Regulation of STEP<sub>61</sub> and tyrosine-phosphorylation of NMDA and AMPA receptors during homeostatic synaptic plasticity

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Abstract

Background: Sustained changes in network activity cause homeostatic synaptic plasticity in part by altering the postsynaptic accumulation of N-methyl-D-aspartate receptors (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), which are primary mediators of excitatory synaptic transmission. A key trafficking modulator of NMDAR and AMPAR is STriatal-Enriched protein tyrosine Phosphatase (STEP<sub>61</sub>) that opposes synaptic strengthening through dephosphorylation of NMDAR subunit GluN2B and AMPAR subunit GluA2. However, the role of STEP<sub>61</sub> in homeostatic synaptic plasticity is unknown.

Findings: We demonstrate here that prolonged activity blockade leads to synaptic scaling, and a concurrent decrease in STEP<sub>61</sub> level and activity in rat dissociated hippocampal cultured neurons. Consistent with STEP<sub>61</sub> reduction, prolonged activity blockade enhances the tyrosine phosphorylation of GluN2B and GluA2 whereas increasing STEP<sub>61</sub> activity blocks this regulation and synaptic scaling. Conversely, prolonged activity enhancement increases STEP<sub>61</sub> level and activity, and reduces the tyrosine phosphorylation and level of GluN2B as well as GluA2 expression in a STEP<sub>61</sub>-dependent manner.

Conclusions: Given that STEP<sub>61</sub>-mediated dephosphorylation of GluN2B and GluA2 leads to their internalization, our results collectively suggest that activity-dependent regulation of STEP<sub>61</sub> and its substrates GluN2B and GluA2 may contribute to homeostatic stabilization of excitatory synapses.

Keywords: STEP, GluN2B, GluA2, Tyrosine phosphorylation, Tetrodotoxin, Bicuculline, Hippocampal neurons, Homeostatic plasticity, Synaptic scaling
synaptic plasticity [9, 10]. Of these activity-regulated transcripts, we identified *PTPN5* that encodes STEP [8]. The present study investigated whether *STEP61* contributes to homeostatic synaptic plasticity.

**Results and discussion**

**Prolonged alterations of hippocampal network activity regulate *STEP61* level and activity**

Prolonged blockade of network activity for 48 h with the sodium channel blocker tetrodotoxin (TTX) induced synaptic scaling in dissociated hippocampal cultured neurons as demonstrated previously [11, 12] (Fig. 1a–d), and reduced *STEP61* mRNA and protein expression compared to CTL treatment (Fig. 1e, f). Conversely, prolonged activity enhancement for 48 h using the GABA$_A$ receptor antagonist bicuculline (BC) increased *STEP61* protein level (Fig. 1g), but did not alter its mRNA level and the miniature excitatory postsynaptic current (mEPSC) (Fig. 1a–e).

To test whether prolonged TTX or BC treatment affects *STEP61* activity, we examined the phosphorylation of *STEP61* at Ser$^{221}$ within its kinase-interactive motif domain, which prevents *STEP61* interaction with all known substrates (Fig. 2a) [13]. TTX treatment for 36–48 h enhanced Ser$^{221}$-phosphorylation of *STEP61*, indicating decreased *STEP61* activity (Fig. 2b, d). In contrast, 36–48 h BC treatment reduced Ser$^{221}$-phosphorylation, indicating increased *STEP61* activity (Fig. 2c, d).

**Prolonged alterations of hippocampal network activity regulate Tyr-phosphorylation of GluN2B and GluA2 in a *STEP61*-dependent manner**

*STEP61* dephosphorylates the NMDAR subunit GluN2B at Tyr$^{1472}$ [5, 6] and reduces Tyr-phosphorylation of the AMPAR subunit GluA2 following group 1 metabotropic glutamate receptor (mGluR) stimulation [3]. Although the specific Tyr residues on GluA2 regulated by *STEP61* are...
unknown, the GluA2 phosphorylation state at Tyr^869, Tyr^873, and Tyr^876 (3Tyr) regulates AMPAR trafficking [14]. To determine if the TTX- or BC-induced changes in STEP^61 alter Tyr-phosphorylation of GluN2B and GluA2, we performed immunoblot analyses using specific antibodies to phosphorylated Tyr^1472 of GluN2B [6] and phosphorylated 3Tyr of GluA2 [14] (Fig. 3).

Consistent with the TTX-induced decrease in STEP^61 level and activity (Fig. 2b, d), prolonged TTX treatment increased the levels of Tyr^1472-phosphorylated GluN2B (GluN2B-pY^1472) and 3Tyr-phosphorylated GluA2 (GluA2-p3Y) compared to CTL treatment without affecting their total protein expression (Fig. 3a–c). In contrast, BC treatment for 24–48 h decreased the levels of GluN2B-pY^1472 and GluA2-p3Y (Fig. 3d–f), concurrently with an increase in STEP^61 level and activity (Fig. 2c, d). Interestingly, total levels of GluN2B and GluA2 were reduced by 48 h BC application (Fig. 3d, e).

We next examined if STEP^61 mediates the TTX- or BC-induced changes in Tyr-phosphorylation of GluN2B and GluA2. Transactivator of transcription (TAT) sequence was fused to STEP^46, allowing the TAT fusion proteins to bind constitutively to substrates but not to dephosphorylate them [13, 15, 16]. Consistently, introduction of TAT-STEP C/S in CTL-treated neurons inactivates STEP^46, allowing it to bind constitutively to substrates but not to dephosphorylate them [13, 15, 16].

Fig. 2 Prolonged alterations of hippocampal network activity regulate STEP^61 activity. a A schematic depicting the regulation of STEP^61 activity by its phosphorylation at Ser^221 within its kinase-interactive motif, a binding site for all STEP substrates. b–d Immunoblot analysis of STEP^61 and Ser^221-phosphorylated STEP^61 (STEP^61-pS^221) in hippocampal cultured neurons following CTL, TTX, or BC treatment for 24–48 h (*n = 3 per treatment). b, d Prolonged TTX treatment reduced STEP^61 protein level and activity. c, d Prolonged BC treatment enhanced STEP^61 protein level and activity. d The relative phosphorylation state of STEP^61, as calculated by the ratio of STEP^61-pS^221 level over total STEP^61 level. Data shown represent the mean ± SEM (*p < 0.05; **p < 0.01; ***p < 0.005).
significantly increased the levels of GluN2B-pY1472 and GluA2-p3Y compared to TAT-myc application (Fig. 4d, e, Additional file 1: Figure S1D). Preincubation with TAT-STEP C/S but not TAT-myc blocked the BC-induced reduction in the level of GluN2B-pY1472, total GluN2B, and total GluA2 but not GluA2-p3Y (Fig. 4d, e). Since specific Tyr residues regulated by STEP remain unknown, our analyses for GluA2-p3Y may not have revealed the effect of TAT-STEP C/S if STEP causes dephosphorylation of only one Tyr. Nonetheless, these results suggest that STEP mediates the BC-induced changes in Tyr1472-phosphorylation of GluN2B and abundance of GluN2B and GluA2.

Enhancement of STEP activity blocks synaptic scaling

Dephosphorylation of Tyr1472 within a conserved endocytic motif of GluN2B [17] via STEP reduces surface NMDAR level [4, 6] by clathrin-mediated internalization [18]. Furthermore, AMPAR internalization can be induced by mGluR stimulation through STEP [3] and by dephosphorylation of GluA2 at 3Tyr [14]. We hypothesized that prolonged activity blockade induces synaptic scaling (Fig. 1a–d) by inhibiting endocytosis of synaptic NMDARs and AMPARs upon STEP reduction (Fig. 1f, Fig. 2b). To test this, we enhanced STEP activity by administering TAT-STEP WT for 30 min prior to recording. In the presence of TAT-myc, 48 h TTX treatment increased the mEPSC amplitude but not frequency compared to CTL application (Fig. 5a–d). However, this TTX-induced synaptic scaling was abolished by TAT-STEP WT preincubation (Fig. 5a–d), indicating that STEP reduction contributes to synaptic scaling.

Interestingly, prolonged activity enhancement increased STEP (Fig. 1g, Fig. 2c) and decreased Tyr-phosphorylation of GluN2B and GluA2 (Fig. 3d, e, Fig. 4d, e) without inducing synaptic down-scaling (Fig. 1a–d), suggesting that this STEP upregulation may cause internalization of extrasynaptic GluN2B and GluA2. Indeed, activity-dependent AMPAR endocytosis requires GluA2 [19] and occurs extrasynaptically [20]. Similarly, GluN2B-containing NMDARs enriched in extrasynaptic sites [21] undergo robust endocytosis [17, 18]. The BC-induced STEP-dependent decrease in GluA2 and GluN2B abundance (Fig. 3d, e, Fig. 4d, e) may provide an additional homeostatic defense

- **Fig. 3** Prolonged alterations of hippocampal network activity regulate Tyr-phosphorylation and levels of GluN2B and GluA2. Immunoblot analysis of hippocampal cultured neurons that were treated for 48 h with CTL, 24–48 h TTX (a–c, or 24–48 h BC treatment (d–f) (n = 6 per treatment). a–c Prolonged TTX treatment increased the level of Tyr1472-phosphorylated GluN2B (GluN2B-pY1472) (a) and the level of GluA2 that were phosphorylated at Tyr869, Tyr873, and Tyr876 (3Tyr) (GluA2-p3Y) (b). d–f Prolonged BC treatment decreased the levels of GluN2B-pY1472 (d) and GluA2-p3Y (e). Total GluN2B and GluA2 levels were reduced at 48 h BC application. f The relative phosphorylation state of GluN2B and GluA2 as calculated by the ratio of phosphorylated proteins over total proteins followed by normalization to GAPDH. Data shown represent the mean ± SEM (*p < 0.05; **p < 0.01)
to limit membrane depolarization and overstimulation of extrasynaptic GluN2B-containing NMDARs, which is shown to cause excitotoxicity [22].

It remains unknown how prolonged activity perturbation regulates STEP$_{61}$. Previous studies have reported that Ser$^{221}$ of STEP$_{61}$ is dephosphorylated by calcium-dependent calcineurin upon NMDAR activation [13] and phosphorylated by protein kinase A (PKA) upon stimulation of dopamine D1 receptor [23]. Interestingly, synaptic scaling is shown to involve reduced calcium influx to the
postsynaptic neuron [9], reduced calcineurin activity [24], and enhanced PKA activity at excitatory synapses [25]. Hence, prolonged activity blockade could increase Ser\(^{221}\)-phosphorylation of STEP\(_{61}\) (Fig. 2b, d) by reduced calcineurin activity and/or enhanced PKA activity, in addition to decreasing STEP\(_{61}\) level by transcriptional down-regulation (Fig. 1e, f). Considering that a loss of PKA from synapses was found during synaptic downscaling [25], reduced PKA activity may contribute to the BC-induced decrease in Ser\(^{221}\)-phosphorylation of STEP\(_{61}\) (Fig. 2c, d).

**Conclusions**
In summary, we demonstrate a bidirectional modulation of STEP\(_{61}\) level and activity by prolonged alterations of hippocampal network activity, resulting in correlative changes in Tyr-phosphorylation of STEP\(_{61}\) substrates, GluN2B and GluA2. We also show that the reduction in STEP\(_{61}\) contributes to synaptic scaling. Future studies should test if this regulation alters NMDAR and AMPAR surface density during homeostatic synaptic plasticity (Fig. 5e). Investigating how prolonged activity perturbation regulates STEP\(_{61}\) should provide mechanistic insights into the dysregulation of STEP\(_{61}\) expression, which are present in multiple neuropathologies [2].

**Materials and methods**

**Hippocampal neuronal culture**
The Institutional Animal Care and Use Committee at the University of Illinois Urbana-Champaign approved all experimental procedures involving animals. Primary dissociated hippocampal cultures were prepared from Sprague–Dawley rat embryos at embryonic day 18 and plated at high density (330 cells/mm\(^2\)) as described [8]. At 10–13 days in vitro, neurons were treated for 24–48 h
with vehicle control (0.1 % dH₂O), TTX (1 μM), and BC (20 μM) (all Tocris).

Electrophysiology
Whole-cell patch-clamp recordings of mEPSCs (>150 events per neuron) were performed at 23–25 °C from pyramidal neurons held at −60 mV in external solution containing 1 μM TTX and 20 μM BC as described [12, 25] using a Multichannel 700B amplifier, Digidata1440A, and the pClamp 10.2 (Molecular Devices). Signals were acquired 3 min after making the whole-cell configuration, filtered at 1 kHz, and sampled at 10 kHz on gap free mode (5 min). The mEPSCs were detected with a 10 pA thresholds and analyzed by Mini Analysis (Synaptosoft).

QPCR
The QPCR was performed with the StepOnePlus real-time PCR system (Applied Biosystems) using total RNA (1–2 μg) as described [8]. The forward and reverse primer sequences for PTTP5 were 5′-GGAGTCCGCCATGAA TACC-3′ and 5′-CAGACGTACCCCTGCTGTGAG-3′ respectively. The primer sequences for GAPDH has been previously described [8]. Following normalization to control GAPDH cDNA levels, the fold change of PTTP5 cDNA levels for each treatment compared to control was determined.

Immunoblot analysis
Neuronal lysate samples were prepared in RIPA buffer supplemented with protease inhibitors and Tyr phosphatase inhibitors (1 mM NaVO₃, 10 mM Na₃O₄P₂, and 50 mM NaF) as described [8] and were subjected to immunoblot analysis with primary antibodies against STEPα₁ (Santa Cruz), STEPα₁-pS²²¹[13]), GluN2B and GluA2 (Millpore), GluN2B-pY¹⁴⁷² (PhosphoSolutions), GluA2-p3Y and GAPDH (Cell Signaling). Densitometric quantification following normalization to GAPDH was performed with ImageJ software (National Institutes of Health).

Immunocytochemistry
Permeabilized immunostaining were performed with anti-myc antibodies (Thermo-Scientific) as described [12, 25]. Fluorescence images of the neurons were acquired using the same exposure time and analyzed with ImageJ to compare their background-subtracted fluorescence intensities.

Statistical analyses
Using Origin 9.1 (Origin Lab), the Student’s t test and one-way ANOVA with Tukey’s and Fisher’s multiple comparison tests were performed to identify the statistically significant difference with a priori value (p) < 0.05 between 2 groups and for >3 groups, respectively.

Additional file
Additional file 1: Figure S1. Membrane-permeable TAT-STEP WT or C/S proteins alter STEPα₁–dependent Tyr-phosphorylation of GluN2B and GluA2. (A) Permeabilized immunostaining of cultured hippocampal neurons at 12 days in vitro that were incubated for 30 min with no fusion proteins (None), TAT-myc, TAT-STEP WT, or TAT-STEP C/S. C.S bars are 20 μm. (B) Background subtracted, mean intensity of myc fluorescence (n = 10–19 images per treatment). AU, arbitrary unit. (C–D) Quantification of the levels of Tyr²⁸⁶-phosphorylated GluN2B (GluN2B-pY²⁸⁶) and the level of GluA2 that were phosphorylated at Tyr 86⁹, Tyr 87³, and Tyr 87⁶ ((GluA2-p3Y) in CTX-treated neurons (from Fig. 4b–e) that were incubated with TAT-fusion proteins for 30 min. (C) TAT-STEP WT decreases basal Tyr-phosphorylation of GluN2B and GluA2, confirming that TAT-STEP WT increases STEP activity. (D) TAT-STEP C/S increases basal Tyr-phosphorylation of GluN2B and GluA2, confirming its ability to block dephosphorylation of STEP substrates. Data shown represent the mean ± SEM (*p < 0.05; **p < 0.01). (PDF 969 kb)

Abbreviations
STEP: STriatal-Enriched protein tyrosine Phosphatase; Ser: Serine; Tyr: Tyrosine; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDAR: N-methyl-D-aspartate receptor; CTL: Control; TTX: Tetrodotoxin; BC: Bicuculline; Pka: Protein kinase A; mEPSC: miniature excitatory postsynaptic current; QPCR: real-time quantitative polymerase chain reaction.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Under HJC’s direction, SSJ and SER designed the study and drafted the manuscript. HJC, SER and SSJ participated in its design and coordination. SSJ, SER, SSJ, and NIH funding MH052711 and MH091037 (PJL). Acknowledgements
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