Proteolytic Degradation of Hippocampal $STEP_{61}$ in LTP and Learning

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Abstract

Striatal-enriched protein tyrosine phosphatase (STEP) modulates key signaling molecules involved in synaptic plasticity and neuronal function. It is postulated that STEP opposes the development of long-term potentiation (LTP) and that it exerts a restraint on long-term memory (LTM). Here, we examined whether $STEP_{61}$ levels are regulated during hippocampal LTP and after training in hippocampal-dependent tasks. We found that after inducing LTP by high frequency stimulation or theta- burst stimulation $STEP_{61}$ levels were significantly reduced, with a concomitant increase of $STEP_{33}$ levels, a product of calpain cleavage. Importantly, inhibition of STEP with TC-2153 improved LTP in hippocampal slices. Moreover, we observed that after training in the passive avoidance and the T-maze spontaneous alternation task, hippocampal $STEP_{61}$ levels were significantly reduced, but $STEP_{33}$ levels were unchanged. Yet, hippocampal BDNF content and TrkB levels were increased in trained mice, and it is known that BDNF promotes STEP degradation through the proteasome. Accordingly, hippocampal $pTrkB^{Tyr816}$, $pPLC\gamma^{Tyr783}$, and protein ubiquitination levels were increased in T-SAT trained mice. Remarkably, injection of the TrkB antagonist ANA-12 (2 mg/Kg, but not 0.5 mg/Kg) elicited LTM deficits and promoted $STEP_{61}$ accumulation in the hippocampus. Also, STEP knockout mice outperformed wild-type animals in an age- and test-dependent manner. Summarizing, $STEP_{61}$ undergoes proteolytic degradation in conditions leading to synaptic strengthening and memory formation, thus highlighting its role as a molecular constrain, which is removed to enable the activation of pathways important for plasticity processes.

Keywords Striatal-enriched protein tyrosine phosphatase · Calpains · BDNF · Proteasome · ANA-12

Introduction

Striatal-enriched protein tyrosine phosphatase (STEP; PTPN5), a neural-specific tyrosine phosphatase highly expressed in the striatum, and at lower levels in the cortex, hippocampus and amygdala, modulates key signaling molecules that are involved in synaptic plasticity and neuronal function. STEP dephosphorylates its target substrates such as the kinases ERK1/2, Pyk2 and Fyn, leading to their inactivation, and NMDA receptor (NMDAR) and AMPA receptor subunits, promoting their endocytosis (reviewed in [1]). Interestingly, STEP administration into the hippocampal CA1 neurons [2] or the lateral amygdala [3] prevents tetanus-induced long-term potentiation (LTP), whereas inhibiting STEP in CA1 neurons enhances transmission and occludes LTP induction [2]. Moreover, genetic deletion of STEP improves LTP in the lateral amygdala [4] and in hippocampal slices when induced by theta-burst stimulation (TBS) [5], but no differences were reported in the case of LTP induced by high frequency stimulation (HFS) [6]. In addition, it has recently been demonstrated that regulation of STEP levels participates in homeostatic synaptic plasticity in primary hippocampal neurons [7].

STEP KO mice display enhanced ERK1/2 phosphorylation in the striatum, hippocampus and central/lateral amygdala compared to wild-type littermates [4, 5, 8, 9]. They also have enhanced tyrosine phosphorylation of the GluN2B subunit of the NMDAR and Pyk2, as well as an increased phosphorylation of ERK1/2 substrates such as CREB and Elk1 in the hippocampus [8]. Moreover, STEP KO mice perform better during a reversal-training task in the Morris water maze [8] and have greater fear memory [4] than wild-type mice. Conversely, administration of TAT-STEP into the lateral amygdala disrupts fear memory consolidation [3].

Accumulating evidence indicates that protein degradation is a critical regulator of memory formation and stability, and both calpain [10–12] and proteasome [13–17] activities have been shown to participate in

synaptic plasticity and learning and memory processes. Remarkably, STEP levels are regulated via cleavage by calpains at the kinase interacting motif domain, the binding site for all STEP substrates. Cleavage at this site produces a truncated STEP product, $STEP_{33}$, which is unable to bind to and dephosphorylate its substrates [18]. Another mechanism for reducing STEP levels is by ubiquitination and degradation through the ubiquitin-proteasome system (UPS) [18–21].

The current functional model postulates that STEP opposes the development of LTP and promotes long-term depression (LTD) and that STEP, by inactivating its substrates, exerts a restraint on long-term memory (LTM). Thus, it is likely that STEP levels are regulated in response to stimuli that promote synaptic strengthening and hippocampal-dependent memory formation. Strikingly, this paradigm has not been directly

addressed. In the present study, we sought to examine whether STEP levels are regulated during hippocampal LTP and after training in hippocampal-dependent tasks. The main finding of this study is that STEP levels are reduced by proteolytic degradation in conditions leading to synaptic strengthening and memory formation, which underscores the idea that protein processing relieves signaling constrains in order to activate downstream pathways that are important for plasticity.

Material and Methods

Mice

Mice were housed under a standard 12:12 h light/dark cycle with access to food and water ad libitum in a colony room kept at 20–24 °C and 40–50% humidity. All animal-related procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committees of Universitat de Barcelona and Universidad de Castilla-La Mancha, following European (2010/63/UE) and Spanish (RD 53/2013) regulations.

Electrophysiology

Transverse slices (350–400 μm thick) of brains from female mice (Crl:NMRI(Han) strain) were cut with a vibratome (VT1200S, Leica Microsystems, Nussloch, Deutschland) and incubated for at least

1 h at room temperature (21– 24 °C) in artificial cerebrospinal fluid (aCSF) that contained (in mM) 124 NaCl, 2.69 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$,2 $CaCl_2$, and 10 glucose, and gassed with a 95% O_2 and 5% CO_2 mixture to attain a pH of 7.2–7.4. Individual slices were transferred to an immersion recording chamber where they were completely submerged into oxygenated aCSF flowing at 2.5 ml/min, and 29.5 °C. Field excitatory postsynaptic potentials (fEPSPs) were recorded via a carbon fiber microelectrode (Carbostar-1, Kation Scientific, Minneapolis, MN) placed in the stratum radiatum of the CA1 pyramidal layer. Evoked fEPSPs were elicited by stim- ulation of the Schaffer collateral fibers with an extracellular bipolar tungsten electrode via a 2100 isolated pulse stimulator (A-M Systems, Inc., Carlsborg, WA). The stimulation inten- sity was adjusted to obtain a fEPSP slope that was approximately 50% of maximal fEPSP size. Data were stored in a Pentium-based PC through an acquisition system PowerLab 4/26 (AD Instruments, Bella Vista, Australia) and the software Scope (AD Instruments) was used to display fEPSPs and measurements of fEPSPs slopes. After recording stable baseline responses for 10 min, LTP was elicited by electrical stimulation of the Schaffer collaterals pathway, using different proto- cols: (1) strong HFS composed of four trains of pulses given at100 Hz, lasting 1 s and spaced 20 s; (2) weak HFS consisted of one single train of pulses given at 100 Hz, lasting 1 s; and (3) the TBS consisted of ten bursts of five pulses given at 100 Hz, spaced 200 ms, and potentiation was measured for 60 min. To test the effect of STEP inhibition, 10 μM TC-2153 [22] was added for 60 min before starting the recording and was present throughout the experiment. All fEPSPs slope values were relativized and expressed as percentage of basal mean slope within each group. To obtain controls for biochemical studies, some slices were subjected to the same protocol in the absence of stimulation. Hippocampi were dissected from the brain slices to perform Western blot analysis. The dissection was made manually by careful removal of surrounding tissues. The slices were always submerged in aCSF at room temperature.

Training in Hippocampal-Dependent Tasks and LTM Assessment

To investigate the effect of training on hippocampal $STEP_{61}$ levels, 3-month-old C57BL/6J male mice were used. Training in the passive avoidance (PA) task was performed as previously described [23].

Mice were placed in the light chamber and the door between the two chambers was opened 5 s later. When the mouse entered the dark chamber, the sliding door was closed and a foot-shock (45 V, 2 s) was delivered through the grid. Twenty seconds later, mice were returned to the home cage and they were sacrificed after 30 min. During training in the T-maze spontaneous alternation task (T-SAT), one arm of the T was closed (novel arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 min, after which they were returned to the home cage. Mice were sacrificed at different time points (15 min to 2 h) after the training trial.

To investigate the effect of TrkB blockade on LTM and STEP levels, mice received an intraperitoneal injection of saline, 0.5 or 2 mg/Kg ANA-12 (Sigma-Aldrich, St Louis, MO), a TrkB antagonist [24], immediately before training in the T- SAT as described above. Some mice were sacrificed 1 h post training, and others were tested 4 h later. For that, mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. The arm preference was measured as the time exploring each arm × 100/total time exploring. Mice were sacrificed after testing and the hippocampi processed for biochemical analysis.

To assess hippocampal-dependent learning and memory in STEP KO mice, we performed the novel object recognition test (NORT), the novel object location test (NOLT) and the T- SAT in 3- and 12-month-old mice. NORT was performed as previously described [25] and T-SAT as detailed above. Briefly, in the NORT, mice were first habituated to the arena in the absence of objects (3 days, 15 min/day). On the fourth day, two similar objects were presented to each mouse during 10 min after which they were returned to their home cage. Twenty-four hours later, mice were tested for 5 min in the arena with a familiar and a new object. Spatial LTM was assessed by using the NOLT as previously described [26]. The device consisted of a square (45 × 45 cm) arena. Mice were first habituated to the arena in the absence of objects (two trials of 15 min, 4 h inter-trial interval). On the following day, two similar objects were presented to each mouse during 10 min. Spatial cues were provided on the wall of the arena, such that animals could detect differences in object location. Twenty-four hours later, mice were tested for 5 min in the arena with the same objects but placement of one

of the objects was changed in respect to the first exposure. The position of the new object (in NORT), the new position of the object (in NOLT), and the new arm (in T-SAT) were counterbalanced between mice. The object/location/arm preference was measured as the time exploring each object/location/arm × 100/ total time exploring. Animals were tracked and recorded with SMART Junior software (Panlab, Spain).

Western Blot Analysis

Non-stimulated hippocampal slices and slices subjected to HFS or TBS were collected and homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$, and 10 mM EGTA plus protease inhibitors [phenylmethylsulphonyl fluoride, PMSF (2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and sodium orthovanadate (1 mM)]. Three to four slices were pooled for each sample number. Hippocampi from in vivo experiments were homogenized in lysis buffer as above. The lysates were centrifuged at 16100 × *g* for 20 min, supernatants were collected, and protein concentration was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA). Western blot analyses were performed as previously described [27]. The primary antibodies used were anti-STEP (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-spectrin (1:2000; Chemicon International, Temecula, CA), anti-BDNF (Santa Cruz Biotechnology), anti- TrkB (BD Transduction Laboratories, San Jose, CA), anti- $pTrkB^{Tyr816}$ (Abcam, Cambridge, UK), anti-$pPLC\gamma^{Tyr783}$ and anti-PLCγ (Cell Signaling, Beverly, MA), and anti-ubiquitin (1:4000; DakoCytomation, Denmark). The relative abundance of $STEP_{61}$ and $STEP_{33}$ is quite different because $STEP_{33}$ levels are very low in control conditions ([19, 28]; see also Fig. 1f, for example). Therefore, representative immunoblots for $STEP_{61}$ and $STEP_{33}$ cannot usually be obtained from the same film exposition since to detect the 33 kDa isoform, signal from $STEP_{61}$ becomes overexposed (Supp. Fig. 1). Moreover, in some cases we needed to load more protein and/or incubate the membrane with less diluted anti-STEP (1:500) in order to detect the $STEP_{33}$ isoform. Loading control was performed by reprobing the membranes with an anti-α-tubulin antibody (1:50000; Sigma-Aldrich) for 15–20 min at room temperature. Then, membranes were washed with TBS-T (Tris-buffered saline containing 0.1% Tween 20), incubated for 1 h (15–20 min for

loading controls) at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody (1:2000; Promega, Madison, WI), and washed again with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics; Warrendale, PA).

Immunoprecipitation Assay

Probes were performed in order to investigate whether immunoprecipitation (IP) of STEP from native or denatured samples gives different ubiquitination patterns related to the detection of ubiquitinated protein(s) associated with immunoprecipitated STEP. Sample denaturation before IP was performed by incubating the extracts in the presence of 1% SDS for 10 min at 100 °C. Then, SDS concentration was reduced to 0.1% by the addition of lysis buffer and samples were incubated for 30 min on ice. Since no differences were found in the ubiquitination patterns of $STEP_{61}$ under native and denaturing conditions (Supp. Fig. 2), the IP was performed in native conditions. Hippocampal protein extracts (200 µg) were diluted in 200 µl ice-cold IP buffer containing 40 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 0.3% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate. Samples were incubated overnight at 4 °C on a tube rotator with 2 µg anti-STEP antibody (Santa Cruz Biotechnology) or AffiniPure mouse anti-human IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA) as a negative control. The immune complexes were precipitated overnight at 4 °C by the addition of 25 µl protein A/G plus- Agarose (Santa Cruz Biotechnology). Beads were collected by centrifugation (5 min, 3300 × *g* at 4 °C) and washed three times with IP buffer and once with wash buffer (50 mM Hepes, pH = 7.5, 40 mM NaCl, 2 mM EDTA). Then, bound proteins were eluted using SDS sample buffer; samples were boiled for 5 min at 100 °C and subjected to SDS-PAGE. Ubiquitinated $STEP_{61}$ was quantified by accounting the total smear (MW > 61 kDa) from each lane.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed by using the unpaired Student's *t* test (95% confidence). Values of $P < 0.05$ were considered as statistically significant.

Results

HFS and TBS Induce $STEP_{61}$ Cleavage in Hippocampal Slices

In order to investigate whether $STEP_{61}$ levels are regulated in response to electrical stimulation of the Schaffer collateral pathway, we collected hippocampal slices 60 min after inducing LTP with strong HFS (4 × 100 Hz trains; Fig. 1a) and analyzed $STEP_{61}$ levels by Western blot. We found that $STEP_{61}$ levels were significantly reduced, with a concomitant increase of $STEP_{33}$ levels (Fig. 1b). The finding that $STEP_{33}$ levels were increased in hippocampal slices upon HFS indicated that calpains were implicated since these proteases cleave $STEP_{61}$ and generate a 33-kDa fragment [18]. Western blot analysis of spectrin breakdown products (SBDPs) at 145–150 kDa, which are specifically generated by calpain-dependent cleavage, confirmed that there was calpain activation in hippocampal slices subjected to HFS (control, 99.99 ± 8.49% and HFS, 182.75 ± 11.29%; $n = 6–10$; $P = 0.0002$; Student's *t* test; Fig. 1b).

It has recently been shown that different signaling path- ways are implicated in LTP induction by different patterns of electrical stimulation [29]. Therefore, we next analyzed STEP levels after inducing LTP by using a single train of 100 Hz for 1 s, or a TBS protocol. Induction of LTP with a 100 Hz, 1 s train (Fig. 1c) reduced $STEP_{61}$ levels, with an increase of $STEP_{33}$ levels (Fig. 1d). Accordingly, there was an almost significant increase of SBDPs levels at 145–150 kDa in hippocampal slices subjected to one train of 100 Hz, 1 s (control, 100.03 ± 10.88% and HFS, 140.58 ± 16.37%; $n = 5–8$; $P = 0.0542$; Student's *t* test; Fig. 1d). Similarly, we found that TBS-induced LTP (Fig. 1e) promoted a significant reduction of $STEP_{61}$ levels, and this effect was paralleled by a significant increase of $STEP_{33}$ levels (Fig. 1f). In agreement, SBDPs levels were also significantly higher in hippocampal slices receiving TBS as compared to control slices (control, 100.03 ± 14.00% and TBS, 166.66 ± 7.40%; $n = 5–$

10; $P = 0.0004$; Student's *t* test; Fig. 1f).

Pharmacological Inhibition of STEP Improves Hippocampal LTP

Acute STEP inhibition in CA1 neurons using an anti-STEP antibody enhances synaptic transmission [2], whereas STEP KO mice have unaffected basal synaptic transmission in the lateral amygdala [4], suggesting that compensatory mechanisms occur in mice with *PTPN5* deletion. Therefore, we test- ed the effect of an acute pharmacological inhibition of STEP on LTP by using TC-2153, a recently characterized STEP inhibitor [22]. For that we pre-incubated hippocampal slices with 10 μM TC-2153 for 60 min and stimulated the Schaffer collateral pathway with a single train of 100 Hz for 1 s. We found that 60 min later LTP was significantly higher in slices exposed to STEP inhibitor than in non-treated ones (control, 107.14 ± 0.92% vs TC-2153, 115.13 ± 1.73%; $n = 9$/group; $P = 0.0008$; Student's *t* test; Fig. 2).

Training in Hippocampal-Dependent Memory Tasks Leads to a Reduction of $STEP_{61}$ Levels

Next, we were interested in addressing whether STEP levels are regulated in a more physiological condition, for instance during memory formation. For that, 3-month-old wild-type mice were trained in the PA and T-SAT, and STEP protein levels were analyzed after training. We observed that 30 min after training in the PA $STEP_{61}$ levels were significantly reduced in the hippocampus as compared to non-trained control mice (Fig. 3a). Similarly, 1 h after training in the T-SAT hippocampal $STEP_{61}$ levels were lower in trained than in control mice (Fig. 3a).

TrkB Antagonism Promotes $STEP_{61}$ and LTM Impairment Accumulation

$STEP_{61}$ levels were reduced due to calpain cleavage (Fig. 1), $STEP_{33}$ levels were not altered in the hippocampus of trained mice (Fig. 3a), indicating that calpains were not responsible for this decrease of $STEP_{61}$ levels. In this line, analysis of SBDPs levels after training also indicated that there was no calpain activation (PA, naïve mice 99.91 ± 9.13% and trained mice 107.08 ± 10.24%, $n = 6–7$/group, $P = 0.6166$, Student's *t* test; T-SAT, naïve mice 100.03 ± 9.22% and trained mice 68.86 ± 10.01%, $n = 5–7$/group, $P = 0.0478$, Student's *t* test). We have recently reported that BDNF promotes $STEP_{61}$ degradation in cultured hippocampal neurons [19]. Thus, we next examined BDNF levels in trained

mice. We found that hippocampal BDNF content was increased in mice trained in PA and T-SAT compared to control animals (Fig. 3b). Moreover, we detected higher TrkB levels in the hippocampus of T-SAT trained mice compared to naïve animals, but no changes were detected in mice trained in the PA (Fig. 3c).

Since BDNF promotes STEP degradation through the UPS in a PLCγ-dependent manner [19], we then analyzed BDNF signaling focusing on the T-SAT. We found increased $pTrkB^{Tyr816}$ (Fig. 4a) and $pPLC\gamma^{Tyr783}$ (Fig. 4b) levels in the hippocampus of trained mice. Interestingly, we also detected increased protein ubiquitination in total extracts obtained from the hippocampus of mice trained in the T-SAT (Fig. 4c). Next, we investigated whether reduced hippocampal $STEP_{61}$ levels after training were due to increased ubiquitination and subsequent proteasome degradation. For that, we immunoprecipitated STEP from hippocampal extracts obtained from naïve and from T-SAT-trained mice, and performed Western blot against ubiquitin. We found that the levels of ubiquitin-conjugated $STEP_{61}$ 1 h after training in the T-SAT were similar in the hippocampus of naïve and trained mice ($P = 0.4948$; Fig. 4d). Thus, we hy pothesized that the pool of $STEP_{61}$ susceptible to UPS-mediated degradation upon training was already fully degraded 1 h post- training, so that differences in ubiquitin-conjugated $STEP_{61}$ levels could no longer be detected. In support of this assumption, we found that 2 h after training $STEP_{61}$ levels were not significantly different from those detected at 1 h (1 h post-training, 73.99 ± 5.77% of naïve values, Fig. 3a; 2 h post-training, 71.11 ± 7.66% of naïve values; $P = 0.7778$, Student's *t* test). Analysis at time-points earlier than 1 h did not show significant changes in $STEP_{61}$ levels in the hippocampus of trained mice ($P = 0.4437$; one-way ANOVA followed by Dunnett's multiple com- parison test; Supp. Fig. 3).

Given that hippocampal BDNF content and signaling was increased after training and was paralleled by reduced $STEP_{61}$ levels, we hypothesized that preventing BDNF signaling would have an impact on $STEP_{61}$ levels and LTM formation. To investigate the effect of TrkB blockade on LTM and STEP levels mice were injected with saline or the TrkB antagonist ANA-12 [24] immediately before training in the T-SAT. We did not detect any difference in $STEP_{61}$ levels 1 h after training when comparing mice

injected with saline or with 2 mg/kg ANA-12 (saline, 100.11 ± 3.78% and ANA-12, 108.52 ± 7.27%; *n* = 9–10/group, *P* = 0.3353, Student's *t* test). In agreement with this result, there was no effect on $pTrkB^{Tyr816}$ levels in ANA-12-treated mice 1 h post- injection plus training (saline, 99.80 ± 4.60% and ANA-12, 92.14 ± 9.69%; *n* = 9–10/group; *P* = 0.5005, Student's *t* test). Given that the effect of ANA-12 on pTrkB levels seems to accumulate over time in different brain regions [24], we decided to analyze LTM in the T-SAT. When we tested mice 4 h after training, we found that mice injected with saline before training explored significantly more on the new arm of the T- maze, an evidence of LTM for the old arm (Fig. 5a). Conversely, mice treated with 2 mg/Kg ANA-12 showed LTM impairment as they explored the old arm significantly more than the new one (Fig. 5a). The levels of $pTrkB^{Tyr816}$ were reduced in ANA-12-injected mice, but statistical significance was not reached (saline, 100.00 ± 16.01% and ANA- 12, 66.95 ± 7.11; *n* = 6/group; *P* = 0.0885; Student's *t* test). Remarkably, this cognitive impairment was accompanied by increased $STEP_{61}$ levels in the hippocampus of ANA-12-injected mice (Fig. 5b), suggesting reduced proteasomal degradation. Accordingly, we found a trend toward reduced $STEP_{61}$ ubiquitination in the hippocampus of ANA-12- injected mice (*P* = 0.0895; Student's *t* test; Fig. 5c). Reinforcing our hypothesis, mice injected with 0.5 mg/Kg ANA-12 showed no LTM impairment in the T-SAT (Supp. Fig. 4a), and their hippocampal $STEP_{61}$ levels were not significantly different from saline-injected mice (Supp. Fig. 4b).

Improved Performance in Hippocampal Memory Tests in STEP KO Mice Is Age- and Test-Dependent

Finally, given that $STEP_{61}$ levels were reduced in the hippo- campus of trained mice (Fig. 3a), suggesting its involvement in memory processes, we performed the NORT, NOLT, and T-SAT in wild-type and STEP KO mice at 3 and 12 months of age. The PA paradigm was not used because we recently reported that STEP plays a regulatory role in nociception, and STEP KO mice have thermal hyperalgesia and mechanical allodynia [30], which would interfere with the interpretation of the results. Mice from both genotypes spent significantly more time exploring the novel object, location, and arm, respectively, at both ages tested. We found that at 3 months of age, STEP KO mice performed better in the NORT, but the performance in the NOLT and T-SAT was similar to their wild-type

littermates (Fig. 6a). At 12 months of age, no differences were found in the preference for the novel object or arm between genotypes, but STEP KO mice performed better than control mice in the NOLT (Fig. 6b). The improved spatial memory of STEP KO mice in the NOLT was not related to worsening of wild-type mice, which performed similarly at hippocampal-dependent tasks. Our results also show that pharmacological inhibition of STEP increases hippocampal LTP, and that blockade of TrkB in vivo induces $STEP_{61}$ accumulation in the hippocampus and impairs LTM formation. Also, we observed improved cognitive performance in STEP KO mice that is age- and test-dependent.

Our findings indicate that hippocampal $STEP_{61}$ is cleaved by calpains after inducing LTP at the Schaffer collateral pathway, either by using a HFS or a TBS protocol. In this line, the three protocols of stimulation tested induced the appearance of the , as well as the generation of SBDPs at 145–150 kDa, a commonly used evidence of calpain activation. Accordingly, several studies using calpain inhibitors and genetic approaches indicate that calpains are implicated in mediating LTP at hippocampal CA1 synapses (reviewed in [12]). HFS of slices from the lateral amygdala does not produce changes in STEP levels [3], and LTP induction in spinal dorsal horn does not affect total $STEP_{61}$ levels, but promotes its phosphorylation-mediated inactivation [31]. Diverse pathways are engaged in LTP induced by different patterns of electrical stimulation [32–35]. For instance, PKA-dependency is limited to LTP induced by using multi-train and temporally spaced, but not massed, stimuli [36–38]. Another example is that TBS-LTP is resistant to inhibition by Aβ42, but LTP and post-tetanic potentiation induced by HFS are significantly blocked by the Aβ42 peptide [39]. Also, inhibition of BDNF/TrkB signaling

Discussion

A major finding of this study is that the tyrosine phosphatase $STEP_{61}$ undergoes proteolytic degradation following LTP in- duction in hippocampal slices and after training in

attenuates LTP induced by TBS, but not by HFS [40–43]. Thus, LTP can be induced by a variety of stimulation protocols that may work through alternative signaling pathways and converge on a common set of mechanisms involved in the maintenance of LTP (reviewed in [44]). Therefore, the use of different stimulation protocols across laboratories might complicate interpretation and direct comparison of results from different groups. Overall, our finding that $STEP_{61}$ is cleaved after electrical stim- ulation leading to LTP at CA1 neurons is compatible with the current model, which postulates that STEP opposes the development of synaptic strengthening. In agreement, we found that or LTD compared to controls [46]. It is likely that the use of different stimulation protocols and experimental conditions con- tribute to these discrepancies. It is noteworthy that although it is unknown whether LTD is paralleled by increased STEP levels, it has been shown that STEP expression increases in hippocampal slices and primary cultures in response to the selective metabotropic glutamate receptor (mGluR) agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) [47]. Since DHPG can induce mGluR-dependent LTD [48, 49], it is likely that LTP and LTD are paralleled by opposite changes in STEP levels. In addition, our results further support the view that protein cleavage relieves signaling or physical constrains to initiate signaling cascades that are relevant for plasticity. For example, in hippocampal slices stimulated with KCl [50] or subjected to TBS [51] there is a calpain-mediated transient downregulation of a negative regulator of the MAPK pathway. Also, upregulation of protein synthesis after TBS has been shown to be mediated by the calpain-dependent cleavage of a translational repressor [52]. Activating transcription factor 4, a repressor of CREB, undergoes robust proteasome-dependent degradation following chemical induction of LTP [53], and proteasome degradation of MOV10 upon KCl depolarization allows protein translation in hippocampal neurons [54].

In the present study, we also demonstrate that $STEP_{61}$ levels were reduced in the hippocampus upon training in the PA and T-SAT. In contrast to the observations in hippocampal slices pharmacological inhibition of STEP with TC-2153 improved LTP induced by a single train of HFS. Moreover, administration of STEP into CA1 neurons [2], the substrate trapping TAT- STEP into the lateral amygdala [3] or the striatum [45], or over- expression of STEP in the spinal dorsal horn [31] prevent LTP. However, although TBS-LTP was reported to be higher in the hippocampus of STEP KO than in wild-type mice [5], HFS-LTP was similar in both genotypes [6]. In addition, studies performing *PTPN5* downregulation and overexpression showed no significant differences in the magnitude of hippocampal LTP subjected to electrical stimulation, this phenomenon was not mediated by calpains as it was not accompanied by the accumulation of the cleaved fragment $STEP_{33}$. However, the reduction of hippocampal $STEP_{61}$ levels after training was paralleled by increased levels of BDNF. In agreement, it is known that BDNF levels increase in the hippocampus after training for memory tasks [55–58]. Remarkably, we have shown that BDNF promotes STEP degradation through the UPS [19], and one of the proposed functions of the UPS is the degradation of neural plasticity inhibitors to remove inhibitory constraints on memory formation [54, 59, 60]. In this line, here, we detect- ed increased total protein ubiquitination after training in the T- SAT, and similar findings were reported after training in inhibitory avoidance [61]. This indicates that in the hippocampus of trained mice, in addition to STEP, other molecular restraints are degraded. The proteasome degradation of these ubiquitinated proteins will follow a protein-dependent time-course as total protein ubiquitination, but not STEP ubiquitination, was found to be increased 1 h after training in the T-SAT. Indeed, no significant changes in $STEP_{61}$ levels were detected at time- points earlier than 1 h, no differences in ubiquitin-conjugated $STEP_{61}$ levels were found1h after training, and $STEP_{61}$ levels at 1 and 2 h post-training were similar. These findings suggest that the pool of $STEP_{61}$ susceptible to UPS-mediated degradation upon training was completely degraded 1 h post-training in a very rapid and plastic manner. Conversely, we found reduced STEP levels and we could detect increased levels of ubiquitin-conjugated STEP in the striatum of aged mice [62], which can be considered a scenario of plastic sustained changes over time. Importantly, we found that in conditions

where blockade of TrkB using ANA-12 promoted $STEP_{61}$ accumulation in the hippocampus there was LTM impairment. Actually, several studies indicate that preventing protein degradation impairs memory consolidation [60, 61, 63–66]. Conversely, injection of 0.5 mg/Kg ANA-12 pre-training induced no accumulation of $STEP_{61}$ in the hippocampus and no cognitive impairment in the T-SAT. Similarly, injection of ANA-12 up to 0.6 mg/Kg pre- acquisition did not induce cognitive deficits in the retention phase of the PA [67]. However, others have reported that hippocampal infusion of ANA-12 in rats impairs memory consol idation in the inhibitory avoidance test [68].

The first indication that experience-dependent plasticity regulates STEP activity was the observation, a decade ago, that STEP was rapidly translated in response to fear conditioning [3]. Here, we found that $STEP_{61}$ levels are downregulated after training in hippocampal-dependent tasks, which is in line with the model where STEP acts as a molecular restraint that needs to be removed in order to allow memory formation. Similarly, other studies show that conditions that promote memory formation stimulate the degradation of certain molecular constrains [50, 52, 69, 70]. Moreover, here, we found that $STEP_{61}$ levels were downregulated through different mechanisms in response to electrical stimulation of hippocampal slices and in vivo, after training in hippocampal-dependent tasks. Interestingly, a recent study shows that the levels of phosphorylated $STEP_{61}$ ($pSTEP_{61}$) at Ser221 increase in the striatum at the second day of training in the accelerating rotarod, whereas total $STEP_{61}$ levels are unchanged during learning [71]. Importantly, inhibition of PKA in the dorsal striatum reduces the levels of $pSTEP_{61}$ (Ser221) and dose- dependently impairs mouse performance in the accelerating rotarod, suggesting that inactivation of $STEP_{61}$ by PKA likely participates in striatal neuronal signaling associated with learning of motor skills [71]. Altogether, this evidence sup- ports a model where STEP activity exerts a restraint on LTM formation, which is removed in response to stimuli that pro- mote synaptic plasticity and memory formation, either through degradation or phosphorylation-mediated inactivation. Actually, several findings point out that $STEP_{61}$ levels/ activity are dynamically regulated in very specific and stimulus-dependent manner [18, 72, 73]. For instance, we demonstrated in primary cortical neurons that a brief depolarization using KCl promotes

$STEP_{61}$ degradation in a TrkB- and proteasome-dependent manner, whereas a sustained incubation with KCl induces its cleavage by calpains [19]. Remarkably, in response to sustained activity blockade there is a concurrent decrease of $STEP_{61}$ mRNA and protein levels, together with increased phosphorylation, which allow enhanced tyrosine phosphorylation of GluN2B and GluA2, and synaptic scaling in cultured hippocampal neurons [7]. Conversely, chronic activation increases $STEP_{61}$ protein levels and reduces its phosphorylation, leading to reduced tyrosine phosphorylation and surface levels of GluN2B and GluA2 [7]. Collectively, these findings and our results in vitro and in vivo highlight that activity-dependent regulation of $STEP_{61}$ levels is a mechanism that contributes not only to homeostatic synaptic plasticity, but likely to synaptic strengthening and memory formation.

Another finding of the present study is that improved performance in hippocampal memory tests in STEP KO mice is age- and test-dependent. STEP KO mice outperformed wild- type mice in the NORT at 3, but not at 12 months of age, and the opposite occurred in the NOLT, while memory for the novel arm in the T-SAT was similar between genotypes at both ages tested. Our results are in line with the reports showing that STEP KO mice have improved performance in the MWM compared to their control littermates at 3–4 [8], but not at 6 [74] months of age, and 6-month-old wild-type mice treated with the STEP inhibitor TC-2153 show no differences com- pared to vehicle-treated mice [22]. Also, in agreement with our findings, wild-type and STEP KO mice perform similarly in the NORT and in the Y-maze at 6 months of age [5, 74]. Interestingly, genetic reduction of STEP levels alleviates age- related hippocampal-dependent memory deficits [74]. Thus, STEP can be linked to certain learning behaviors like the MWM and fear conditioning [3, 4, 8], but other types of memory can be independent, or at least less dependent, on STEP levels/activity, or its contribution evident only under certain experimental conditions.

In agreement with the current functional model that STEP opposes the development of LTP, the main finding of the present study is that $STEP_{61}$ levels are downregulated due to calpain-mediated cleavage in response to stimuli that induce hippocampal LTP. Moreover, $STEP_{61}$ levels are reduced after training in hippocampal-dependent tasks in a TrkB-dependent manner, and preventing its UPS-mediated

degradation con- tributes to memory impairment.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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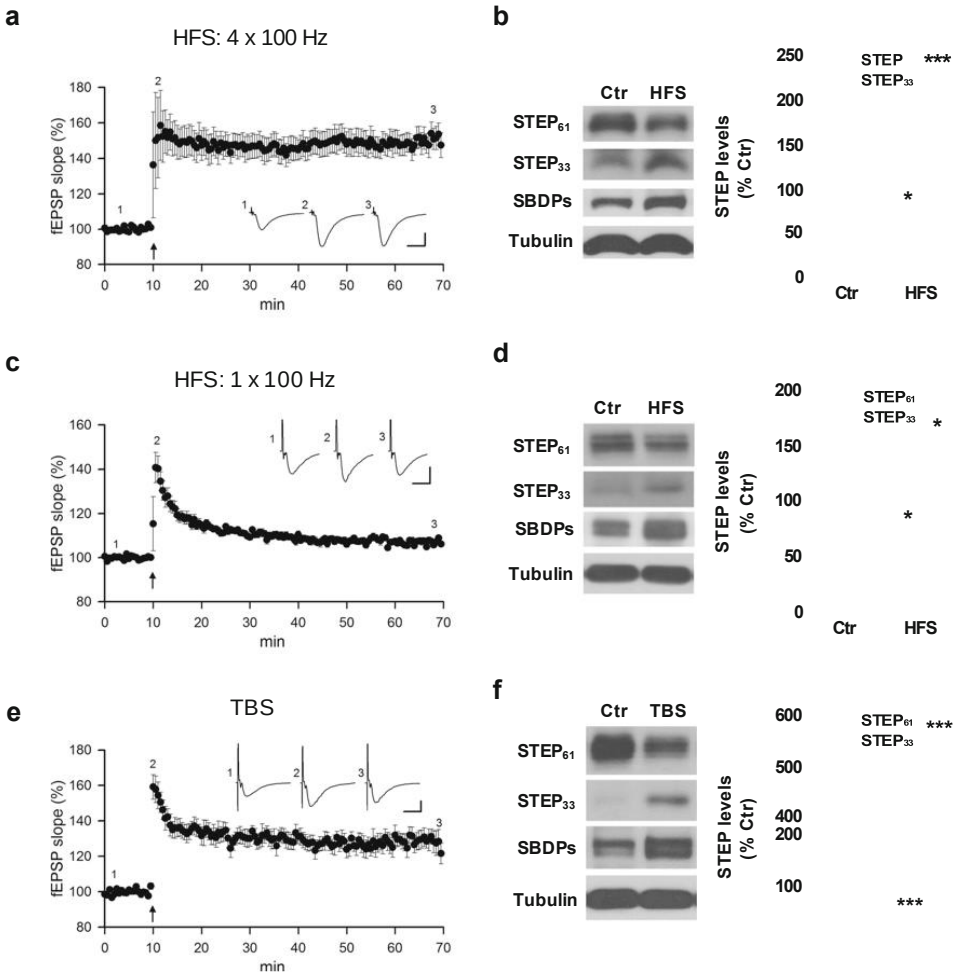


Fig. 1 $STEP_{61}$ is cleaved upon HFS and TBS. a, c, and e Time-course of relative fEPSPs slope in basal conditions and following (arrow) a strong electrical stimulation, c weak electrical stimulation, or e TBS of hippo- campal slices. In each experiment, *n* = 9 slices obtained from 4 to 5 mice. Values are expressed as mean ± SEM. Trace insets show representative fEPSPs recorded during periods indicated by corresponding numbers in the graphic (1, 2, and 3). Scale bars: vertical 0.25 mV, horizontal 10 ms (for a and e) and vertical 0.5 mV, horizontal 10 ms (for c). b, d, and f The levels of STEP and spectrin breakdown products (SBDPs) at 145–150 kDa in hippocampal slices were analyzed by Western blot of protein extracts obtained 60 min after b 4 × 100 Hz HFS, d 1 × 100 Hz HFS or f TBS. Representative immunoblots are shown. The possibility of showing representative immunoblots for $STEP_{61}$ and $STEP_{33}$ from the same film exposition was hampered by their different relative abundance. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of control slices and shown as mean ± SEM (*n* = 5–10). Data were analyzed by Student's *t* test. **P* < 0.05 and ****P* < 0.001 as compared to control

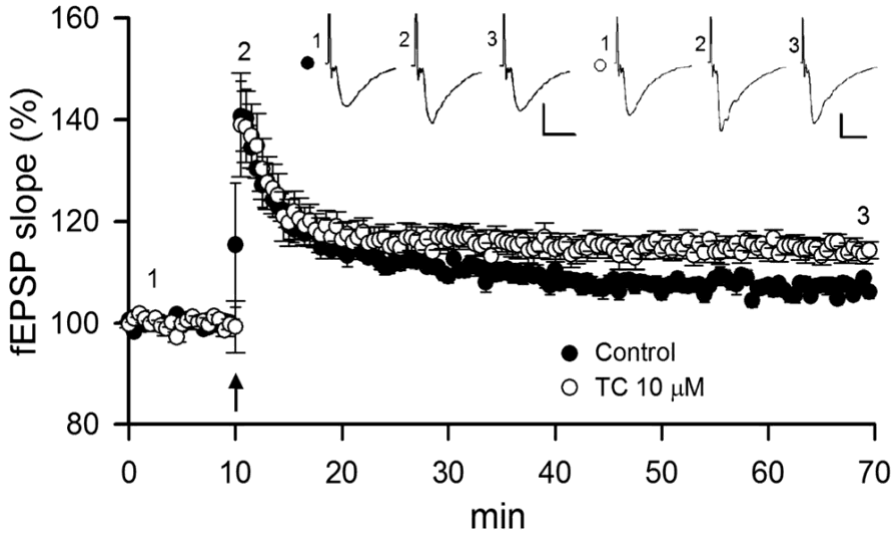


Fig. 2 Inhibition of STEP improves hippocampal LTP. Time-course of relative fEPSPs slope in basal conditions and following weak HFS (arrow) in control slices (black dots, *n* = 9 slices obtained from four mice) and in slices incubated with 10 μM TC-2153 (white dots, *n* = 9 slices obtained from three mice). Values are expressed as mean ± SEM. Traces inset shows representative fEPSPs recorded during periods indicated by the corresponding numbers in the graphic (1, 2, and 3). Scale bars: vertical 0.5 mV, horizontal 10 ms. Data were analyzed by Student's *t* test. $^{**}P < 0.01$ as compared to control slices

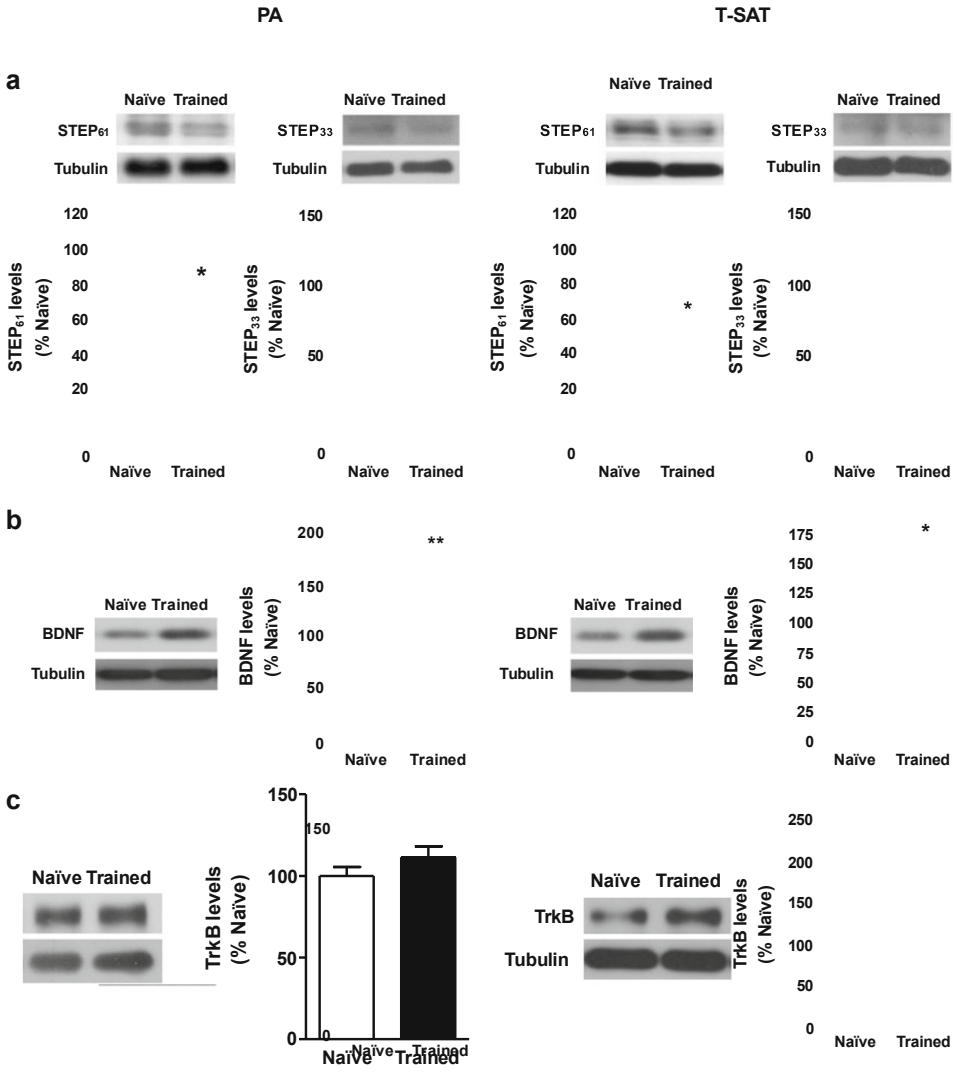
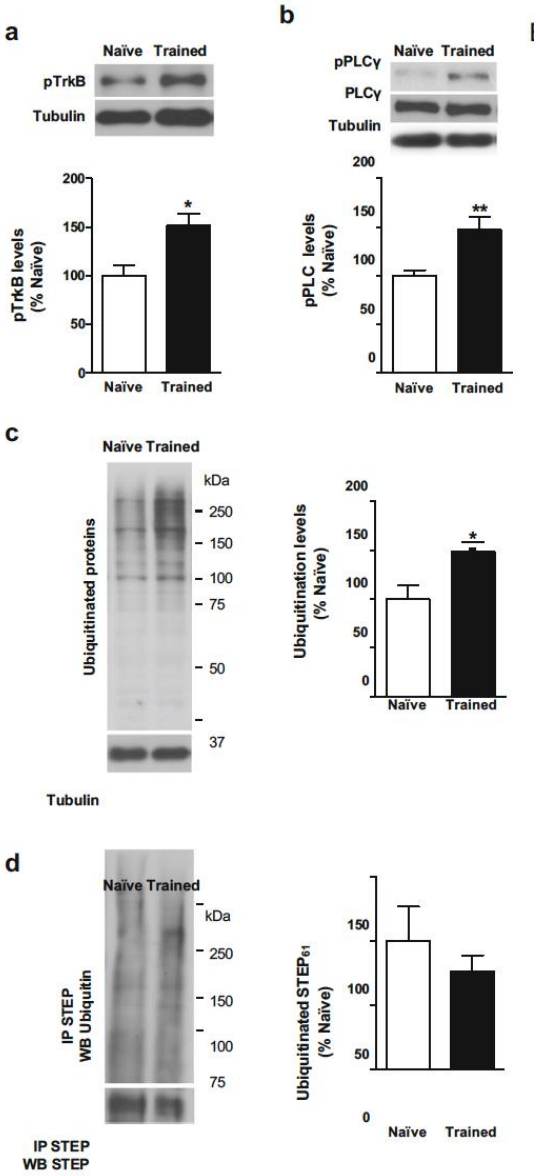


Fig. 3 Training in the PA and T-SAT leads to a decrease of hippocampal $STEP_{61}$ levels. a $STEP_{61}$, b BDNF, and c TrkB levels were analyzed by Western blot of protein extracts obtained from the hippocampus of mice trained in the PA and T-SAT and sacrificed 30 min or 1 h later, respectively. Representative immunoblots are shown. The different relative abundance of $STEP_{61}$ and $STEP_{33}$ precluded the possibility of showing representative immunoblots for $STEP_{61}$ and $STEP_{33}$ from the same film exposition. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of naïve mice and shown as mean ± SEM (n = 8–12). Data were analyzed by Student's t test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as compared to naïve miceImproved Performance in Hippocampal Memory Tests in STEP KO Mice Is Age- and Test-Dependent



EFig. 4 Analysis of TrkB signaling, protein ubiquitination, and $STEP_{61}$- ubiquitin conjugates after T-SAT training. The levels of a $pTrkB^{Tyr816}$, b $pPLC\gamma^{Tyr783}$, and c protein ubiquitination were analyzed by Western blot of protein extracts obtained from the hippocampus of naïve mice and mice trained in the T-SAT and sacrificed 1 h later. Representative immu- noblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of naïve mice and shown as mean ± SEM (a, *n* = 8–11; b, *n* = 9–10; c, *n* = 5–6). Data were analyzed by Student's *t* test. **P* < 0.05 and ***P* < 0.01 as compared to naïve mice. d Levels of $STEP_{61}$-ubiquitin conjugates in hippocampal protein extracts obtained from naïve mice and mice trained in the T-SAT and sacrificed 1 h later. The levels of $STEP_{61}$-ubiquitin conjugates in the hippocampus after training were determined by performing immunoprecipitation (IP) of STEP and Western blot (WB) against ubiquitin. Representative immuno- blots are shown. The ratio of ubiquitin-conjugated $STEP_{61}$/IP $STEP_{61}$ was calculated for each sample, and data are expressed as percentage of naïve mice and shown as mean ± SEM (*n* = 5–7). Data were analyzed by Student's *t* test

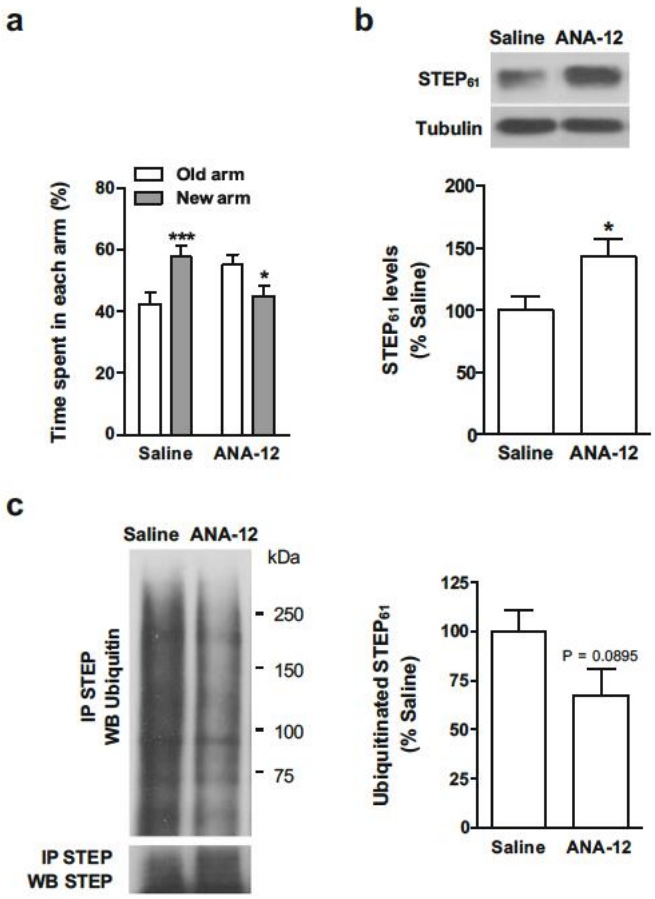


Fig. 5 Blockade of TrkB induces $STEP_{61}$ accumulation and LTM impairment in the T-SAT. a Mice received an intraperitoneal injection of saline or 2 mg/Kg ANA-12 and were trained in the T-SAT immediately after. LTM was assessed 4 h later. The graph shows the percentage of arm preference (mean ± SEM; *n* = 13/group). Data were analyzed by Student's *t* test. **P* < 0.05 and ****P* < 0.001 as compared to old arm. b $STEP_{61}$ levels were analyzed by Western blot of protein extracts obtained from the hippocampus of mice injected with saline or 2 mg/Kg ANA-12 before T-SAT training and subjected to LTM assessment. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of saline-treated mice and shown as mean ± SEM (*n* = 6/group). Data were analyzed by Student's *t* test. **P* < 0.05 as compared to saline-injected mice. c Levels of $STEP_{61}$- ubiquitin conjugates in hippocampal protein extracts obtained from mice injected with saline or 2 mg/Kg ANA-12, trained in the T-SAT and tested 4 h later. Representative immunoblots are shown. The ratio of ubiquitin- conjugated $STEP_{61}$/IP $STEP_{61}$ was calculated for each sample and data are expressed as percentage of saline-injected mice and shown as mean ± SEM (*n* = 5–6). Data were analyzed by Student's *t* test

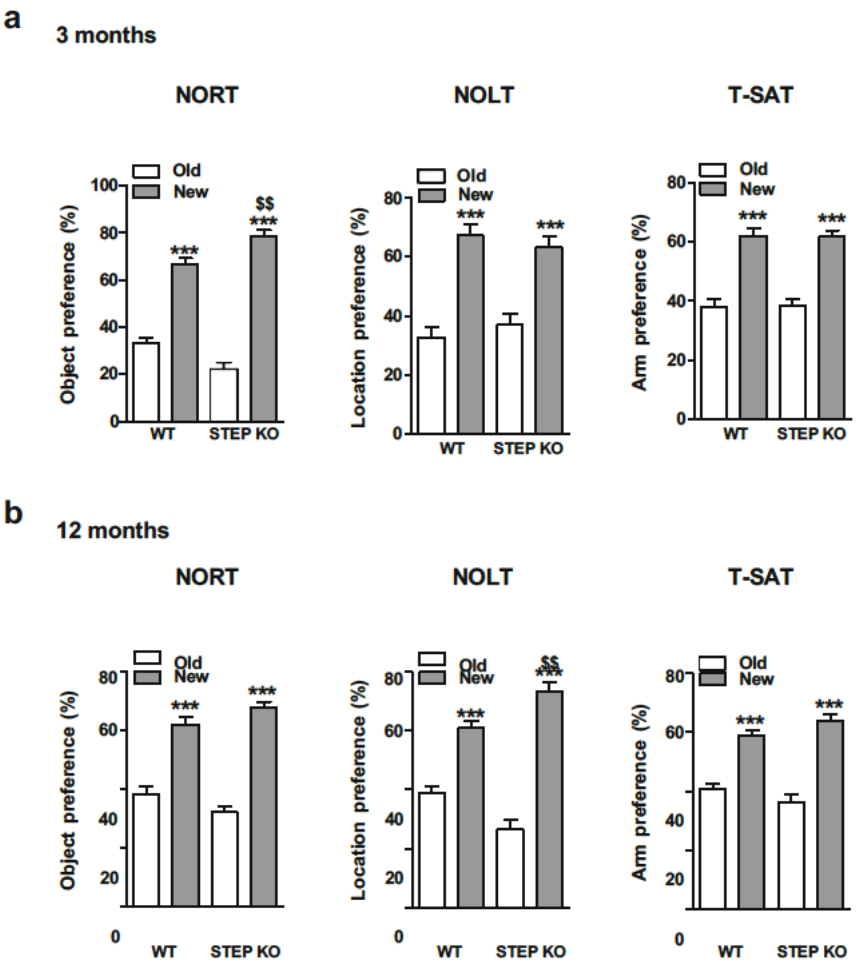


Fig. 6 Improved performance in the NORT and NOLT in young and older STEP KO mice, respectively. Wild-type (WT) and STEP KO mice were subjected to the NORT, NOLT and T-SAT. LTM was assessed 24 h (NORT and NOLT) or 4 h (T-SAT) after the training trial. Graphs show the percentage of object (NORT), lo- cation (NOLT) and arm (T-SAT) preference (mean ± SEM; n = 8– 16/group at 3 months and n = 11– 17/group at 12 months of age) in WT and STEP KO mice at a 3 andn b 12 months of age. Data were analyzed by Student's t test. ***P < 0.001 as compared to old ob- ject/location/arm and $^{\$\$}P$ < 0.01