, $n = 4$, Fig. 5a) and the increased tyrosine phosphorylation of GluN2B (1.21 ± 0.09 , $P > 0.05$, $n = 4$, Fig. 5a). These results suggest that degradation of STEP₆₁ after bicuculline treatment is sufficient to enhance NMDAR trafficking to surface membranes.

To visualize the changes in surface GluN1/GluN2B, we staining neurons with well-validated antibodies against the extracellular domains of GluN1 (clone 54.1) and GluN2B (clone N59/20) prior to cell permeabilization. Synaptic regions were then labeled by anti-synapsin I antibody. Co-localized GluN1 or GluN2B puncta with synapsin puncta was quantitated using ICA ImageJ plug-in, the fluorescence intensity of PDM was used to compare changes in surface GluN1/GluN2B levels. In agreement with the surface biotinylation assay, bicuculline treatment led to increased surface GluN1 (Supplementary Fig. 4) and GluN2B (Fig. 5b), which was blocked by MK-801.

Bicuculline leads to degradation of STEP₆₁ and increased tyrosine phosphorylation of STEP substrates *in vivo*

To examine whether bicuculline was able to induce changes in STEP₆₁ and its substrates levels *in vivo*, we administrated bicuculline intraperitoneally (i.p.) in mice. Male WT mice were injected with 0.5, 1 or 2 mg/kg (i.p.) of bicuculline and synaptic membranes from frontal cortex were collected 30 min later and analyzed. We found reduced STEP₆₁ levels after bicuculline injection at 1 or 2 mg/kg (1 mg/kg: 0.69 ± 0.06 ; 2 mg/kg: 0.61 ± 0.08 , $P < 0.05$, $n = 6$, Supplementary Fig. 5a) but not 0.5 mg/kg injection (0.86 ± 0.06 , $P > 0.05$, $n = 6$, Supplementary Fig. 5a). As expected, removal of STEP₆₁ led to increased tyrosine phosphorylation of STEP substrates (at 1 mg/kg, pGluN2B: 1.66 ± 0.11 ; pPyk2: 1.50 ± 0.14 ; pERK1/2: 1.51 ± 0.15 , $P < 0.05$, $n = 6$) and increased expression of GluN2B and GluN1 in synaptic membranes (at 1 mg/kg, GluN2B: 1.57 ± 0.15 ; GluN1: 1.51 ± 0.12 , $P < 0.05$, $n = 6$, Supplementary Fig. 5a).

Next we assessed the kinetics of bicuculline-induced degradation of STEP₆₁. Mice were treated with 1 mg/kg (i.p.) bicuculline for 15–60 min and synaptic membrane fractions were analyzed. Bicuculline treatment induced a rapid degradation of STEP₆₁ at 15 and 30 min (15 min: 0.75 ± 0.06 ; 30 min: 0.68 ± 0.11 , $P < 0.05$, $n = 6$, Supplementary Fig. 5b), increased tyrosine phosphorylation of STEP substrates (at 30 min, pGluN2B: 1.47 ± 0.17 ; pPyk2: 1.43 ± 0.18 ; pERK1/2: 1.48 ± 0.18 , $P < 0.05$, $n = 6$, Supplementary Fig. 5b) and increased expression of GluN2B and GluN1 in synaptic membranes (at 30 min, GluN2B: 1.42 ± 0.12 ; GluN1: 1.49 ± 0.15 , $P < 0.05$, $n = 6$). Together, these results indicate that bicuculline results in the rapid degradation of STEP₆₁ in mice and concomitant increases in the tyrosine phosphorylation of substrates, and the localization of GluN1/GluN2B subunits to synaptic membranes.

Activation of synaptic NMDAR by D-serine leads to ubiquitination and degradation of STEP₆₁

Hypofunction of NMDAR signaling is one mechanism proposed to occur in the pathophysiology of SZ [14,56,57] and D-serine has been tested clinically as a potential treatment for the disorder [58,59]. As D-serine primarily activates synaptic NMDARs [53],

we tested whether D-serine activation of NMDAR signaling would lead to ubiquitination and degradation of STEP₆₁. Treatment of cortical cultures with D-serine for 30 min to 2 h led to a significant degradation of STEP₆₁ (at 30 min: 0.49 ± 0.15 , $P < 0.05$, $n = 4$, Fig. 6a) and increased tyrosine phosphorylation of its substrates (at 30 min: pGluN2B: 1.42 ± 0.13 ; pPyk2: 1.41 ± 0.09 ; pERK1/2: 1.60 ± 0.16 , $P < 0.05$ or $P < 0.01$, $n = 4$, Fig. 6a). There was also a significant increase in STEP₆₁ ubiquitination after D-serine treatment (Fig. 6b). D-serine treatment also led to significant increases in surface GluN1 and GluN2B levels (GluN1: 1.91 ± 0.19 ; GluN2B: 1.90 ± 0.17 , $P < 0.05$, $n = 4$, Fig. 6c) as well as an increased tyrosine phosphorylation of GluN2B at membrane surfaces (1.62 ± 0.12 , $P < 0.05$, $n = 4$) and in RIPA extracts (1.54 ± 0.11 , $P < 0.05$, $n = 4$, Fig. 6c), which was blocked by MK-801 treatment (Fig. 6c). No changes were seen in surface GABA_Aβ (2/3) receptors or total receptors in RIPA extracts (Fig. 6c).

Next, we determined whether D-serine administration resulted in STEP₆₁ degradation *in vivo*. Male C57BL/6 mice were administered with D-serine (600 mg/kg, i.p.) and frontal cortices were collected at several time points (30 min, 1 h and 3 h) for biochemical analyses. D-serine administration led to decreased STEP₆₁ (at 1 h: 0.66 ± 0.07 , $P < 0.05$, $P < 0.05$, $n = 4$) and increased tyrosine phosphorylation of STEP₆₁ substrates (at 1 h: pGluN2B: 1.30 ± 0.11 ; pPyk2: 1.58 ± 0.14 ; pERK1/2: 1.57 ± 0.15 , $P < 0.05$ or $P < 0.01$, $n = 4$, Fig. 7a). Reduction of STEP₆₁ by D-serine led to increased synaptic localization of GluN1 and GluN2B (at 1 h: GluN1: 1.50 ± 0.15 ; GluN2B: 1.36 ± 0.11 , $P < 0.05$, $n = 4$).

Previously, we showed that STEP₆₁ level was elevated upon administration of psychotomimetics including MK-801 [17]. To test whether D-serine could normalize STEP₆₁ levels after MK-801 treatment, we pretreated mice with D-serine (600 mg/kg, i.p.) for 30 min, followed by MK-801 administration (0.15 mg/kg, i.p.) for 1 h. There was a significant accumulation of STEP₆₁ in frontal cortex after MK-801 treatment (1.55 ± 0.19 , $P < 0.05$, $n = 4$, Fig. 7b), decreased tyrosine phosphorylation of STEP substrates (pGluN2B: 0.51 ± 0.10 ; pPyk2: 0.57 ± 0.14 ; pERK1/2: 0.62 ± 0.10 , $P < 0.05$, $n = 4$, Fig. 7b), and decreased GluN1 and GluN2B levels in synaptic membranes (GluN1: 0.58 ± 0.14 ; GluN2B: 0.66 ± 0.15 , $P < 0.05$, $n = 4$, Fig. 7b). D-serine pretreatment decreased STEP₆₁ back to baseline levels and normalized deficits in the downstream targets of STEP₆₁ (Fig. 7b).

We next examined whether the reduction in STEP₆₁ level caused by D-serine was associated with reversal of motor and cognitive deficits in a SZ model. For these studies, we used an MK-801 mouse model, which is a widely validated pharmacological model of SZ and shows hyperactivity and cognitive deficits in several tasks [60–63,34]. Mice were administered vehicle or MK-801 treatment (0.15 mg/kg, i.p.) for 1 h. A third group of mice were pretreated with D-serine (600 mg/kg, i.p.) for 30 min, followed by MK-801 challenge. A two-way ANOVA with repeated measurement analysis showed a significant time $\times$ treatment interaction ($F(22,154) = 6.64$, $P < 0.001$). Post hoc Bonferroni's test revealed that MK-801 injection significantly increased locomotor activity (Veh/MK vs Veh/Veh: $P < 0.01$, $n = 8$ per group, Fig. 8a), consistent with previous findings [60,64]. D-serine reversed the MK-801-induced hyperactivity (DS/MK vs Veh/MK: $P < 0.01$, $n = 8$ per group, Fig. 8a). D-serine also significantly attenuated total distance traveled and total stereotypic counts seen upon MK-801 administration (Fig. 8b, c).

Next we used spontaneous alternation in the Y-maze task to assess working memory. Consistent with previous findings [64,65], MK-801 impaired alternation behavior, which was rescued by D-serine administration (Fig. 8d). A number of studies have demonstrated that MK-801 administration impairs long-term memory in rodents [61,34,62,63], and we examined the efficacy of D-serine to reverse deficits in a novel object recognition task. We confirmed impaired long-term memory (24 h post training) by MK-801, and D-serine was effective in reversing the cognitive deficits (Fig. 8e).

As bicuculline treatment induced degradation of STEP₆₁ in mice (Supplementary Fig. 5a, b), we next tested whether bicuculline was able to reverse MK-801-induced motor and cognitive deficits. We treated another cohort of mice with bicuculline (1 mg/kg, i.p.) followed by MK-801 treatment for 1 h. MK-801 induced robust hyperactivity, and two-way ANOVA with repeated measurement analysis showed a significant time $\times$ treatment interaction ($F(22,154) = 12.12$, $P < 0.001$). Post hoc Bonferroni's test revealed a significant difference with or without MK-801 treatment (Veh/MK vs Veh/Veh: $P < 0.001$, $n = 8$ per group, Supplementary Fig. 6a), while pretreatment with bicuculline significantly reduced the hyperlocomotion (Bic/MK vs Veh/MK: $P < 0.01$, $n = 8$ per group, Supplementary Fig. 6a). Bicuculline pretreatment normalized overall activity (Supplementary Fig. 6b) and stereotypic behavior in mice (Supplementary Fig. 6c). Bicuculline also rescued the MK-801-induced cognitive deficits in the Y-maze (Supplementary Fig. 6d) and novel object recognition tasks (Supplementary Fig. 6e).

Discussion

In this study we demonstrated that bicuculline and picrotoxin-induced neuronal activity results in the ubiquitination and degradation of STEP₆₁. The reduction in STEP₆₁ resulted in increased tyrosine phosphorylation of three STEP substrates (GluN2B, Pyk2 and ERK1/2) and increased localization of GluN1 and GluN2B subunits to synaptic membranes as assessed by immunoblotting and immunofluorescence staining. We also showed that activation of NMDAR signaling by D-serine induced similar biochemical changes in STEP₆₁ and its substrates, and reversed motor and cognitive deficits in MK-801-treated mice.

The details of STEP₆₁ ubiquitination are still not fully understood. Our results suggest that it is likely regulated by NMDAR signaling. Previous findings have shown that activation of the NMDAR is required for the ubiquitination and degradation of several synaptic proteins [43,46]. We speculate that ubiquitination of STEP₆₁ is controlled by pathways downstream of NMDAR activation, or by modulation the UPS activity [66,67]. Activation of NMDAR by glycine is relevant to this work. We showed that glycine binding is required for the degradation of STEP₆₁, whereas bicuculline failed to induce STEP₆₁ degradation in the absence of glycine or in the presence of a glycine site antagonist in the ECS buffer. Since PSD-95 modulates NMDAR gating and trafficking at synaptic sites [68] and a recent study showed that palmitoylation of PSD-95 regulates STEP₆₁ stability [54], we set out to determine whether PSD-95 is required for STEP₆₁ degradation in response to bicuculline. Loss of PSD-95 palmitoylation by 2-BP treatment or shRNA-mediated knockdown of PSD-95 did not block bicuculline-induced STEP₆₁ degradation in our experiment,

suggesting PSD-95 did not play a predominant role in this process. However, we cannot exclude the involvement of PSD-95 due to residual levels of PSD-95 present at synaptic membranes. Further studies are needed to investigate the mechanisms involved in the ubiquitination and degradation of STEP₆₁ and the role of PSD-95 using PSD-95 knockout mouse cultures and brains.

Disruption of the glutamatergic system has been proposed to cause hypofunction of the prefrontal cortex (PFC) [69]. Recent work on glycine site modulators such as D-serine, D-cycloserine and sarcosine has shown that they are able to improve not only positive and negative symptoms but also cognitive deficits [32,33,58]. Add-on treatment of D-serine with neuroleptics also showed improved efficacy in several clinical evaluations [36,59], although some studies fail to support clinical efficacy [59]. Here we demonstrated that STEP₆₁ is regulated by D-serine, i.e. activation of the synaptic NMDARs by D-serine led to STEP₆₁ ubiquitination and degradation, this in turn increased surface expression of the NMDARs. We used MK-801 to mimic glutamatergic deficits in mice, which has been widely used [60–63,34]. We confirmed that MK-801 induced hyperactivity, an endophenotype often seen in SZ patients and animal models, which was reversed by D-serine administration. For cognitive performance, we used a novel object recognition task, which is based on the animals' natural interest for investigating their novel environment. The test is quite similar to tasks used in humans and should therefore have a significant level of predictive validity [70]. D-serine or bicuculline preserved intact memory when administrated prior to MK-801 treatment, consistent with previous findings that inhibition of STEP₆₁ is able to reverse other psychotomimetic-induced cognitive deficits [17,19].

Due to the critical roles of NMDARs in neurodevelopment and cognition, modulators of NMDARs represent promising therapeutic targets. STEP negatively regulates the NMDAR signaling by promoting internalization of the NMDARs, whereas genetic or pharmacological inhibition of STEP rescue membrane NMDARs and downstream signaling [39,40,19]. In summary, we show that removal of STEP₆₁ by D-serine restores synaptic NMDARs, which may contribute to its therapeutic effects. These findings suggest that direct targeting of STEP activity may have therapeutic benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2-BP	2-bromepalmitate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance

BDNF	Brain-derived neurotrophic factor
Bic	bicuculline
DIV	Day in vitro
DMSO	Dimethyl sulfoxide
ERK1/2	Extracellular signal-regulated kinase 1 and 2
GRIP1	Glutamate receptor interacting protein 1
ICA	intensity correlation analysis
i.p.	Intraperitoneal
KO	Knock out
NMDA	N-Methyl-D-aspartic acid
PAGE	Polyacrylamide electrophoresis
PCP	Phencyclidine
PDM	product of the relative differences from the mean
Pyk2	Proline-rich tyrosine kinase 2
RIPA buffer	Radio-immunoprecipitation assay buffer
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
STEP₆₁	STriatal-Enriched protein tyrosine Phosphatase, 61 kDa
WT	Wild-type

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Fig. 1. Bicuculline-induced neuronal activity leads to the rapid loss of STEP₆₁ in primary cortical neurons. **a** Primary cortical neurons were treated with Bic (10 μ M) for 15–60 min. STEP₆₁ and phosphorylation of STEP substrates (GluN2B, Pyk2 and ERK1/2) were probed with phospho-specific and pan-antibodies. Phospho-levels were normalized to pan protein levels and then to β -actin as loading control. Data were expressed as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (* P < 0.05, ** P < 0.01, n = 4). **b** After bicuculline treatment, cortical neurons were fixed in

paraformaldehyde and stained with anti-STEP and anti-MAP2 antibodies, followed by incubation with Alexa Fluor 488- and 594-conjugated secondary antibodies. STEP₆₁ fluorescence intensities along dendrites were quantitated using the ImageJ plug-in (see Methods for details). Data were expressed as mean $\pm$ SEM. Statistical significance was determined using Student t-test (***) $P < 0.001$, $n = 49$ for Ctl, $n = 50$ for Bic), scale bar, 10 μm .

Fig. 2. Bicuculline leads to ubiquitination and degradation of STEP₆₁ in primary cortical neurons. Cortical neurons were pre-treated without or with MG-132 (MG, 10 μ M) for 30 min followed by without or with Bic treatment (10 μ M) for 5–30 min. Total STEP was immunoprecipitated with anti-STEP (23E5) antibody and probed with STEP, anti-ubiquitin (a); FK1 and FK2 (b) or Lys⁴⁸ and Lys⁶³ antibody (c) antibodies. Representative blots were from three independent experiments.

Fig. 3.

Activation of NMDAR and the glycine co-agonist are required for bicuculline-induced degradation of STEP₆₁. **a** Primary cortical neurons were pretreated with various receptor blockers followed by Bic (10 μ M, 15 min) stimulation. **b** Neurons were washed three times in ECS buffer and preincubated in ECS for 1h, followed by Bic or Bic/glycine (Gly, 100 μ M) treatment. Some cells were pretreated with L-689560 (1 μ M) for 30 min, followed by Bic/Gly treatment. After treatments, neurons were lysed in RIPA buffer and RIPA extracts were examined for STEP₆₁ and tyrosine phosphorylation of STEP substrates (GluN2B, Pyk2

and ERK1/2) levels with phospho-specific and pan-antibodies. Phospho-levels were normalized to pan protein levels and then to β -actin as loading control. Data were expressed as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (* $P < 0.05$, ** $P < 0.01$, $n = 6$ for **a**, $n = 4$ for **b**).

phospho-specific and pan-antibodies. Phospho-levels were normalized to pan protein levels and then to β -actin as loading control. Data were expressed as mean $\pm$ SEM. Statistical significance was determined using oneway ANOVA with post hoc Tukey test for multiple comparison between groups (* P < 0.05, ** P < 0.01, n = 4 for **a**, n = 6 for **b**).

Fig. 5. Bicuculline-induced degradation of STEP₆₁ results in increased localization of GluN1 and GluN2B subunits in cell surface. **a** Primary cortical neurons were pretreated with vehicle or MK- 801 (10 μ M, 30 min) followed by Bic (10 μ M, 15 min) stimulation. Surface proteins were labeled with 1 mg/ml EZ link sulfo-NHS-SS-biotin in PBS and pulled down with NeutrAvidin-agarose beads. Samples of surface proteins and in RIPA extracts were subjected to SDS-PAGE and examined for phospho-GluN2B, GluN2B, GluN1 and GABA_Aβ2/3 using specific antibodies. Surface protein levels were normalized to GABA_Aβ2/3 levels, whereas

targets in RIPA extracts were normalized to β -actin as loading control. Data were expressed as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (* $P < 0.05$, ** $P < 0.01$, $n = 4$). **b** Surface GluN2B levels were measured using a validated anti-GluN2B antibody (clone N59/20) against the extracellular domain of the subunit. After GluN2B labeling, neurons were permeabilized in PBS+0.1% Triton X-100, followed by staining with anti-synapsin I antibody. Co-localized GluN2B and synapsin puncta were counted using the ImageJ ICA plug-in (see Methods for details). Fluorescence intensity was normalized to control group. Data were expressed as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (*** $P < 0.01$, $n = 35$ for Ctl, $n = 37$ for Bic, $n = 42$ for Bic/MK), scale bar, 10 μ m.

Fig. 6.

Activation of synaptic NMDAR by D-serine results in the ubiquitination and degradation of STEP₆₁ in cortical neurons. **a** Primary cortical neurons were treated with D-serine (DS, 100 μM) for 30 min-2 h. STEP₆₁ and phosphorylation of STEP substrates (GluN2B, Pyk2 and ERK1/2) were probed with phospho-specific and pan-antibodies. Phospho-levels were normalized to pan protein levels and then to β-actin as loading control. **b** Neurons were pre-treated with MG-132 (MG, 10 μM) for 30 min followed by D-serine treatment for 30 min in the presence or absence of MK-801 (10 μM). Total STEP was immunoprecipitated with anti-STEP (23E5) antibody and probed with anti-STEP or anti-ubiquitin (PA1-10023) antibodies. Representative blots were from three independent experiments. **c** Neurons were pretreated with MK-801 (10 μM for 30 min) followed by D-serine treatment for 30 min. Surface proteins were labeled with 1 mg/ml EZ link sulfo-NHS-SS-biotin in PBS and pulled down with NeutrAvidin-agarose beads. Phospho-GluN2B, GluN2B, GluN1 and GABA_Aβ2/3 levels were examined in biotin-labeled samples and in RIPA extracts using specific

antibodies. Surface protein levels were normalized to GABA_Aβ2/3 levels, whereas protein levels in RIPA extracts were normalized to β-actin as loading control. Data were expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (* $P < 0.05$; ** $P < 0.05$, n = 4).

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Fig. 7.

D-serine leads to STEP₆₁ degradation in mice. **a** Male C57BL/6J mice were administrated with D-serine (DS, 600 mg/kg, i.p.) for 30 min, 1 h or 3 h. **b** Male C57BL/6J mice were administered D-serine (DS, 600 mg/kg, i.p.) for 30 min, followed by MK-801 treatment (0.15 mg/kg, i.p.) for 1 h. Frontal cortices were collected after treatment and processed to obtain synaptic membranes (P2). Samples were subjected to SDS-PAGE and examined for STEP₆₁, tyrosine phosphorylation of STEP substrates (GluN2B, Pyk2 and ERK1/2) and GluN1 levels with phospho-specific and pan-antibodies. Data were expressed as mean ±

SEM. Statistical significance was determined using one-way ANOVA with post hoc Tukey test (***) $P < 0.01$, $n = 4$).

Fig. 8. D-serine reverses MK-801-induced motor and cognitive deficits in mice. **a-c** Male C57BL/6 mice (3–6 months old) were administered vehicle (Veh) or D-serine (DS, 600 mg/kg, i.p.) followed by control (Veh/Veh) or MK-801 challenge (0.15 mg/kg, i.p.) for 1 h. Distance traveled every 5 min during the 90 min test period (**a**), total distance traveled (**b**) and total stereotypic counts (**c**) were recorded and analyzed using an activity monitor (MED Associates). **d** Mice were tested in the Y-maze 30 min post MK-801 injection. Spontaneous alternations were recorded and calculated using an ANY-Maze video tracking system. **e**

Mice were trained with two identical objects 30 min post MK-801 injection. Twenty-four hours post training, mice were tested in the choice phase with one familiar object and one novel object. Time spent with either object recorded and calculated using an ANY-Maze video tracking system. Data were expressed as mean $\pm$ SEM and statistical significance determined using two-way ANOVA with repeated measures followed by post hoc Bonferroni's test for the data shown in panel **a** or one-way ANOVA with post hoc Bonferroni's test for panels **b-d** or two-tailed Student's t-test for panel **e** (* P < 0.05; ** P < 0.01, n = 8 per group).