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Striatal-enriched protein tyrosine phosphatase modulates nociception: evidence from genetic deletion and pharmacological inhibition

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Abstract

The information from nociceptors is processed in the dorsal horn of the spinal cord by complex circuits involving excitatory and inhibitory interneurons. It is well documented that GluN2B and ERK1/2 phosphorylation contributes to central sensitization. *Striatal-enriched protein tyrosine phosphatase* (STEP) dephosphorylates GluN2B and ERK1/2, promoting internalization of GluN2B and inactivation of ERK1/2. The activity of STEP was modulated by genetic (STEP knockout mice) and pharmacological (recently synthesized STEP inhibitor, TC-2153) approaches. $STEP_{61}$ protein levels in the lumbar spinal cord were determined in male and female mice of different ages. Inflammatory pain was induced by complete Freund's adjuvant injection. Behavioral tests, immunoblotting, and electrophysiology were used to analyze the effect of STEP on nociception. Our results show that both genetic deletion and pharmacological inhibition of STEP induced thermal hyperalgesia and mechanical allodynia, which were accompanied by increased $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$ levels in the lumbar spinal cord. *Striatal-enriched protein tyrosine phosphatase* heterozygous and knockout mice presented a similar phenotype. Furthermore, electrophysiological experiments showed that TC-2153 increased C fiber-evoked spinal field potentials. Interestingly, we found that $STEP_{61}$ protein levels in the lumbar spinal cord inversely correlated with thermal hyperalgesia associated with age and female

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gender in mice. Consistently, *STEP* knockout mice failed to show age-related thermal hyperalgesia, although gender-related differences were preserved. Moreover, in a model of inflammatory pain, hyperalgesia was associated with increased phosphorylation-mediated $STEP_{61}$ inactivation and increased $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$ levels in the lumbar spinal cord. Collectively, the present results underscore an important role of spinal STEP activity in the modulation of nociception.

Keywords

$STEP_{61}$; Thermal hyperalgesia; Mechanical allodynia; pGluN2B; pERK1/2; Age; Gender; CFA

1. Introduction

Primary sensory neurons detect pain-producing stimuli.[22] There are different types of nociceptors,[15]most of them terminating in the dorsal horn of the spinal cord with a distribution pattern that is determined by their sensory modality and the region of the body that they innervate. In the spinal cord, the information is processed by complex circuits involving excitatory and inhibitory interneurons and is transmitted by projection neurons to several brain areas.[40]

Afferent inputs to dorsal horn neurons are mediated by glutamate through the activation of AMPA and NMDA receptors.[25] The NMDA receptor (NMDAR) subunit GluN2B plays a critical role in central sensitization. Noxious stimuli rapidly induce GluN2B phosphorylation (pGluN2B) at Tyr1472 causing its redistribution to the membrane of spinal dorsal horn neurons.[11,28,48,51,55] After the activation of glutamate receptors, influx of extracellular calcium activates multiple intracellular protein kinase cascades, including extracellular signal-regulated kinases 1/2 (ERK1/2).[20,21,44] Like GluN2B, ERK1/2 phosphorylation (pERK1/2) is implicated in central sensitization.[7,8,12,20,23]

Striatal-enriched protein tyrosine phosphatase (STEP) is a neural-specific phosphatase that opposes the development of synaptic strengthening.[14] Striatal enriched protein tyrosine phosphatase has 2 major splicing isoforms (the membrane-associated $STEP_{61}$ and the cytosolic $STEP_{46}$), with $STEP_{61}$ being the only isoform expressed in the dorsal spinal cord neurons.[34] Multiple posttranslational modifications regulate STEP levels and activity, including phosphorylation/dephosphorylation.[14] Phosphorylation by cAMP-dependent protein kinase (PKA) of a regulatory serine residue within the binding domain for all STEP substrates (the kinase interacting motif) inactivates STEP isoforms,[33] whereas activation of NMDARs results in the dephosphorylation and activation of STEP through a calcineurin/PP1 pathway.[32,41] Striatal-enriched protein tyrosine phosphatase dephosphorylates the glutamate receptor subunits GluN2B and GluA2, leading to their endocytosis, and the kinases ERK1/2, p38, Fyn, and Pyk2, thereby controlling the duration of their signal.[14] Consistent with these findings, mice null for *STEP* have higher levels of pERK1/2 in the striatum, amygdala, and hippocampus[42,43] and increased surface expression of GluN2B in the hippocampus.[42] Importantly, in addition to GluN2B and ERK1/2 (references as above), also Fyn and p38 have been implicated in the regulation of

nociception.[1,20,30] Evidence supports that STEP levels and activity are downregulated or upregulated in distinct brain areas in multiple neurodegenerative and psychiatric disorders.[14] However, to the best of our knowledge, spinal STEP levels and the possible contribution of their alteration to impaired pain processing have not been investigated in the context of these disorders. In fact, its role in the spinal cord is now beginning to be unraveled. While we were preparing this article, another group reported that $STEP_{61}$ acts as an intermediary for GABAergic inhibition to regulate mechanical nociception and pain sensitization.[29] Moreover, $STEP_{61}$ signaling downstream the activation of noradrenergic α2 receptor attenuates ERK1/2 activation and inflammatory pain.[50] We used STEP knockout (KO) mice[43] and a recently synthetized STEP inhibitor[49] to show that $STEP_{61}$ activity in the lumbar spinal cord modulates physiological nociception and inflammatory pain, likely through the regulation of pGluN2B^{Tyr1472} and pERK1/2$^{Thr202/Tyr204}$ levels.

2. Materials and methods

2.1. Animals

Male and female *STEP* KO (*STEP*$^{-/-}$),[43] heterozygous (*STEP*$^{+/-}$), and wild-type (*STEP*$^{+/+}$) mice (C57BL/6J background), and male Sprague Dawley rats (200–250 g) were housed in cages lined with sawdust under a standard 12/12 hours light/dark cycle (lights on at 08:00 AM) with food and water available ad libitum. Every effort was made to minimize animal suffering and to use the minimum number of animals per group and experiment. Experimental procedures were approved by the Local Ethical Committee of the Universities of Barcelona and the Basque Country, following European (2010/63/UE) and Spanish (RD 53/2013) regulations for the care and use of laboratory animals.

2.2. Drug preparation and delivery

The STEP inhibitor (TC-2153; benzopentathiepin 8-(trifluoro-methyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride)[49] was dissolved in 2% DMSO and injected (10 mg/kg; intraperitoneal, i.p.) 1 hour before the behavioral assessment. For spinal application, stock solutions were obtained by diluting the drug powder in DMSO, and working solutions were prepared in artificial cerebrospinal fluid (aCSF; in mM: 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 24 $NaHCO_3$, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 10 D-(þ) glucose; pH 7.4) immediately before delivery. Small volumes (10–15 μL) of either aCSF or drug solution were applied by controlled superfusion through a silicone, 40- to 50-mm^2 pool attached to the dorsal surface of the spinal cord. To induce inflammatory pain, 10 μL of complete Freund's adjuvant (CFA; Sigma, St. Louis, MO) were injected into the plantar surfaces of both hind paws of 3-month-old wild-type and *STEP* KO male mice.

2.3. Hargreaves test

To allow acclimation to the testing environment, animals were placed in the examination room 30 minutes before analysis. Then, animals were placed in Plexiglas enclosures with glass floors suspended 30 cm from the table top and allowed to habituate for 15 minute before testing. The hind paws were individually stimulated from below using a halogen heat source from the Hargreaves apparatus (Ugo Basile, Varese, Italy).[18] The intensity of the beam (40 W for mice and 80 W for rats) was selected to produce an average baseline

threshold of approximately 8 seconds. A 20-second cut-off was used to prevent tissue damage in nonresponsive subjects. The latency to produce a nocifensive paw-withdrawal response was used to measure thermal hypersensitivity. Each hind paw was targeted 3 times in alternating order, producing 6 scores of nociception that were averaged and analyzed.

2.4. Mechanical sensitivity

To assess mechanical sensitivity, the withdrawal threshold to punctate mechanical stimulation of the hind paw was determined by the application of calibrated von Frey filaments (North Coast Medical, Inc., Morgan Hill, CA). The von Frey filaments (3.92, 5.88, 9.80, 19.60, 39.21, 58.82, 78.43, and 147.05 mN; equivalent to 0.4, 0.6, 1, 2, 4, 6, 8, and 15 g) were applied vertically to the plantar surface of the hind paw and gently pushed to the bending point. The 50% withdrawal threshold was determined using the up–down method as previously described.[9] A brisk hind paw lift in response to von Frey filament stimulation was considered a withdrawal response.

2.5. Electrophysiological recording

To measure the ability of STEP to modulate C-fiber–evoked spinal field potentials, electrophysiological recordings were performed during spinal superfusion with successively increasing, cumulative concentrations of STEP inhibitor TC-2153 (10 nM-10 mM). The electrophysiological setup was essentially as described previously.[2] Briefly, the left sciatic nerve was exposed, gently freed from connective tissue, and placed onto platinum hook electrodes for bipolar electrical stimulation. Bilateral dorsal laminectomies were performed at vertebrae T13–L1, the vertebral column was immobilized to a rigid frame and the duramater overlaying the lumbosacral spinal segments was carefully removed. Single monophasic, square wave electrical pulses were delivered as test stimuli to the sciatic nerve trunk at a midthigh level on a per-minute basis by means of a computer-controlled stimulus isolator, and the elicited spinal field potentials were amplified (analog band-pass set at 1–550 Hz), displayed on an oscilloscope, and digitized to a PC-based computer at a 10-kHz sampling rate through an A/D converter card (MIO16; National Instruments, Austin, TX). Field potentials evoked in superficial laminae of the spinal dorsal horn by the activation of C fibers (3–3.5 mA pulses of 0.5 milliseconds duration) were extracted from 90- to 200-milliseconds latency bands (<1.2 m/s conduction velocity) and quantified as described previously.[5]

2.6. Western blot

Animals were killed and the lumbar spinal cord rapidly removed on ice. Tissue was homogenized in lysis buffer (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) and protease inhibitors (phenylmethylsulphonyl fluoride [2 mM], aprotinin [1 μg/mL], leupeptin [1 μg/mL], and sodium orthovanadate [1 mM]) and centrifuged at 16,100*g* for 20 minutes. The supernatants were collected and the protein concentration was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis was performed as previously described.[37] The following primary antibodies were used: anti-STEP (23E5; 1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pERK1/2$^{Thr202/Tyr204}$ (1:1000) and anti-

pGluN2B^{Tyr1472} (1:500) (Cell Signaling Technology, Beverly, MA), anti-pSTEP^{Ser221} (1:1000; Millipore, Temecula, CA), and anti-tubulin (1:50,000; Sigma). The anti-STEP antibody is raised against an 18-aa sequence mapping at the N terminus of $STEP_{46}$ of rat origin. Since $STEP_{46}$ sequence is entirely contained within $STEP_{61}$, this antibody recognizes both $STEP_{61}$ and $STEP_{46}$. In our hands, it easily detects both $STEP_{61}$ and $STEP_{46}$ and sometimes lower molecular weight STEP isoforms in striatal extracts, and each isoform can be identified based on its molecular weight. In agreement with previous studies,[34] we only detected the $STEP_{61}$ isoform in spinal cord homogenates. Then, membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), incubated for 1 hour (15 minutes for loading controls) at room temperature with the corresponding horseradish peroxidase-conjugated 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).

2.7. Statistical analysis

Experimental data were analyzed using the GraphPad Prism (v. 5.01, GraphPad Software, Inc, La Jolla, CA). Data are presented as mean ± SEM. Statistical analyses were performed using the unpaired Student's *t* test (95% confidence), one-way or two-way analysis of variance (ANOVA) with Bonferroni's post hoc test, and simple linear regression, as appropriate, and indicated in the figure legends. In all cases, a difference was considered significant if $P < 0.05$.

3. Results

3.1. STEP knockout mice have thermal hyperalgesia and mechanical allodynia

As a first approach to study the role of STEP in thermal nociception, we subjected 3-month-oldmale $STEP^{+/+}$, $STEP^{+/-}$, and $STEP^{-/-}$ mice to the Hargreaves test. The results showed that both STEP dosage reduction (heterozygous mice) and deletion (KO mice) produced a lower paw-withdrawal latency (one-way ANOVA; $F_{(2,43)} = 34.83$; $P < 0.001$). Interestingly, the lack of 1 allele of STEP produced the same effect as the total deletion (Fig. 1A). We next asked whether STEP could also be involved in other types of nociception, namely, a response to a mechanical stimulus. For that, we analyzed the threshold of evoked mechanical pain in response to von Frey filaments in 3-month-old wild-type ($STEP^{+/+}$) and *STEP* KO ($STEP^{-/-}$) male mice. As for thermal stimulus, we observed that the lack of STEP also reduced the mechanical threshold (Fig. 1B). Western blot analysis confirmed the lack of $STEP_{61}$ protein expression in STEP KO mice and a reduction of 46.7% in heterozygous mice compared to controls (one-way ANOVA; $F_{(2,13)} = 28.8$; $P < 0.001$; Fig. 1C). As a readout of STEP activity, we analyzed the phosphorylation status of 2 of its substrates implicated in nociception, pGluN2B^{Tyr1472} and pERK1/2. Consistent with the reduction or lack of $STEP_{61}$ expression we found higher levels of pGluN2B^{Tyr1472} (one-way ANOVA; $F_{(2,13)} = 15.19$; $P < 0.01$; Fig.1D), pERK1 (one-way ANOVA; $F_{(2,13)} = 6.02$; $P < 0.05$; Fig. 1E), and pERK2 (one-way ANOVA; $F_{(2,13)} = 22.18$; $P < 0.001$; Fig. 1E), whereas no changes were observed in the total GluN2B or ERK1/2 level (data not shown). In line with

the results obtained in the Hargreaves test, there were no differences in pGluN2B^{Tyr1472} (Fig. 1D) and pERK1/2$^{Thr202/Tyr204}$ (Fig. 1E) levels between *STEP*$^{+/-}$ and *STEP*$^{-/-}$ mice.

3.2. Pharmacological inhibition of STEP contributes to thermal hyperalgesia and mechanical allodynia

To investigate whether an acute inhibition of STEP activity promotes thermal hyperalgesia, and mechanical allodynia, and biochemical alterations similar to those observed in STEP-deficient mice, we tested the effect of pharmacological inhibition of STEP on thermal and mechanical nociception. To this end, we injected 3-month-old wild-type male mice with TC-2153 (10 mg/kg, i.p.), a pharmacological inhibitor of STEP,[49] and explored thermal and mechanical nociception after 1 hour (Fig. 2A). Pharmacological inhibition of STEP produced a significant decrease in both paw-withdrawal latency (Fig. 2B) and mechanical threshold (Fig. 2C). These phenotypes were accompanied by increased pGluN2B^{Tyr1472} (Fig. 2D) and pERK1/2$^{Thr202/Tyr204}$ (Fig. 2E) levels in the lumbar spinal cord of TC-2153-treated animals.

3.3. Inhibition of STEP increases C-fiber–evoked spinal potentials

To further characterize the effect of STEP inhibition, we treated male Sprague Dawley rats with TC-2153 (10 mg/kg, i.p.) and subjected them to the Hargreaves test. As in mice, inhibition of STEP produced lower paw-withdrawal latency compared to vehicle-treated rats (Fig. 3A). This lower latency was accompanied by increased levels of pGluN2B^{Tyr1472} (Fig. 3B) andpERK1/2$^{Thr202/Tyr204}$ (Fig. 3C) in the lumbar spinal cord of rats treated with TC-2153. Next, we performed electrophysiological studies to determine how STEP inhibition affects neuronal functioning. Spinal superfusion with TC-2153 at 10 μM significantly increased the C-fiber–evoked spinal potentials by 18.5 ± 0.5% (16.37 ± 0.42 V milliseconds area from a 13.81 ± 0.48 V milliseconds control baseline area during superfusion with aCSF), and reached 42.28 ± 1.02% for control during administration of 1 mM of TC-2153 (19.65 ± 0.55 V milliseconds from a 13.81 ± 0.48 V milliseconds baseline; Fig. 3D).

3.4. Spinal STEP$_{61}$ levels are reduced with age and correlate with thermal hyperalgesia

Several studies have shown age-related alterations in nociception,[52] and STEP$_{61}$ levels are reported to change with age.[4,54] As our results indicated that reduced STEP$_{61}$ levels/activity promoted thermal hyperalgesia, we next characterized thermal nociception and STEP$_{61}$ levels in the lumbar spinal cord of male and female mice from 3 to 15 months of age. The Hargreaves test showed reduced paw-withdrawal latency with age in males (one-way ANOVA, $F_{(3, 56)} = 15.39$; $P < 0.001$; Fig. 4A). Interestingly, spinal STEP$_{61}$ levels also decreased with age (one-way ANOVA, $F_{(3, 20)} = 12.2$; $P < 0.001$; Fig. 4B), and there was a correlation between paw-withdrawal latency and STEP$_{61}$ levels ($r^2 = 0.48$; $P < 0.001$; Fig. 4C). Female mice showed the same pattern, including reduced paw-withdrawal latency (one-way ANOVA, $F_{(3, 56)} = 4.39$; $P < 0.01$; Fig. 4D) and decreased STEP$_{61}$ levels with age (one-way ANOVA, $F_{(3, 20)} = 8.46$; $P < 0.001$; Fig. 4E), along with a correlation between both parameters ($r^2 = 0.42$; $P < 0.001$; Fig. 4F).

3.5. Gender differences in thermal nociception and $STEP_{61}$ levels

Data from animal studies show that female rodents have a lower thermal-pain threshold.[19] When analyzing the paw-withdrawal latency in the Hargreaves test at different ages we detected significant differences between male and female mice (two-way ANOVA, sex effect; $F_{(1, 75)} = 10.51$; $P < 0.01$). Analysis of the data by age showed that 3- and 6-month-old female animals presented a lower paw-withdrawal latency compared to males, a difference that was lost in older mice (Fig. 5A). As we found a correlation between thermal hyperalgesia and changes in $STEP_{61}$ levels in the spinal cord with age (Figs. 4C and F), we next investigated the potential differences in spinal $STEP_{61}$ levels between male and female mice. Consistent with the results from the Hargreaves test, western blot analysis revealed that 3-month-old female mice had significantly less $STEP_{61}$ levels in the spinal cord compared to age-matched males (Fig. 5B), whereas no significant differences in $STEP_{61}$ expression were detected between 15-month-old male and female mice (Fig. 5C). To further characterize the implication of $STEP_{61}$ levels in the differences in thermal nociception between male and female mice, we performed the Hargreaves test in male and female $STEP^{-/-}$ mice at different ages. When comparing the latency in *STEP* KO mice, we did not observe any significant difference between 3-, 6- and 12-month-old mice of either gender. Conversely, for age-matched $STEP^{-/-}$ mice, there was a gender effect (two-way ANOVA, gender effect; $F_{(1, 75)} = 17.09$; $P < 0.001$; Fig. 5D).

3.6. $STEP_{61}$ activity is decreased during inflammatory pain

Next, we sought to analyze whether STEP was also involved in the regulation of inflammatory pain. To this end, we performed the Hargreaves test before and 24 hours after the injection of saline or 10 μL of CFA into the plantar surfaces of both hind paws in 3-month-old wild-type and *STEP* KO mice. No differences were detected in the paw-withdrawal latency in saline-injected mice of either genotype (Fig. 6A). However, both wild-type and *STEP* KO mice injected with CFA displayed thermal hyperalgesia, without differences between genotypes (Fig. 6A). We further analyzed the molecular changes associated with CFA-induced hyperalgesia in the lumbar spinal cord of wild-type animals. Western blot analysis showed that 24 hours after CFA-induced inflammation, there were no differences in the total $STEP_{61}$ levels (saline: 100.08 ± 9.15% and CFA: 94.16 ± 8.75%, n = 5–6, $P = 0.66$, Student's *t* test). Phosphorylation of $STEP_{61}$ at Ser221 by PKA blocks its activity.[33] Thus, we analyzed whether $pSTEP_{61}^{Ser221}$ levels in the lumbar spinal cord were altered by CFA injection. We found that there were increased levels of $pSTEP_{61}^{Ser221}$ in CFA-injected mice compared to saline-injected animals (Fig. 6B). Importantly, phosphorylation-mediated $STEP_{61}$ inactivation was accompanied by increased $pGluN2B^{Tyr1472}$ (Fig. 6C) and $pERK1/2^{Thr202/Tyr204}$ (Fig. 6D) levels in the lumbar spinal cord of CFA-treated animals.

4. Discussion

Phosphorylation and dephosphorylation of specific proteins in dorsal horn neurons is critical to nociception.[47] The role of several protein kinases in pain modulation has been extensively studied.[6,27] However, less is known about the role of protein phosphatases in this process. Here, we provide functional evidence that $STEP_{61}$ levels and activity modulate nociception,

both under physiological and pathological conditions, and that GluN2B and ERK1/2 are important downstream players.

Our results show that adult *STEP*$^{+/-}$ and *STEP*$^{-/-}$ male mice present thermal hyperalgesia. Interestingly, the lack of one allele of *STEP* produced the same effect as the total deletion, indicating that it is not necessary to completely block STEP to modulate the response to a thermal stimulus. This is in accordance with the results obtained after pharmacological inhibition of STEP, which also induced thermal hyperalgesia, not only in mice, but also in rats. Moreover, both genetic deletion and pharmacological inhibition of STEP promoted mechanical allodynia. Remarkably, although there is controversy on the effect of age on pain sensitivity, with some studies reporting either increased, decreased, or no changes in the sensitivity with advancing age,[52] here we show that age-dependent thermal hyperalgesia correlated with reduced $STEP_{61}$ levels in the lumbar spinal cord in both male and female mice. Nevertheless, cortical and hippocampal $STEP_{61}$ levels increase with age,[4,54] suggesting that tissue-specific transcriptional and/or posttranslational modifications regulate $STEP_{61}$ levels with age. Further supporting an important role of STEP in this process, we observed that this age effect on thermal hyperalgesia was lost in *STEP* KO mice. Also, in agreement with our proposal that STEP plays a role in nociception, we found that 3-month-old female mice presented lower spinal $STEP_{61}$ levels and paw-withdrawal latency in the Hargreaves test than age-matched male mice, whereas, at 15 months of age, $STEP_{61}$ levels and thermal threshold were similar between genders. Our results are in accordance with previous reports showing sex differences in response to thermal noxious stimuli both in humans and in laboratory animals.[19,39] However, a gender-related difference in latency was observed in *STEP*$^{-/-}$ mice indicating that, in addition to STEP, other mechanisms, such as sexual hormones,[13] contribute to sex-dependent response to a thermal stimulus.

The mechanism underlying thermal hyperalgesia and mechanical allodynia after STEP inhibition is likely related to the activation of GluN2B and ERK1/2 in the spinal cord, similar to what occurs in different brain areas of *STEP* KO mice[42,43] and in cortical neurons treated with TC-2153 in vitro and in vivo.[49] Higher levels of pGluN2B^{Tyr1472},[16] and pERK1/2[8,12] have been found in conditions associated with nociception and pain hypersensitivity. In fact, a recent study shows that intrathecal administration of a recombinant-adenovirus–encoding $STEP_{61}$ blocks GluN2B phosphorylation and pain sensitivity upon GABAergic inhibition.[29] Moreover, spinal expression of a $STEP_{61}$ mutant, which cannot be phosphorylated and inactivated, reduces ERK1/2 phosphorylation and inflammatory pain.[50] Therefore, these reports provide a direct link between STEP, the regulation of GluN2B, and ERK1/2 phosphorylation in neurons from dorsal spinal cord and pain sensitization. Central sensitization that is produced by the phosphorylation of these proteins results from an activity-dependent increase in the excitability of dorsal horn neurons[21,46] and altered gene transcription in the spinal cord.[23] Remarkably, it was recently found that the transcriptional signature of *STEP* KO mice is consistent with enhanced ERK signaling and NMDAR activity.[35] Moreover, a number of activity-dependent genes, including *c-fos*, are upregulated in *STEP* KO mice and in STEP-shRNA–transduced neurons.[35] Our data are in line with recently reported findings that suggest that a tonic level of STEP activity suppresses ERK1/2- and Fyn-signaling pathways, thereby increasing the

synaptic availability of GluN2B and promoting central sensitization.[29] In addition, we show that selective inhibition of STEP results in significantly increased field potentials that are evoked in the spinal dorsal horn by C-fiber input, supporting the fact that STEP may tonically repress nociceptive neurotransmission at the spinal level. The hypersensitivity resulting from STEP inhibition is consistent with the view that distinct protein phosphatases may modulate acute nociception, probably by repressing NMDAR-mediated excitatory neurotransmission in the spinal dorsal horn.[10,36] Indeed, recombinant STEP depresses NMDAR-mediated single-channel currents in dorsal horn neurons.[34] Thus, our findings support the view that STEP opposes synaptic strengthening in the spinal cord, and that genetic deletion or pharmacological inhibition facilitates central sensitization and nociceptive responses.

Finally, we explored the role of $STEP_{61}$ in a model of inflammatory pain. Although $STEP_{61}$ levels were unchanged in CFA-injected wild-type mice, which is in agreement with previous reports,[29,51] we found that CFA-induced hyperalgesia was accompanied by decreased $STEP_{61}$ activity, as evidenced by higher levels of its phosphorylated form and increased phosphorylation of GluN2B and ERK1/2 in the lumbar spinal cord. Accordingly, CFA-induced phosphorylation of GluN2B[17] and ERK1/2[29] was previously reported in rodents. Interestingly, increased $pGluN2B^{Tyr1472}$ levels upon CFA-induced inflammation were attributed to reduced $STEP_{61}$/Fyn interaction.[51] Our results show that phosphorylation-induced $STEP_{61}$ inactivation could explain its reduced interaction with Fyn, and the increased $pGluN2B^{Tyr1472}$ levels. Nevertheless, we observed that thermal hyperalgesia on CFA injection was similar in wild-type and *STEP* KO mice, and thus, in addition to STEP inactivation, other mechanisms contribute to inflammatory pain.

Here, we demonstrate that STEP participates in the regulation of nociception. Therefore, it would be interesting to explore whether changes in STEP levels and activity after stroke and ischemia[3] contribute to poststroke pain,[24] and whether the inactivation of STEP produced by drug abuse[41] participates in the increased pain prevalence observed in drug users.[31] Inhibition of STEP has been proposed as a promising therapeutic approach to fight synaptic deficits and cognitive impairment in pathological conditions.[49,53] However, our results highlight that caution needs to be taken because inhibiting STEP could lead to thermal hyperalgesia and mechanical allodynia and aggravate existing pain symptoms in affected individuals. Interestingly, as STEP modulates the activity of both NMDAR and ERK, hence, targeting STEP to manage pain may have additional benefits over other proposed phosphatases such as protein phosphatase 2A, which regulates the function of glutamate receptors,[45] or MAPK phosphatase-3, which dephosphorylates ERK.[26,38]

In summary, our behavioral, molecular, and electrophysiological data indicate that spinal $STEP_{61}$ plays a regulatory role in nociception, both under physiological and pathological conditions, likely through the dephosphorylation of GluN2B and ERK1/2. Thus, STEP might constitute a valuable therapeutic target for pain management.

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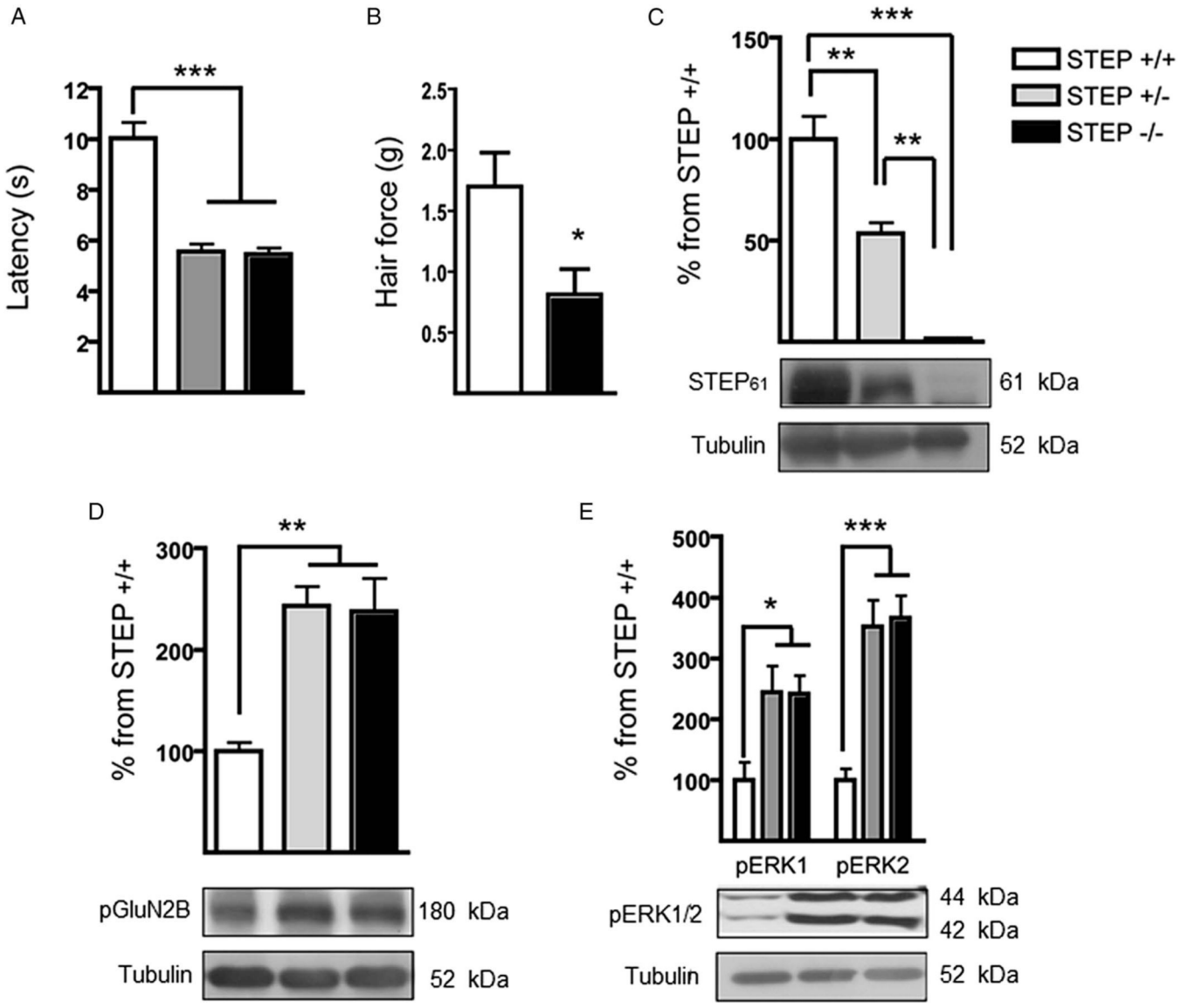

Figure 1.

Lack or reduction of STEP decreases the paw-withdrawal latency and mechanical threshold and increases the phosphorylation level of GluN2B and ERK1/2. (A) Paw-withdrawal latency in the Hargreaves test in $STEP^{+/+}$, $STEP^{+/-}$, and $STEP^{-/-}$ mice (n = 15 per genotype). (B) Mechanical threshold in the von Frey test in $STEP^{+/+}$ and $STEP^{-/-}$ mice (n = 10–12 per group). (C) $STEP_{61}$, (D) $pGluN2B^{Tyr1472}$ and (E) $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of $STEP^{+/+}$, $STEP^{+/-}$, and $STEP^{-/-}$ mice (n = 5 per genotype). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of $STEP^{+/+}$ (wild-type) mice and are shown as mean ± SEM. Data were analyzed by one-way ANOVA with Bonferroni's test as a post hoc. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$.

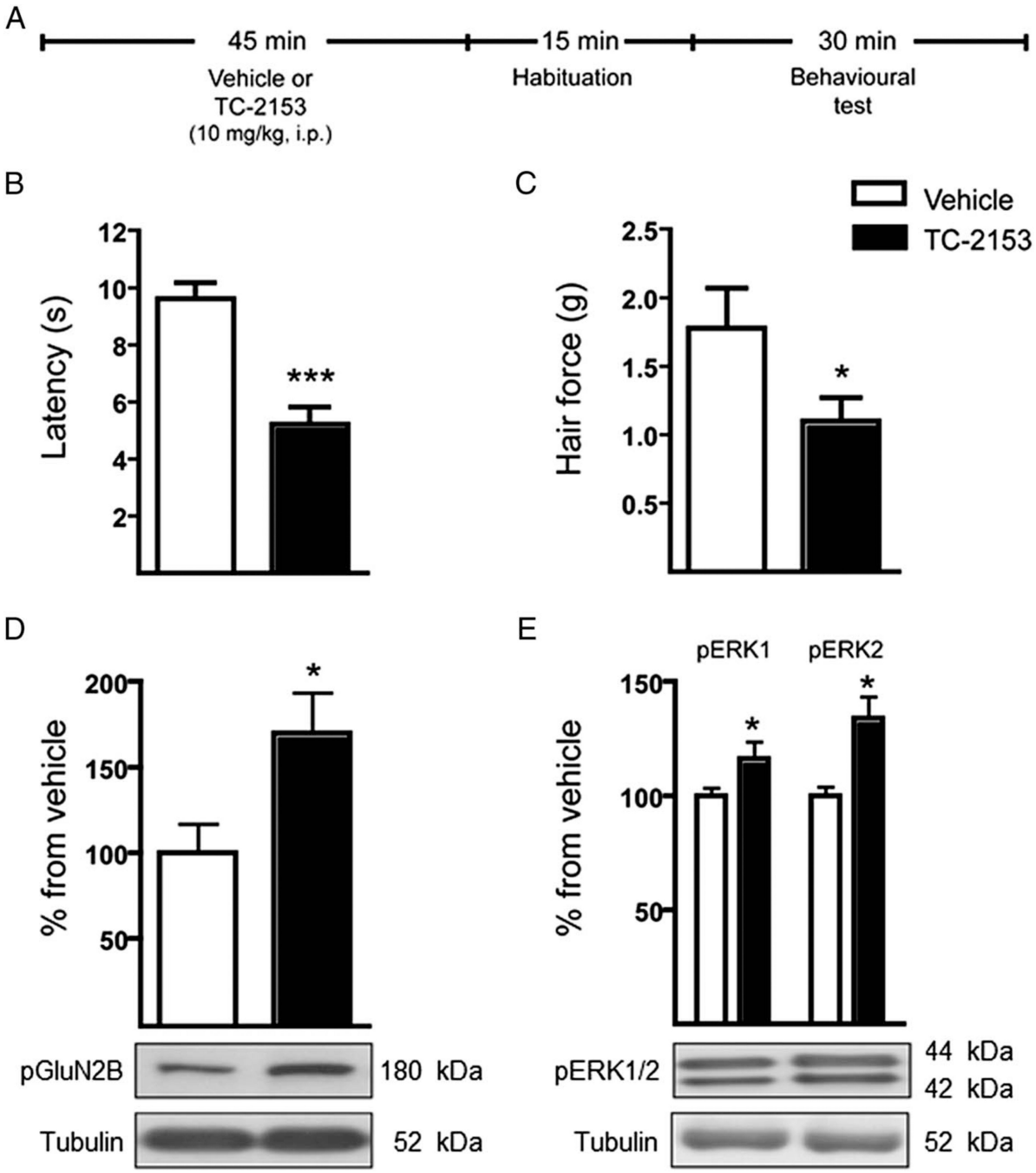

Figure 2.

Pharmacological inhibition of STEP causes thermal hyperalgesia and mechanical allodynia in mice. (A) Schematic representation of the experimental design. (B) Paw-withdrawal latency in the Hargreaves test and (C) mechanical threshold in the von Frey test in vehicle- and TC-2153–treated wild-type mice (n = 10–12 per group). (D) pGluN2B^{Tyr1472} and (E) pERK1/2$^{Tyr202/Tyr204}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of vehicle- and TC-2153–treated 3-month-old male mice (*n* = 4 per group). Representative immunoblots are shown. Values obtained by densitometric

analysis of western blotting data are expressed as percentage of vehicle-treated mice and shown as mean ± SEM. Data were analyzed by Student's *t* test. *: $P < 0.05$ and ***: $P < 0.001$, as compared with vehicle-treated mice.

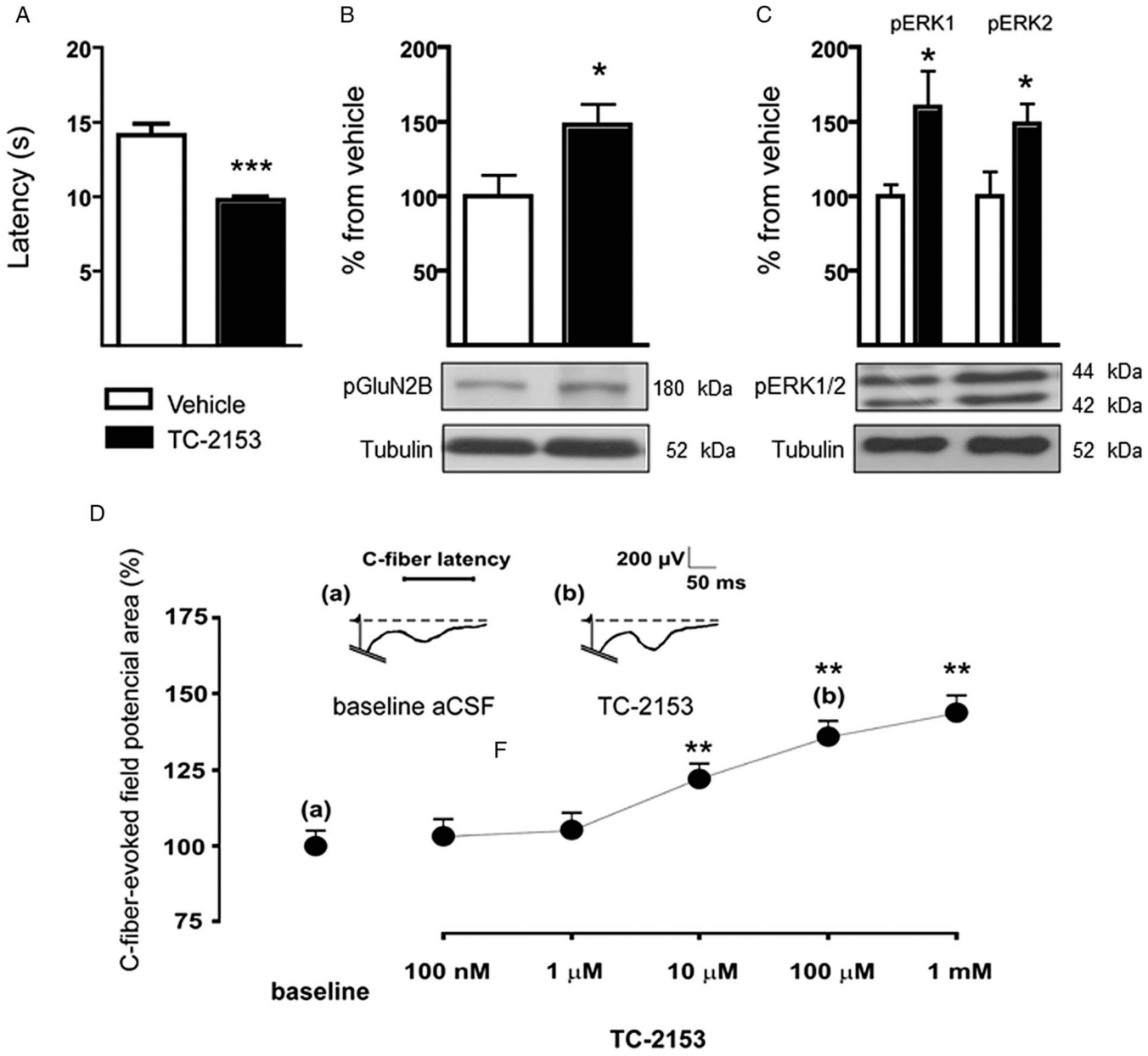

Figure 3.

Pharmacological inhibition of STEP causes thermal hyperalgesia and increases C-fiber–evoked spinal field potentials in rats. (A) Paw-withdrawal latency in the Hargreaves test in vehicle- and TC-2153–treated Sprague Dawley male rats (n = 10–11). (B) pGluN2B^{Tyr1472} and (C) pERK1/2$^{Thr202/Tyr204}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of vehicle- and TC-2153–treated rats (n = 5 per group). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of vehicle-treated rats, and data were analyzed by the Student's *t* test. (D) Diagram showing the mean field potential areas that are evoked by unmyelinated afferents during spinal superfusion with either aCSF (baseline control) or increasing cumulative concentrations of the STEP inhibitor, TC-2153 (n = 6).

Each circle represents the mean area of 10 spinal field potentials, and data were analyzed by one-way ANOVA followed by Bonferroni's post hoc test. In all graphs data are expressed as mean ± SEM. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$.

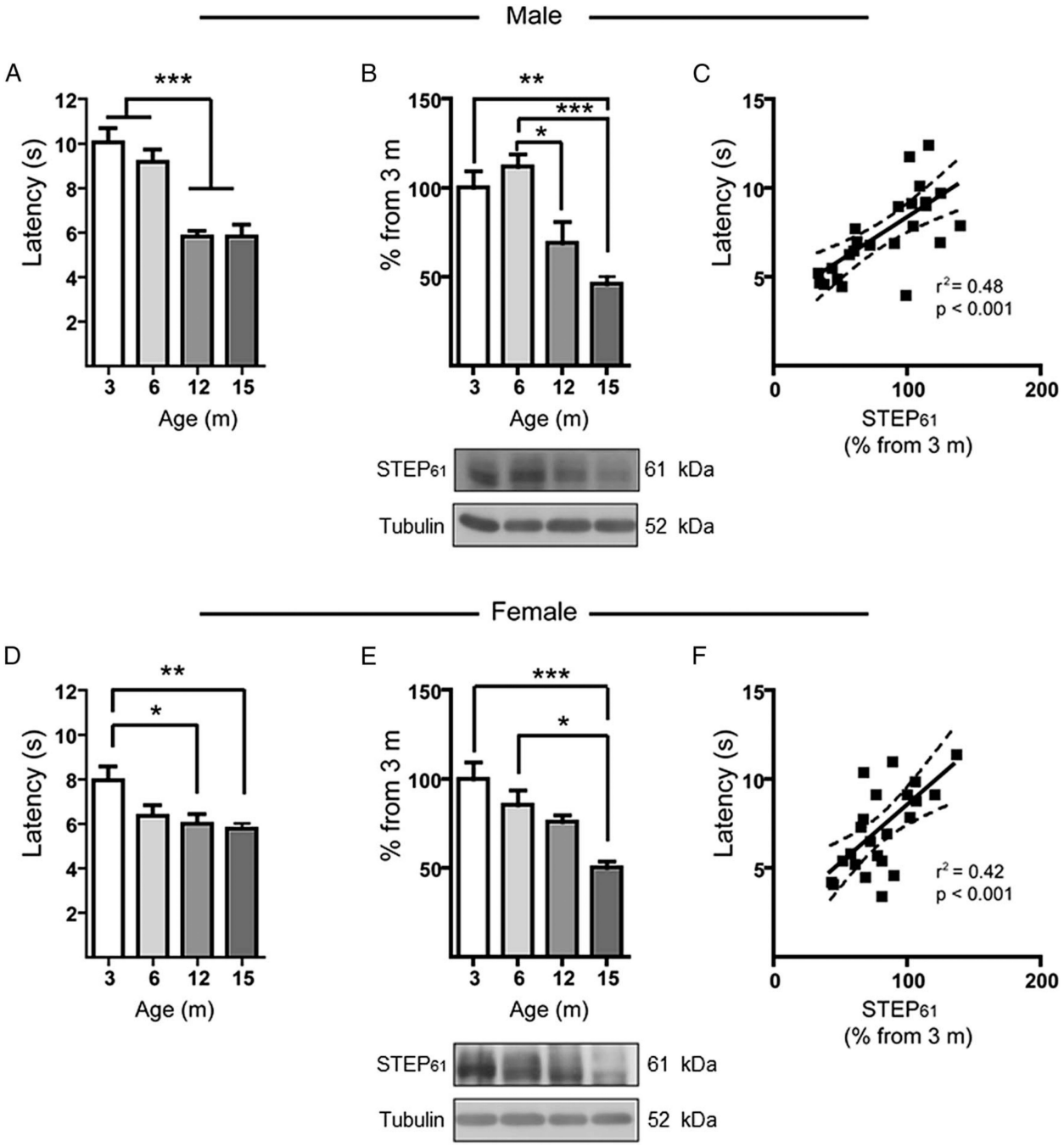

Figure 4.

Thermal nociception and $STEP_{61}$ levels are altered during aging. (A) Paw-withdrawal latency in the Hargreaves test in male mice at different ages (m, months; n = 9–15). (B) $STEP_{61}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of $STEP^{+/+}$ male mice of different ages (n = 6 per age). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of 3-month-old male mice. (C) Correlation between paw-withdrawal latency and $STEP_{61}$ levels in males (n = 6 per group). (D) Paw-withdrawal

latency in the Hargreaves test in female mice at different ages (n = 15 per group). (E) $STEP_{61}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of $STEP^{+/+}$ female mice at different ages (n = 6 per age). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of 3-month-old female mice. (F) Correlation between paw-withdrawal latency and $STEP_{61}$ levels in female mice (n = 6 per group) as determined by simple linear regression. (A, B, D, and E) Graphs show mean ± SEM, and data were analyzed by one-way ANOVA with Bonferroni's test as post hoc. Graphs (C) and (F) were determined by simple linear regression. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$.

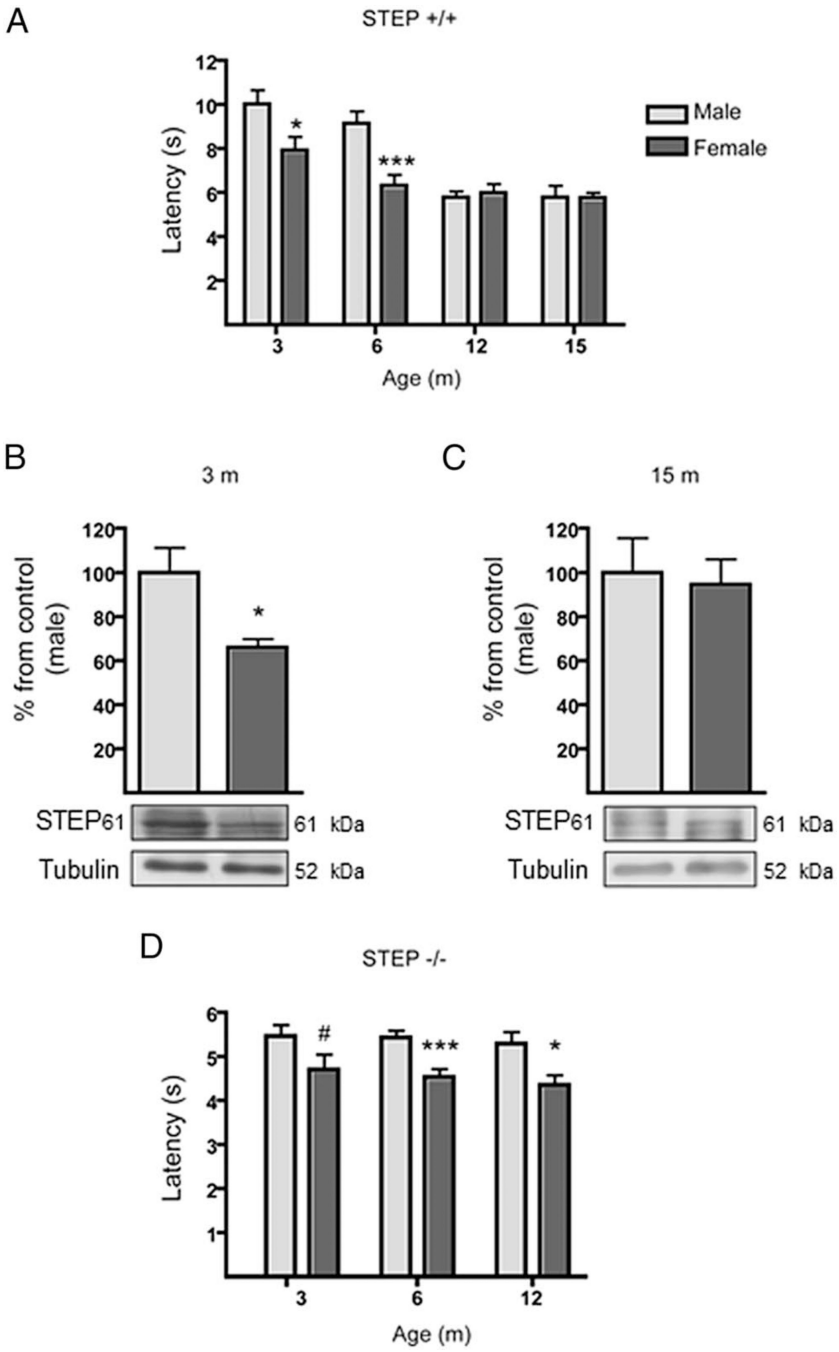

Figure 5.

Gender differences in thermal nociception and $STEP_{61}$ levels. (A) Paw-withdrawal latency in the Hargreaves test in male and female C57BL6/J mice at different ages (m, months; n = 9–15). $STEP_{61}$ levels in the lumbar spinal cord were compared between male and female $STEP^{+/+}$ mice at (B) 3 months and (C) 15 months of age (n = 5–6 per group). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of 3- and 15-month-old males, respectively. (D) Paw-withdrawal latency in the Hargreaves test in $STEP^{-/-}$ male vs female mice at different ages.

In all graphs, data are shown as mean ± SEM. Data were analyzed by two-way ANOVA analysis of variance with Bonferroni's test as post hoc in graphs (A and D) and by the Student's *t* test in graphs (B and C). #: $P = 0.06$, *: $P < 0.05$, and ***: $P < 0.001$, as compared with male mice.

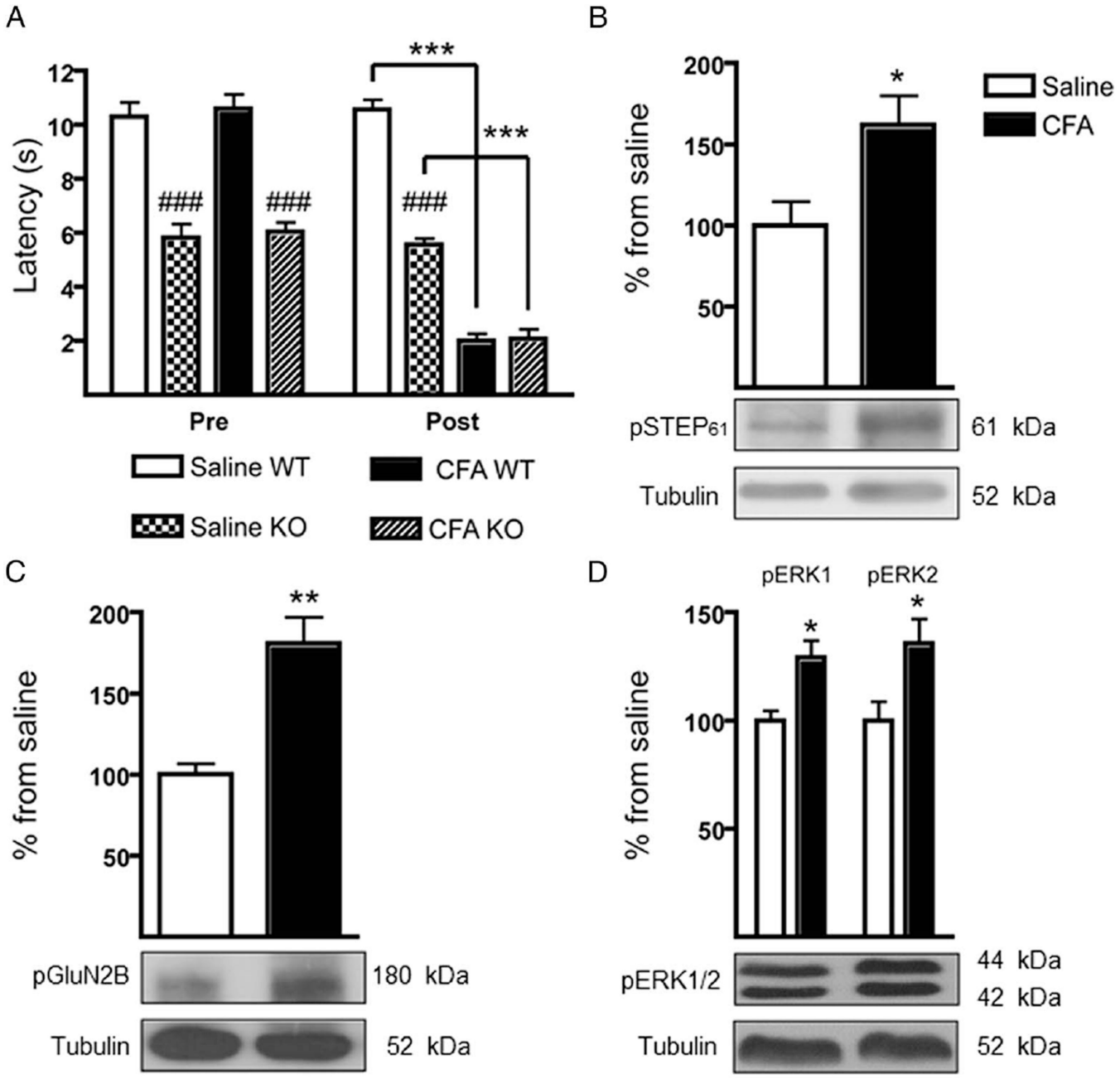

Figure 6.

CFA-induced inflammatory pain correlates with decreased $STEP_{61}$ activity. (A) Paw-withdrawal latency in the Hargreaves test before and after CFA injection in 3-month-old wild-type (WT) and *STEP* KO (KO) male mice (n = 5–6 per group). Data were analyzed by one-way analysis of variance with Bonferroni's test as post hoc. ***: $P < 0.001$ ###: $P < 0.001$, as compared with WT mice. (B) $pSTEP_{61}^{Ser221}$, (C) $pGluN2B^{Tyr1472}$ and (D) $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by western blotting of protein extracts obtained from the lumbar spinal cord of saline- and CFA-treated WT mice (n = 5 per group). Representative immunoblots are shown. Values obtained by densitometric analysis of western blotting data are expressed as percentage of saline-injected mice and represent the

mean ± SEM. Data were analyzed by the Student's *t* test. *: $P < 0.05$ and **: $P < 0.01$, as compared with saline-injected mice.