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STEP modulates nociception: evidences 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 contribute to central sensitization. STriatal-Enriched protein tyrosine Phosphatase (STEP) dephosphorylates GluN2B and ERK1/2, promoting internalization of GluN2B and inactivation of ERK1/2. STEP activity was inhibited by genetic (STEP knockout mice) and pharmacological (recently synthesized STEP inhibitor, TC-21539) 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. Interestingly, STEP 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 the increased thermal hyperalgesia associated with age and female gender in mice. Consistently, STEP knockout mice failed to show age-related thermal hyperalgesia, while 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, present results underscore an important role of spinal STEP activity in the modulation of nociception.

STEP modulates nociception: evidences 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 contribute to central sensitization. STriatal-Enriched protein tyrosine Phosphatase (STEP) dephosphorylates GluN2B and ERK1/2, promoting internalization of GluN2B and inactivation of ERK1/2. STEP activity was inhibited by genetic (STEP knockout mice) and pharmacological (recently synthesized STEP inhibitor, TC-21539) 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. Interestingly, STEP 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 the increased thermal hyperalgesia associated with age and female gender in mice. Consistently, STEP knockout mice failed to show age-related thermal hyperalgesia, while 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, 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

INTRODUCTION

Primary sensory neurons detect pain-producing stimuli [22]. There are different types of nociceptors [15] and the majority of them terminate 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 via activation of AMPA and NMDA receptors [25]. The NMDA receptor (NMDAR) subunit GluN2B plays a critical role in central sensitization. Noxious stimuli rapidly induces GluN2B phosphorylation (pGluN2B) at Tyr1472 causing its redistribution to the membrane surface of spinal dorsal horn neurons [11; 29; 48; 51; 55]. After the activation of glutamate receptors, there is a large influx of extracellular calcium, which, in turn, 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) has also been implicated in central sensitization [7; 8; 12; 20; 23].

STriatal-Enriched protein tyrosine Phosphatase (STEP) is a neural specific phosphatase that normally opposes the development of synaptic strengthening [14]. STEP has two 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 [28; 34; 51]. Multiple post-translational 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]. STEP 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]. Accumulating evidence supports that STEP levels and activity are down- or up-regulated in multiple neurodegenerative and psychiatric disorders [14]. In contrast, its role in the spinal cord is now beginning to be unraveled. While we were preparing this manuscript, another group reported that $STEP_{61}$ acts as an intermediary for GABAergic inhibition to regulate mechanical nociception and pain sensitization [28]. Moreover, $STEP_{61}$ signaling downstream the activation of noradrenergic α2 receptor attenuates ERK1/2 activation and inflammatory pain [50]. Here, we used STEP knockout (KO) mice [44] and a recently synthetized STEP inhibitor [49] and we extend these findings by showing that $STEP_{61}$ activity in the lumbar spinal cord modulates physiological nociception, as well as inflammatory pain likely through the regulation of $pGluN2B^{Tyr1472}$ and $pERK1/2^{Thr202/Tyr204}$ levels.

MATERIALS AND METHODS

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 h 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.

Drug preparation and delivery

STEP inhibitor (TC-2153; benzopentathiepin 8-(trifluoromethyl)-1,2,3,4,5-benzopentathiepin-6-amine hydrochloride) [49] was dissolved in 2% DMSO and injected (10 mg/kg; intraperitoneal, i.p.) 1 h before the behavioral assessment. For spinal application, stock solutions were obtained by diluting 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 via a silicone, 40-50 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, USA) were injected into the plantar surfaces of both hind paws of 3-month-old wild-type and *STEP* KO male mice.

Hargreaves test

To allow acclimation to the testing environment, animals were placed in the examination room 30 min 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 min prior to 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 80W for rats) was selected to produce an average baseline threshold of approximately 8 s. A 20 s cut-off was employed to prevent tissue damage in non-responsive subjects. The latency to produce a nocifensive paw withdrawal response was used to measure thermal hypersensitivity. Each hind paw was targeted three times in alternating order, producing six scores of nociception that were averaged and analyzed.

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, USA). 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 by 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.

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 the 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 lumbosacral spinal segments were carefully removed. Single monophasic, square wave electrical pulses were delivered as test stimuli to the sciatic nerve trunk at a mid-thigh level on a per-min 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 via an A/D converter card (MIO16, National Instruments, Austin, TX, USA). Field potentials evoked in superficial laminae of the spinal dorsal horn by activation of C fibers (3-3.5 mA pulses of 0.5 ms duration) were extracted from 90-200 ms latency bands (<1.2 m/s conduction velocity) and quantified as described previously [5].

Western blot

Animals were sacrificed 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] plus protease inhibitors [phenylmethylsulphonyl fluoride (2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and sodium orthovanadate (1 mM)] and centrifuged at 16,100 x *g* for 20 min. The supernatants were collected and the protein concentration was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA, USA). Western blot analysis was performed as previously described [37]. The following primary antibodies were used: anti-STEP (23E5; 1:1,000) (Santa Cruz Biotechnology, CA, USA), anti-pERK1/2$^{Thr202/Tyr204}$ (1:1,000) and anti-pGluN2B^{Tyr1472} (1:500) (Cell Signaling Technology, Beverly, MA, USA), anti-pSTEP^{Ser221} (1:1,000; Millipore Temecula, CA, USA) and anti-tubulin (1:50,000; Sigma, St. Louis, MO, USA). 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 [28; 34; 51] 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 h (15 min for loading controls) at room temperature with the corresponding horseradish peroxidase-conjugated antibody (1:2,000; Promega, Madison, WI, USA) 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).

Statistical analysis

Experimental data were analyzed using the GraphPad Prism (v. 5.01, GraphPad Software, Inc). Data are presented as mean ± SEM. Statistical analysis was performed by using the unpaired Student's t-test (95% confidence), one-way or two-way 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 to be significant if $p < 0.05$.

RESULTS

STEP knock-out mice have thermal hyperalgesia and mechanical allodynia

As a first approach to study the role of STEP in thermal nociception, we subjected 3-month-old male *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 one 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, such as 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. Similarly to that observed for thermal stimulus, we observed that the lack of STEP also reduced 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 readout of STEP activity we analyzed the phosphorylation status of two 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 total GluN2B or ERK1/2 (data not shown). In line with the results obtained in the Hargreaves test, there were no differences in pGluN2B^{Tyr1472} and pERK1/2$^{Thr202/Tyr204}$ levels between *STEP*$^{+/-}$ and *STEP*$^{-/-}$ mice.

Pharmacological inhibition of STEP contributes to thermal hyperalgesia and mechanical allodynia

In order to discard the contribution of any developmental or compensatory mechanisms to the hyperalgesia and allodynia 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 male mice with TC-2153 (10 mg/kg, i.p.), a pharmacological inhibitor of STEP [49], and explored thermal and mechanical nociception after 1 h (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.

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) and pERK1/2$^{Thr202/Tyr204}$ (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 C fiber-evoked spinal potentials by 18.5 ± 0.5% (16.37 ± 0.42 V ms area from a 13.81 ± 0.48 V ms control baseline area during superfusion with aCSF), and reached 42.28 ± 1.02% of control during administration of 1 mM TC-2153 (19.65 ± 0.55 V ms from a 13.81 ± 0.48 V ms baseline; Fig. 3D).

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; 53]. 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 that paw withdrawal latency was reduced with age in males (one-way ANOVA, $F_{(3, 56)} = 15.39$; $p < 0.001$; Fig. 4A). Interestingly, spinal STEP$_{61}$ levels were reduced with age (one-way ANOVA, $F_{(3, 20)} = 12.2$; $p < 0.001$; Fig. 4B), and there was correlation between paw latency withdrawal and STEP$_{61}$ levels ($r^2 = 0.48$; $p < 0.001$; Fig. 4C). Female mice showed the same pattern, including reduced 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), as well as a correlation between both parameters ($r^2 = 0.42$; $p < 0.001$; Fig. 4F).

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-months-old female animals presented a lower paw withdrawal latency compared with 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 (Fig. 4C and F), we next investigated 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$_{61}$ 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 in 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).

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 pre- and 24 h post-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 molecular changes associated with CFA-induced hyperalgesia in the lumbar spinal cord of wild-type animals. Western blot analysis showed that 24 h after CFA-induced inflammation there were no differences in 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.

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 both in male and female mice. Nevertheless, cortical and hippocampal $STEP_{61}$ levels increase with age [4; 53], suggesting that tissue-specific transcriptional and/or post-translational 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 still 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 activation of GluN2B and ERK1/2 in the spinal cord, similar to what occurs in different brain areas from *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 [28]. Moreover, spinal expression of a STEP$_{61}$ mutant that cannot be phosphorylated and inactivated reduces ERK1/2 phosphorylation and inflammatory pain [52]. 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 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 up-regulated in *STEP* KO mice and in STEP shRNA-transduced neurons [35]. Our data are in line with recently reported findings suggesting that a tonic level of STEP activity suppresses ERK1/2 and Fyn signaling pathways, thereby increasing synaptic availability of GluN2B and promoting central sensitization [28]. In addition, we show that selective inhibition of STEP results in significantly increased field potentials evoked in the spinal dorsal horn by C fibers

input, supporting 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, NMDAR-mediated single-channel currents recorded in dorsal horn neurons are depressed by recombinant STEP [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. Even though $STEP_{61}$ levels were unchanged in CFA-injected wild-type mice, which is in agreement with previous reports [28; 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 [28] 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 showing phosphorylation-induced $STEP_{61}$ inactivation could explain its reduced interaction with Fyn, and the increased $pGluN2B^{Tyr1472}$ levels. Nevertheless, we observed that thermal hyperalgesia upon 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 post-stroke pain [24], and if 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; 54]. However, our results highlight that caution needs to be taken since 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, 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 LEGENDS

Fig. 1. Lack or reduction of STEP levels decreases 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 Von Frey test (n = 10-12 per group). (C) $STEP_{61}$, (D) $pGluN2B^{Tyr1472}$ and (E) $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by Western blot 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 blot data are expressed as percentage of $STEP^{+/+}$ (wild-type) mice and shown as mean ± S.E.M. 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.

Fig. 2. Pharmacological inhibition of STEP causes thermal hyperalgesia in mice. (A) Schematic representation of the experimental design. (B) Paw withdrawal latency in the Hargreaves test and (C) mechanical threshold in Von Frey Test in vehicle- and TC-2153-treated mice (n = 10-12 per group). (D) $pGluN2B^{Tyr1472}$ and (E) $pERK1/2^{Tyr202/Tyr204}$ levels were analyzed by Western blot 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 blot data are expressed as percentage of vehicle-treated mice and shown as mean ± S.E.M. Data were analyzed by Student's t-test. *p < 0.05 and ***p < 0.001 as compared with vehicle-treated mice.

Fig. 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 blot 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 blot data are expressed as percentage of vehicle-treated rats, and data were analyzed by Student's t test. (D) Diagram showing mean field potential areas 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 ten spinal field potentials, and data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test. In all graphs data are expressed as mean ± S.E.M. *p < 0.05, **p < 0.01 and ***p < 0.001.

Fig. 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 blot 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 blot 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 blot 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 blot 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 data are shown as mean ± S.E.M. and data were analyzed by one-way ANOVA with Bonferroni's test as *post-hoc*. C and F graphs were determined by simple linear regression. *p < 0.05, **p < 0.01 and ***p < 0.001.

Fig. 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). Graphs showing the comparison between (B) $STEP_{61}$ levels in male and female $STEP^{+/+}$ mice at 3 months of age and at (C) 15 months of age (n = 5-6 per group). Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of 3- and 15-month-old males, respectively. (D) Paw withdrawal latency in the Hargreaves test in $STEP^{-/-}$ male versus female mice at different ages. In all graphs, data are shown as mean ± S.E.M. Data were analyzed by two-way ANOVA with Bonferroni's test as *post-hoc* in A and D and by Student's t-test in B and C. # p = 0.06, *p < 0.05 and ***p < 0.001 as compared with male mice.

Fig. 6. CFA-induced inflammatory pain correlates with decreased $STEP_{61}$ activity. (A) Paw withdrawal latency in the Hargreaves test pre- and post-CFA injection in 3-month-old wild-type and *STEP* KO male mice (n = 5-6 per group). Data was analyzed by one-way ANOVA with Bonferroni's test as *post-hoc*. ***p < 0.001 as compared with saline-injected wild-type mice and ### p < 0.001 as compared with *STEP* KO mice. (B) $pSTEP_{61}^{Ser221}$ and $STEP_{61}$, (C) $pGluN2B^{Tyr1472}$ and (D) $pERK1/2^{Thr202/Tyr204}$ levels were analyzed by Western blot of protein extracts obtained from the lumbar spinal cord of saline and CFA-treated mice (n = 5 per group). Representative immunoblots are shown. Values obtained by densitometric analysis of Western blot data are expressed as percentage of saline-inject mice and represent the mean ± S.E.M. Data was analyzed by Student's t-test. *p < 0.05, **p < 0.01 as compared with saline-injected mice.

Summary

In this work, we demonstrate by using genetic (STEP KO mice) and pharmacological (administration of the STEP inhibitor, TC-2153) approaches that STEP61 levels/activity modulates nociception (mechanical allodynia, thermal algesia and inflammatory-induced pain) likely through the regulation of pGluN2BTyr1472 and pERK1/2Thr202/Tyr204 levels in the spinal cord. We also found that STEP61 protein levels in the lumbar spinal cord inversely correlate with the increased thermal hyperalgesia associated with age and female gender in mice. In addition, we provide electrophysiological evidence that pharmacological inhibition of STEP increases C fiber-evoked spinal field potentials in rats. Therefore, taken together our results suggest an important role of STEP61 levels/activity in the modulation of nociception both under physiological and pathological conditions.

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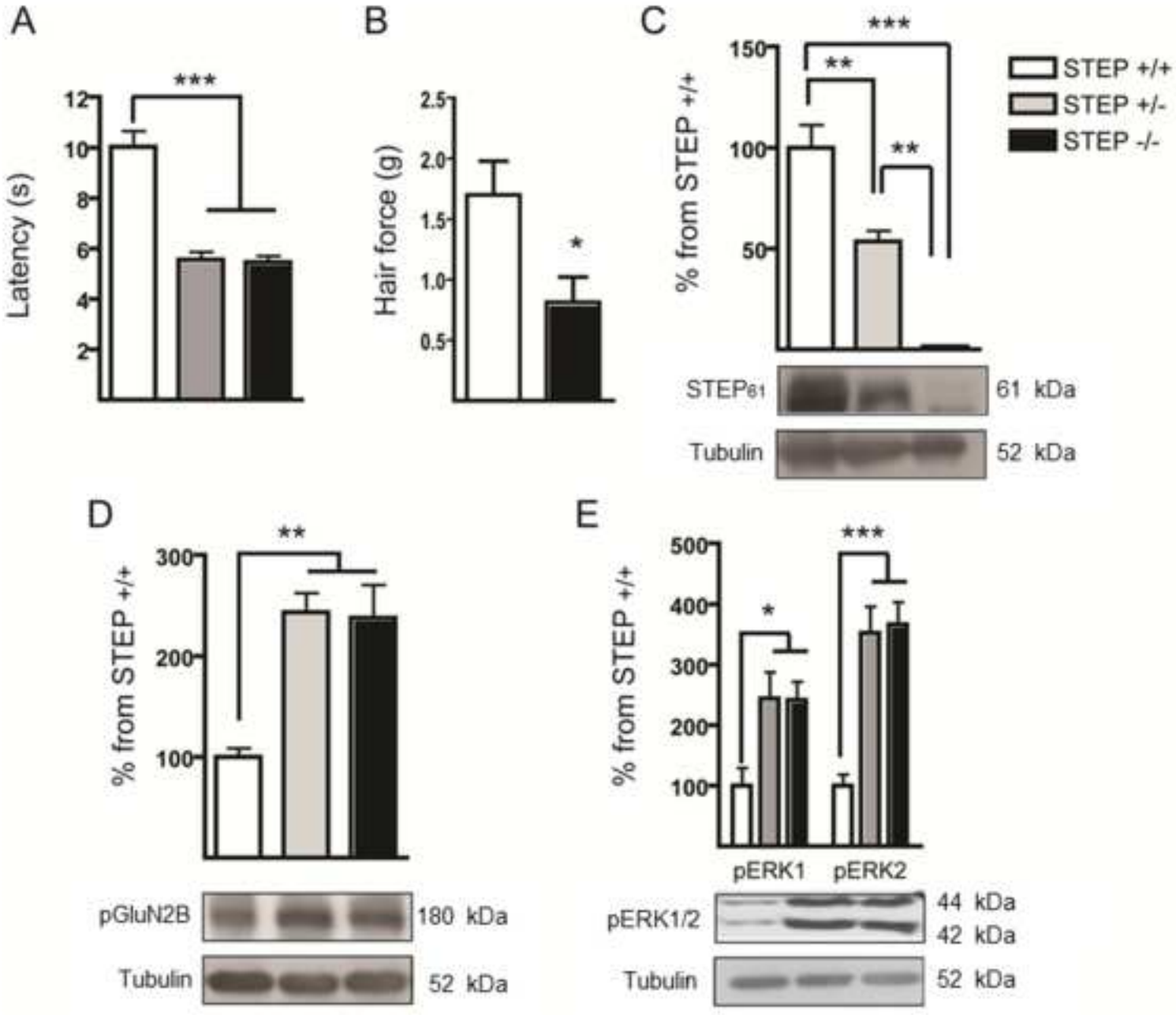

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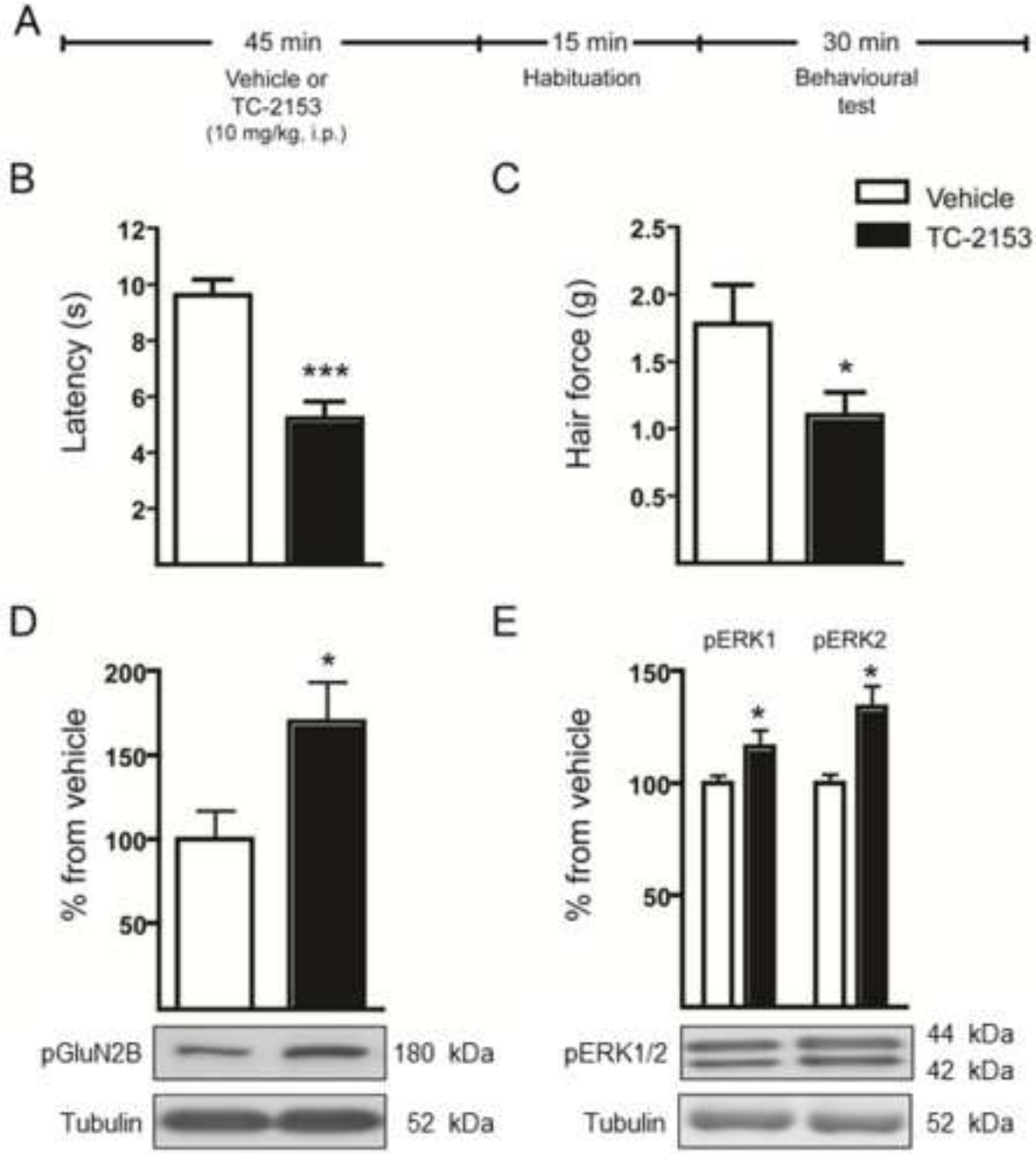

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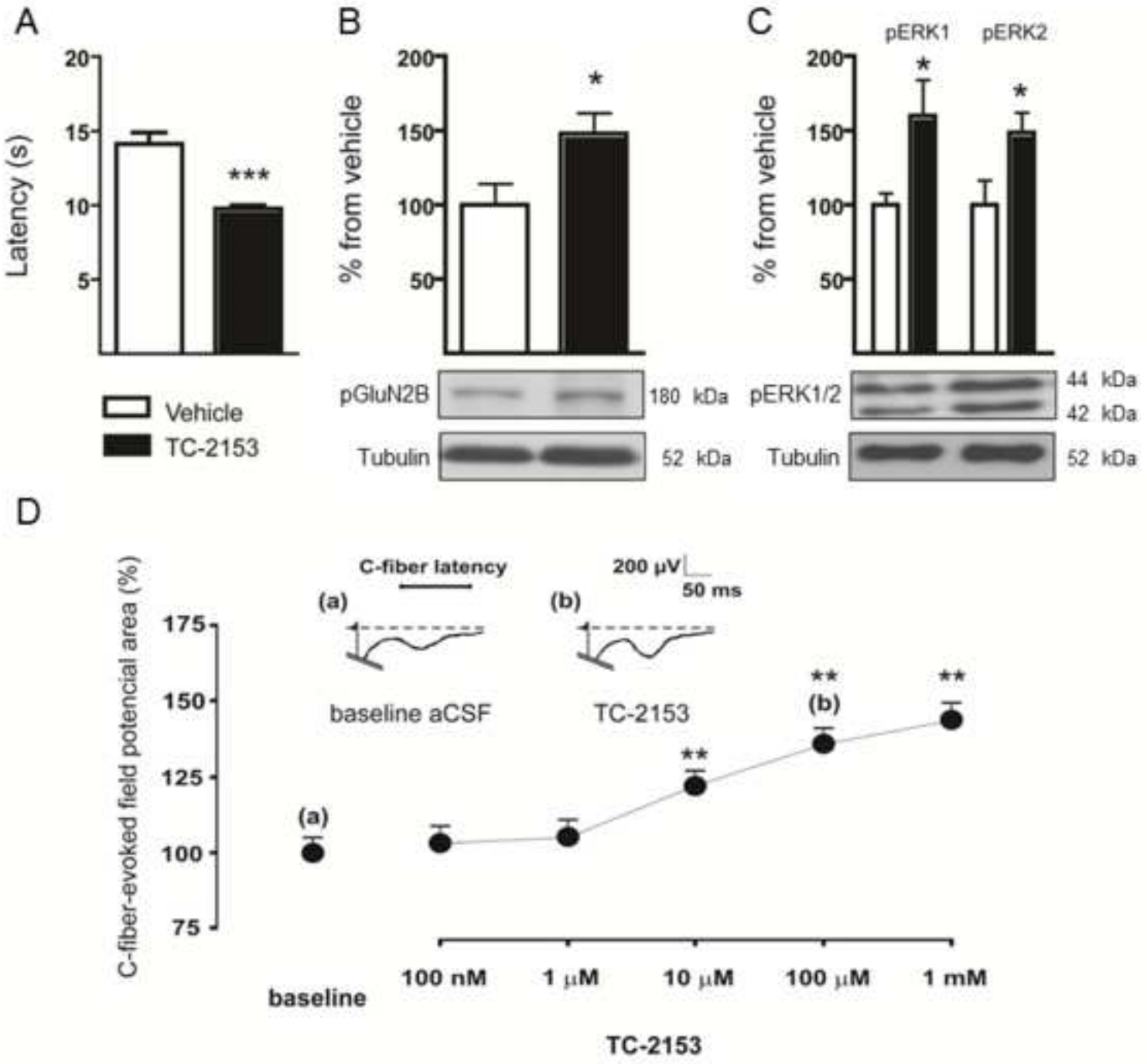

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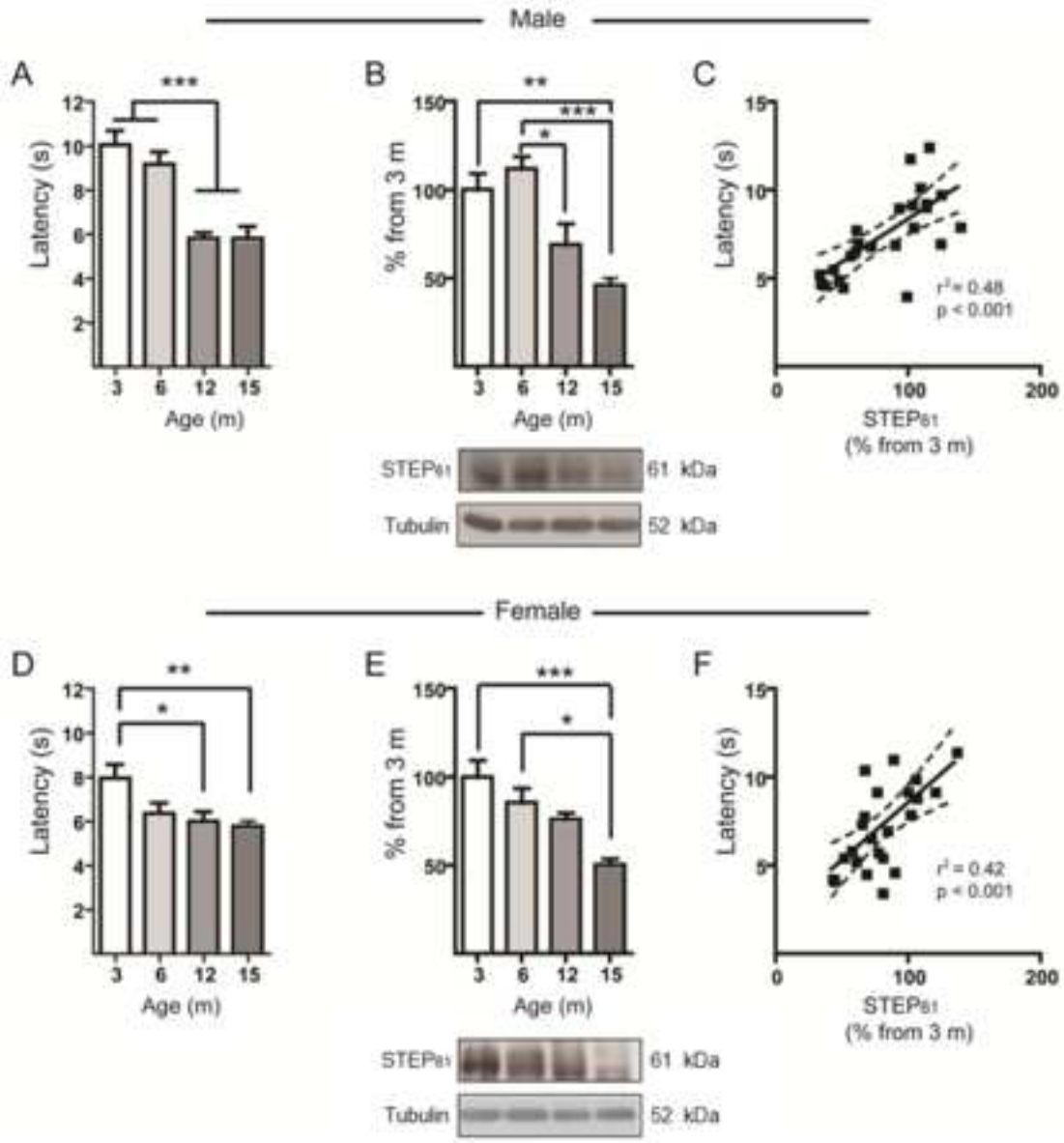

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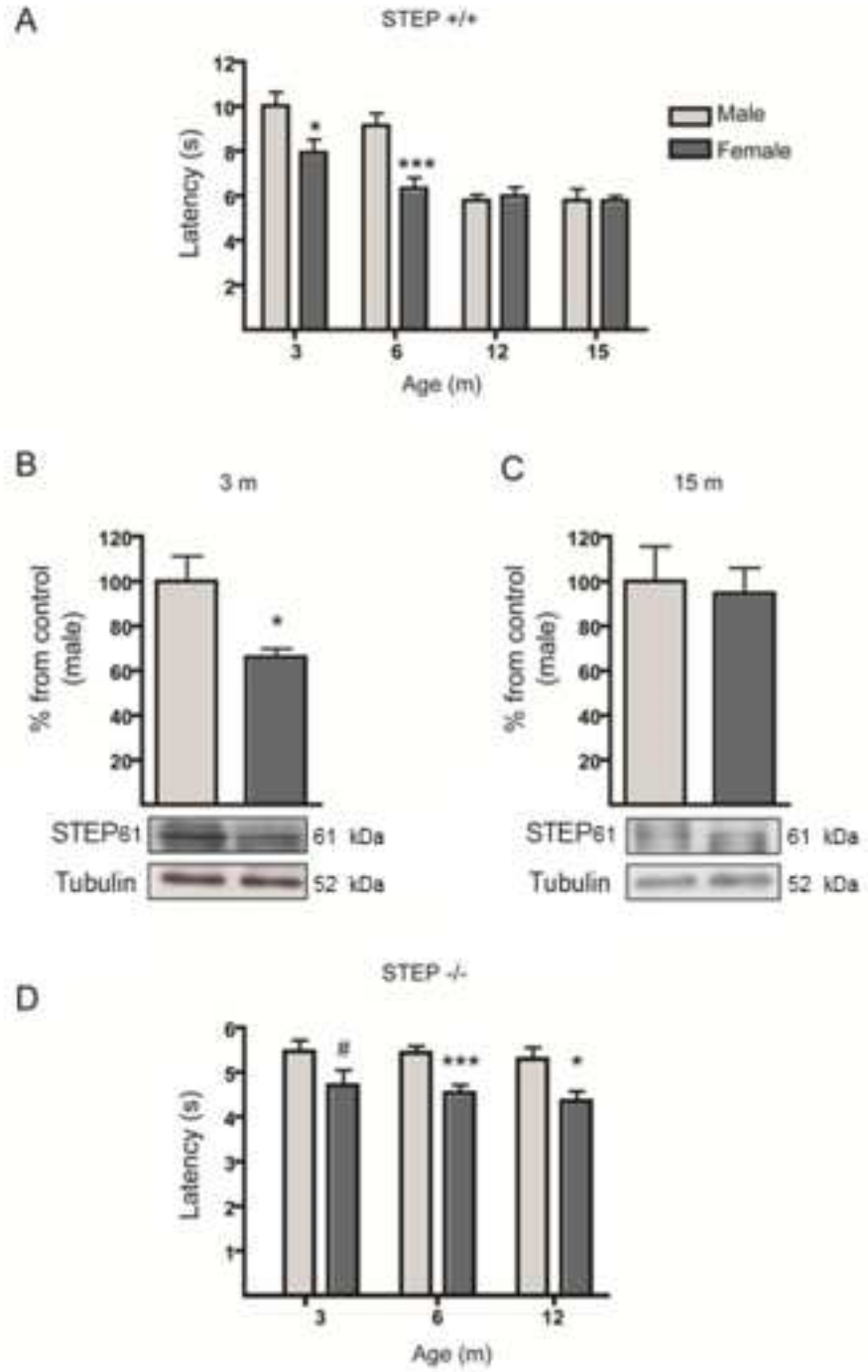

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