

Published in final edited form as:

FEBS Lett. 2006 December 11; 580(28-29): 6629–6634.

DESCENDING SEROTONERGIC FACILITATION OF SPINAL ERK ACTIVATION AND PAIN BEHAVIOR

Camilla I. Svensson, Thao K. Tran, Bethany Fitzsimmons, Tony L. Yaksh, and Xiao-Ying Hua
Department of Anesthesiology, University of California - San Diego, La Jolla, CA 92093-0818, USA

Abstract

Serotonin (5-HT) derived from bulbo-spinal projection is released by nociceptive input into the spinal dorsal horn. Here we report that formalin injection in the paw produced pain behavior (flinching) and phosphorylation of spinal ERK1/2 (P-ERK1/2, indicating activation) in rats. Depletion of spinal 5-HT by intrathecal (IT) 5,7-DHT, a serotonergic neurotoxin, profoundly reduced formalin evoked flinching and the increase in P-ERK1/2. Ondansetron (a 5-HT₃ receptor antagonist) at IT doses that inhibited flinching also attenuated spinal ERK activation. These findings reveal that primary afferent-evoked activation of spinal ERK requires the input from an excitatory 5-HT descending pathway.

Keywords

Serotonin; Descending facilitation; ERK; Spinal cord; Primary afferent; Nociception

1. Introduction

Several reports indicate that extracellular signal-regulated kinases 1 and 2 (ERK1/2) expressed in the spinal cord are involved in pain signal processing. ERK1/2 are activated in the spinal dorsal horn following peripheral inflammation and tissue/nerve injury. Pharmacologically blocking this activation reduces the hypersensitivity otherwise observed in these experimental models [1–5]. Current thinking has been that this activation of ERK is directly mediated by a local afferent evoked excitation of the dorsal horn neurons. This assertion, while reasonable, ignores the possible contribution of other dorsal horn inputs that are believed to regulate dorsal horn excitability. The excitatory serotonergic pathway, which arises from the brainstem medullary 5-HT containing nuclei of the caudal midline raphe [6–8], plays a significant role in controlling spinal excitability through activation of spinal 5-HT₃ receptors [6,9–11]. These observations led us to advance the hypothesis that spinal ERK1/2 activation observed in the face of noxious afferent stimulation is in fact mediated by this descending pathway.

2. Materials and methods

2.1. Animals

All experiments were carried out according to protocols approved by the Institutional Animal Care Committee of University of California, San Diego. Male Holzman Sprague-Dawley rats (300–350 g) were used. Chronic lumbar intrathecal (IT) catheters (PE-5, 8.5 cm) were

Corresponding author: Xiao-Ying Hua, Department of Anesthesiology, University of California - San Diego, La Jolla, CA 92093-0818, USA, Tel: 619-543-2007; Fax: 619-543-6070; E-mail: xyhua@ucsd.edu.

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implanted through a cisternal exposure under isoflurane anesthesia [12]. PD98059 (Calbiochem, San Diego, CA), 5,7-dihydroxy-tryptamine (5,7-DHT, Sigma), and Ondansetron hydrochloride (Zofran^R, GlaxoSmithKline, UK) were prepared for IT delivery in 10 μ l followed by 10 μ l saline. PD98059 was dissolved in 25% DMSO, 0.5% Tween-80 in saline, and 5,7-DHT in 45% cyclodextrin.

2.2. Western blot

Spinal cords harvested 5, 30 and 60 minutes after hind paw formalin (50 μ l, 2.5%) injection were homogenized by sonication in extraction buffer (50 mM Tris buffer, containing 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, Protease inhibitor cocktail (P-8340, Sigma 1:100), phosphatase inhibitor cocktail I and II (Sigma 1:100)). The samples were subjected to 4–12% Bis-Tris gel electrophoresis (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (Invitrogen). After 1 hour incubation in 5% low-fat milk the membranes were probed with antibodies overnight at 4°C followed by HRP-conjugated secondary antibody for 1 hour at room temperature. The signal was detected with chemiluminescent reagents (SuperSignal, Pierce, Rockford, IL). The nitrocellulose membranes were then stripped with Re-Blot Western blot recycling kit (Chemicon, Temecula, CA) and incubated with different antibodies. Antibodies used in this study: phosphorylated ERK1/2 (1:1000), total ERK1/2 (1:1000) (Cell Signaling Technology, Beverly, MA) and β -actin (1:25,000) (Sigma). Intensity of immunoreactive bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). ERK1/2 immuno-positive bands were normalized relative to β -actin.

2.3. Nociceptive models

To quantify formalin induced flinching, an automated sensing system was employed [13]. Briefly, a soft metal band was placed on the hind paw of the animal being tested, and 2.5% formalin (50 μ l) was injected into the dorsal side of the banded paw. Nociceptive flinching behavior was counted for 1-min periods for 60 minutes.

2.4. Measurements of tissue content of 5-HT

High performance liquid chromatography (HPLC) with a fluorescent detector was employed to determine the content of 5-HT in the spinal cord, sciatic nerve and skin of 5,7-DHT and vehicle treated rats [14].

2.5. Statistics

For measurements of protein levels by Western blotting and formalin test, four to seven animals were included per group. Differences between groups were compared with one-way ANOVA and Bonferroni *post hoc* test.

3. Results

3.1. Injection of formalin into the paw evokes phosphorylation of spinal ERK1/2

The formalin model is frequently used to study tissue injury induced facilitated pain states in rodents [13]. Formalin-induced hyperalgesia (i.e., phase II flinches, see below) is thought to represent spinal facilitation initiated by afferent input [15]. In agreement with the immunocytochemical studies [1,16], we showed by western blots that formalin paw injection produced an increase in phosphorylation of spinal ERK1/2. Both P-ERK1 and P-ERK2 increased profoundly in the ipsilateral spinal cord 5 min after formalin injection with the peak effects at 30 min (4–7 fold, $p < 0.05$), and returned towards baseline levels at 60 min (Fig. 1A, B, E). There were no significant changes in P-ERK1/2 in the contralateral side (Fig. 1A, B, E) or total protein expression of ERK1/2 at the time points examined (Fig. 1C, D).

3.2. Inhibition of spinal ERK1/2 attenuates formalin induced flinching

After formalin injection, the total number of flinches in the vehicle group was 224 ± 25 in phase 1 (1–9 min), and 1360 ± 111 in phase 2 (10–60 min) (Fig. 2A). Pretreatment with the MEK1 inhibitor PD98059 (IT, 1–30 μ g, 15 min prior to formalin injection) produced a dose-dependent inhibition of the phase 2 flinching (Fig. 2 A, B). The highest dose of IT PD98059 (30 μ g) reduced the second phase response in comparison with the vehicle group (786 ± 103 flinches, $p < 0.05$ vs. vehicle group) without causing any sign of sedation or motor weakness.

3.3. Depletion of spinal 5-HT prevents formalin-evoked spinal ERK1/2 phosphorylation and hyperalgesia

Two days after IT treatment with 5,7-DHT (20–60 μ g), the 5-HT level in rat spinal cord was significantly reduced, i.e., 5-HT (nmol/mg tissue): 2.8 ± 0.5 after vehicle treatment, 0.6 ± 0.2 and 0.5 ± 0.1 after 5,7-DHT 20 μ g and 60 μ g respectively (78–84% reduction, $p < 0.05$). 5,7-DHT (20 μ g, IT) which effectively depleted the 5-HT in the spinal cord, did not affect the 5-HT content in two peripheral tissues examined, sciatic nerve and skin (data not shown). No abnormal behavior or motor functions were observed in 5,7-DHT or vehicle treated animals. In rats pretreated with IT vehicle there was a robust increase in spinal P-ERK1/2 observed 30 min after injection of formalin into the paw. This elevation of spinal P-ERK1/2 was diminished in rats treated with 5,7-DHT (60 μ g, $p < 0.05$, Fig. 3). In parallel with the potent inhibitory effect on activation of spinal ERK1/2, formalin-induced second phase flinching, but not first phase, was profoundly attenuated in 5,7-DHT treated animals ($p < 0.05$, Fig. 4).

3.4. Inhibition of spinal 5-HT₃ receptors attenuates formalin-induced ERK activation and pain behavior

Intrathecal ondansetron inhibited both pain behavior and ERK activation, in agreement with the 5,7-DHT data. Ondansetron, at a dose of 1–3 μ g, inhibited the second phase of formalin (but not the first phase) flinching by 30–40% compared to saline group ($P < 0.05$, Fig. 6). Expression of P-ERK1/2 was attenuated by ondansetron (1 μ g), however, only inhibition of P-ERK2 reached statistical significance (Fig. 5).

4. Discussion

The present studies indicate that injury-evoked spinal P-ERK expression is dependent on the presence of an excitatory descending 5-HT pathway. It is known that serotonergic innervation of the spinal cord is derived largely from supraspinal sources [17]. Thus, 5,7-DHT, a neurotoxin selective for serotonergic terminals [18], at IT doses that produced a 70–80% reduction in dorsal horn 5-HT, prevented both the formalin-evoked increase in P-ERK in the dorsal horn and the phase II flinching behavior. The involvement of spinal 5-HT₃ receptors is supported by the attenuation of P-ERK after IT delivery of ondansetron, a selective 5-HT₃ antagonist, at a dose that suppressed formalin-evoked flinching.

Immunocytochemical studies [1,16] have demonstrated that shortly after peripheral formalin injection there is a marked increase in the number of neurons in the dorsal horn ipsilateral to the stimulus containing the activated (phosphorylated) form of ERK1/2. An important issue relates to the organization of the input that initiates phosphorylation of dorsal horn ERK1/2. It has been shown in a variety of neuronal systems that membrane depolarization triggered by excitatory transmitters activates ERK [8]. Therefore, in the present context, it may be argued that activation of ERK in the dorsal horn is evoked by substance P and glutamate released from the central terminals of primary afferents. This suggestion is supported by the observations that afferent-evoked activation of spinal ERK1/2 is blocked by antagonism of neurokinin 1 (NK1) and N-methyl-D-aspartate receptors [1,16]. While it is evident that ERK activation could be initiated by primary afferent input, spinal neuronal excitability is also regulated by

input from supraspinal sources [8]. Apart from the presence of descending inhibitory pathways altering pain thresholds [17,19,20], there is a growing body of data indicating the importance of descending facilitatory pathways [6,17,21]. The descending serotonergic system arises in the rostroventral medulla, projects to the dorsal horn and exerts a potent facilitatory effect through dorsal horn 5-HT₃ receptors. Thus, formalin-induced spinal neuronal activation (assessed as cFOS expression) and nocifensive behaviors are significantly reduced after either functional elimination of 5-HT₃ receptors [10] or by spinal delivery of the 5-HT₃ receptor antagonists [6,7]. It is currently thought that this facilitatory drive is initiated by small afferent input leading to activation of superficial dorsal horn NK1 receptor-bearing neurons projecting to the periaqueductal grey and parabrachial area [22]. These areas connect to spinopetal serotonergic projections via the rostral ventromedial medulla [6,9] and serotonin is released in the spinal cord through this spino-bulbo-spinal loop upon high intensity afferent input [23]. These observations along with our present finding that 5,7-DHT abolish formalin-induced ERK activation highlights the importance of descending facilitatory systems and suggests that enhancement of afferent-initiated spinal ERK activation as one mechanism underlying this descending facilitation.

Although there are conflicting reports, spinal 5-HT₃ receptors are considered to have predominantly excitatory functions [6,17,24]. Immunocytochemical and *in situ* hybridization studies have revealed that 5-HT₃ receptors are present on superficial dorsal horn neurons and terminals [10,25–28], a finding consistent with the processing of nociceptive information in the spinal cord. A proportion of the 5-HT₃ receptors are located on terminals of primary afferents while others are on dorsal horn interneurons, in particular on a subgroup of excitatory interneurons in lamina II that uses glutamate as neurotransmitter. It is known that 5-HT₃ receptor channels enhance calcium permeability and phospholipase C activity and both events can reinforce ERK activation that is initially triggered by neurotransmitters released from sensory afferents [1,16,17]. Our data indicate that the 5-HT₃ receptors are possibly involved in descending 5-HT-mediated facilitation of spinal ERK signaling.

In conclusion, the present findings emphasize the role of a spino-bulbo-spinal serotonergic pathway activated by nociceptive input in initiating a facilitated state of processing at the level of spinal dorsal horn. This spinopetal input acts by the release of spinal 5-HT which works through a local 5-HT₃ receptor to activate ERK-containing cell systems. These spinal ERK systems then serve to enhance nociceptive transmission. These studies thus show the importance of an extra-spinal linkage in mediating afferent evoked changes in local dorsal horn excitability. It is evident that this loop pathway must now be routinely considered in interpreting the effects of afferent stimulation on dorsal horn function and particularly those indices such as ERK, which mediate facilitated processing.

ACKNOWLEDGEMENT

This work was supported by NIH NS 16541. The authors wish to thank Alan Moore and Steve Rossi for performing 5-HT assay.

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Abbreviations

ERK, extracellular signal-regulated protein kinase; IT, intrathecal; NK1, neurokinin 1; P-ERK, phosphorylated extracellular signal-regulated protein kinase; SP-SAP, substance P and saporin; 5-HT, 5-hydroxytryptamine.

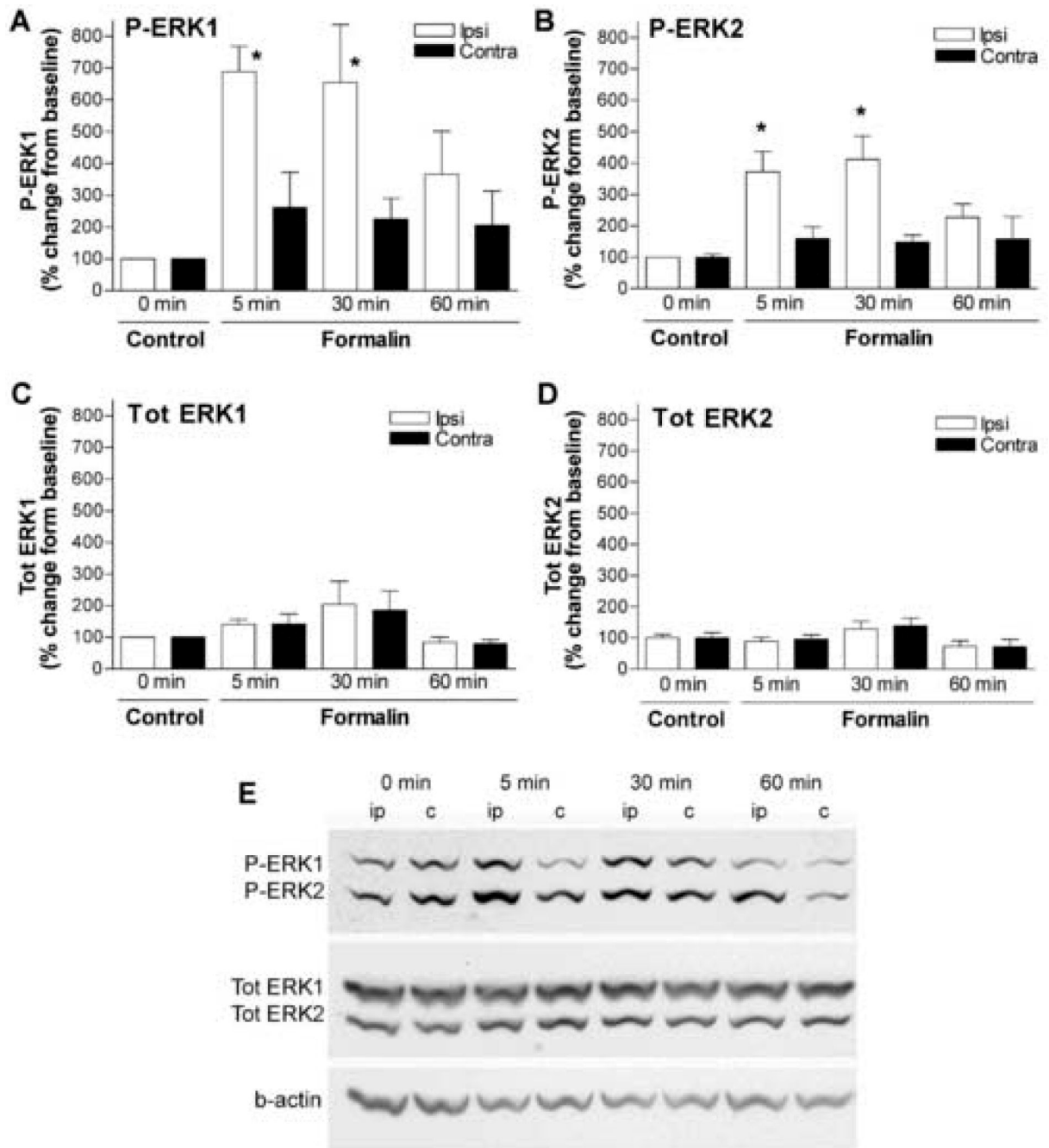


Figure 1.

Injection of formalin to the paw activates ERK1/2 in the spinal cord. Percentage change of phosphorylated (P) ERK1 (A), P-ERK2 (B), Total (Tot) ERK1 (C) and Tot-ERK2 (D) in the lumbar dorsal cord ipsilateral (ipsi) or contralateral (contra) to formalin paw injection at 5, 30 and 60 min compared to naïve control. The data were normalized against beta-actin (b-actin) expressed in each sample. N=4–7/group, * $p < 0.05$, 5 or 30 min vs. 0 min, one-way ANOVA. (E) Representative western blots showing the levels of P-ERK1/2, tot ERK1/2 and b-actin in ipsilateral (ip) and contralateral (c) spinal cords after formalin injections at the time points of 5, 30 and 60 min (0 min = naïve rat).

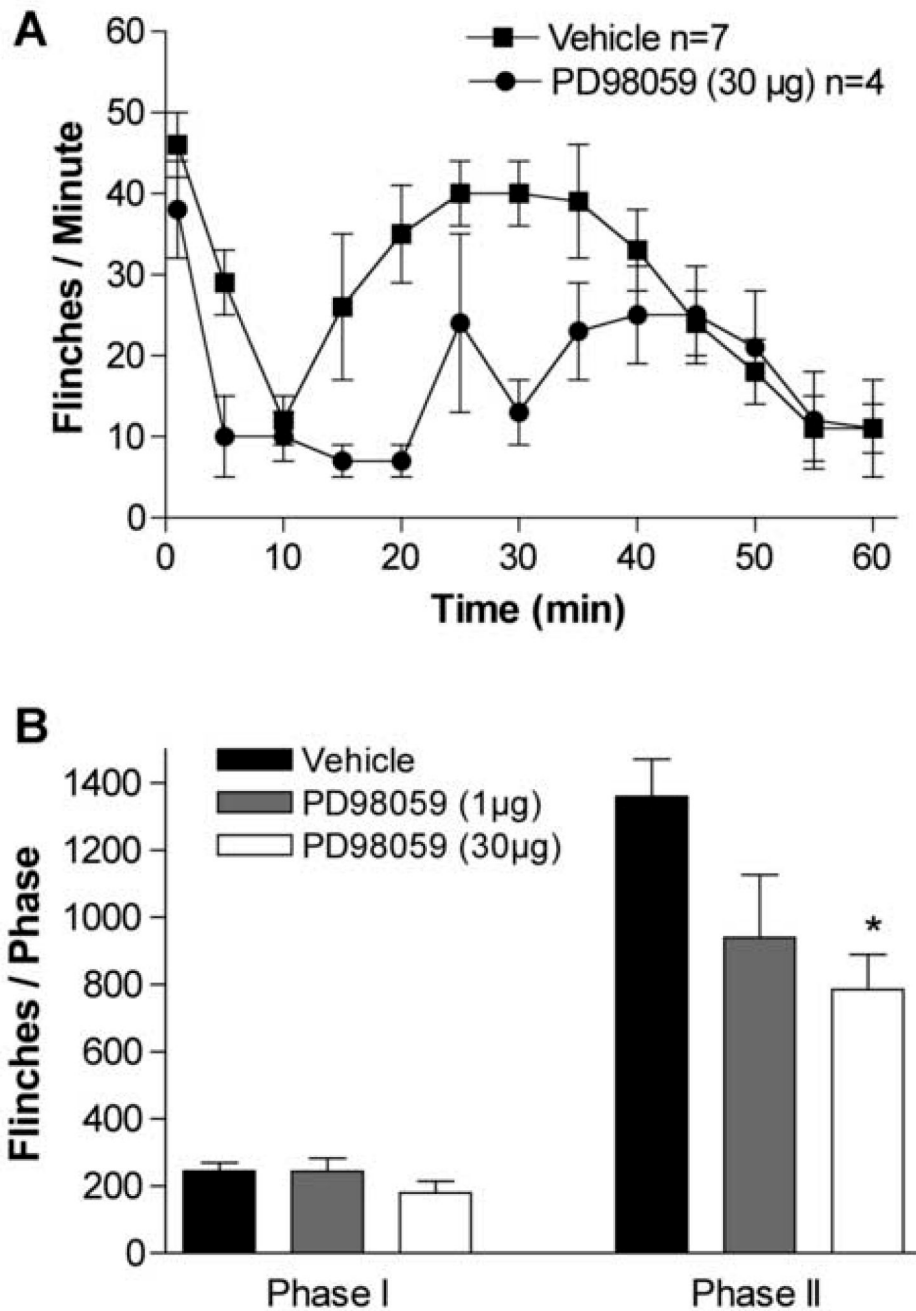


Figure 2. Inhibition of ERK1/2 activity by blocking MEK1 activity attenuates formalin evoked formalin induced flinching. **(A)** Number of flinches/minute plotted versus time following injection of formalin to the paw and IT injection of PD98059 (30 µg), or vehicle (10 µl). **(B)** Histogram showing the dose-response effect of IT PD98059 (1–30 µg) on formalin test phase I and phase II flinching behavior. The bars represent the total number of flinches in each phase: Phase I, 1–9 min; Phase II, 10–60 min. The data are presented as mean ± SEM of 4–7 rats/group. * $p < 0.05$, PD98059 (30 µg) vs. vehicle, one-way ANOVA.

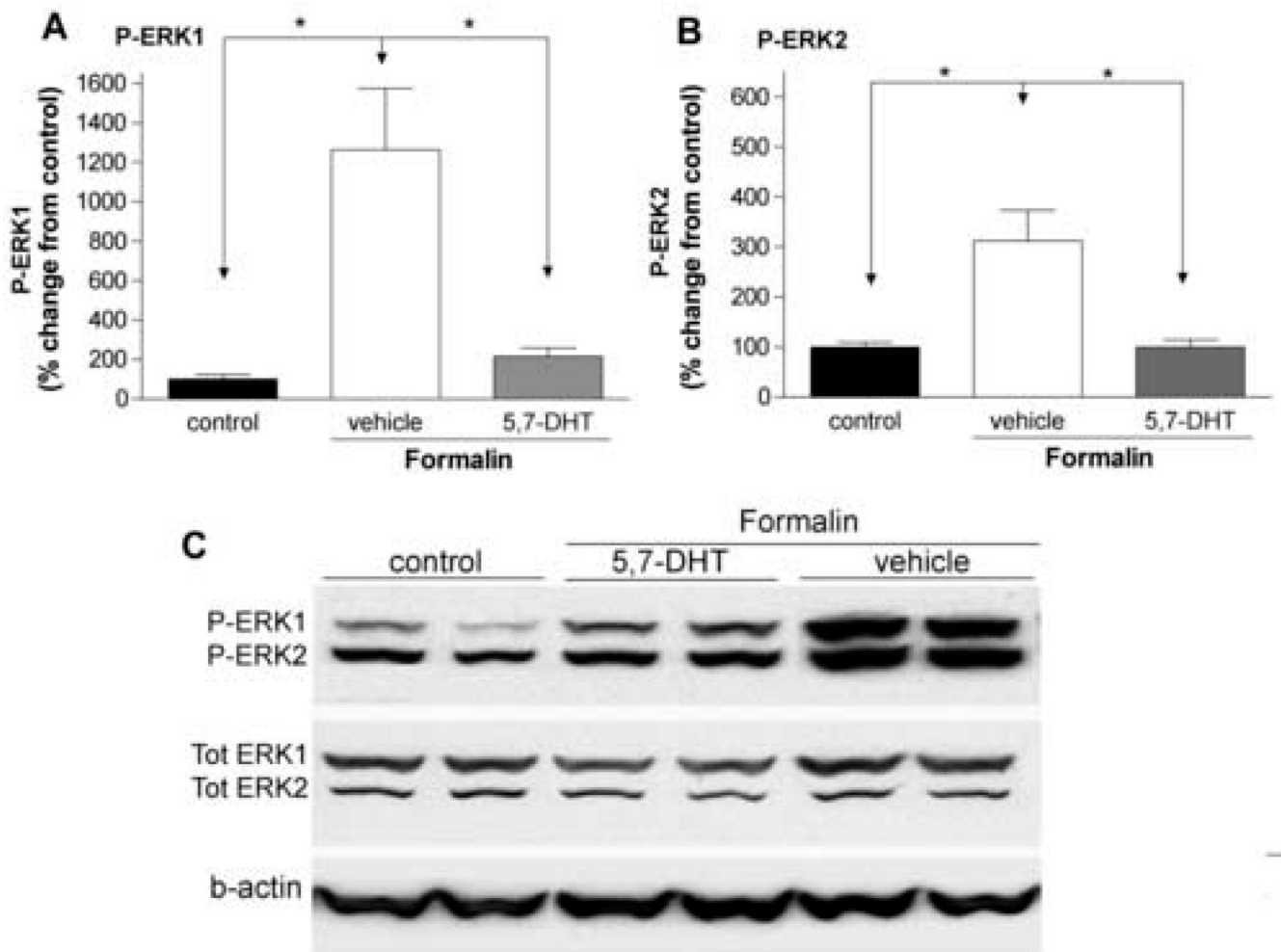


Figure 3. Intrathecal pretreatment with 5,7-DHT blocks formalin-evoked phosphorylation of spinal ERK1/2. Percentage change of P-ERK1 (**A**), P-ERK2 (**B**) in the ipsilateral lumbar spinal cord 30 min after formalin paw injection in the control (no formalin injection), vehicle (10 μ l) and 5,7-DHT (60 μ g) group. The data were normalized against beta-actin (b-actin) expressed in each sample and presented as mean \pm SEM of 4–7 rats/group. * $p < 0.05$, one-way ANOVA. (**C**) Representative western blots showing the levels of P-ERK1/2, tot ERK1/2 and b-actin in ipsilateral spinal cords after formalin injections in rats.

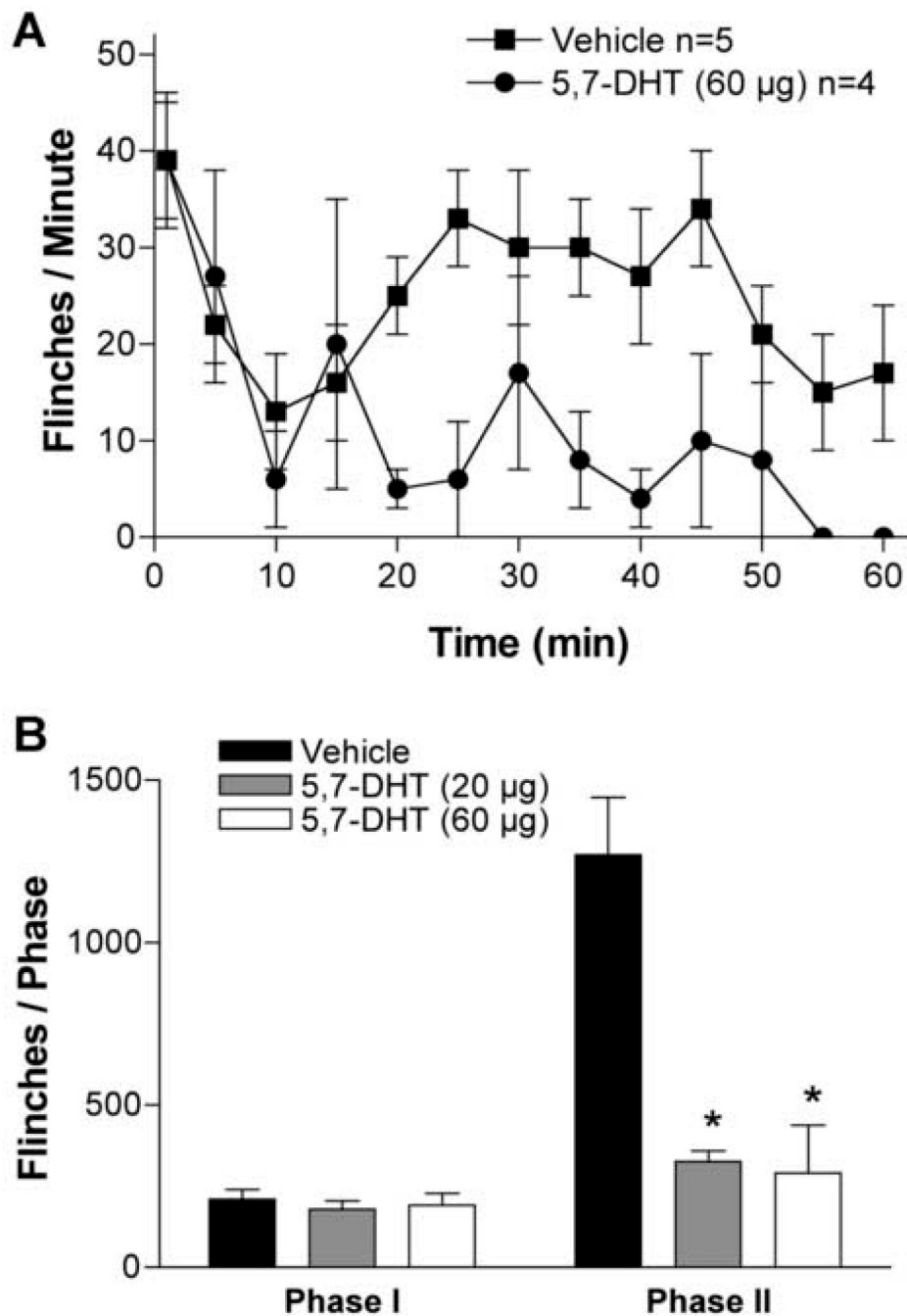


Figure 4. Depletion of spinal 5-HT prevents formalin-evoked hyperalgesia. **(A)** Flinches/minute plotted versus time after injection of formalin in rats treated with 5,7-DHT (60 µg, IT) or vehicle (10 µl, IT). **(B)** Histogram showing the dose-response effect of 5,7-DHT (20–60 µg, IT) on formalin test phase I and phase II flinching behavior. The bars represent the total number of flinches in each phase. The data are presented as mean ± SEM of 4–5 rats /group. * $p < 0.05$, 5,7-DHT vs. vehicle, one-way ANOVA.

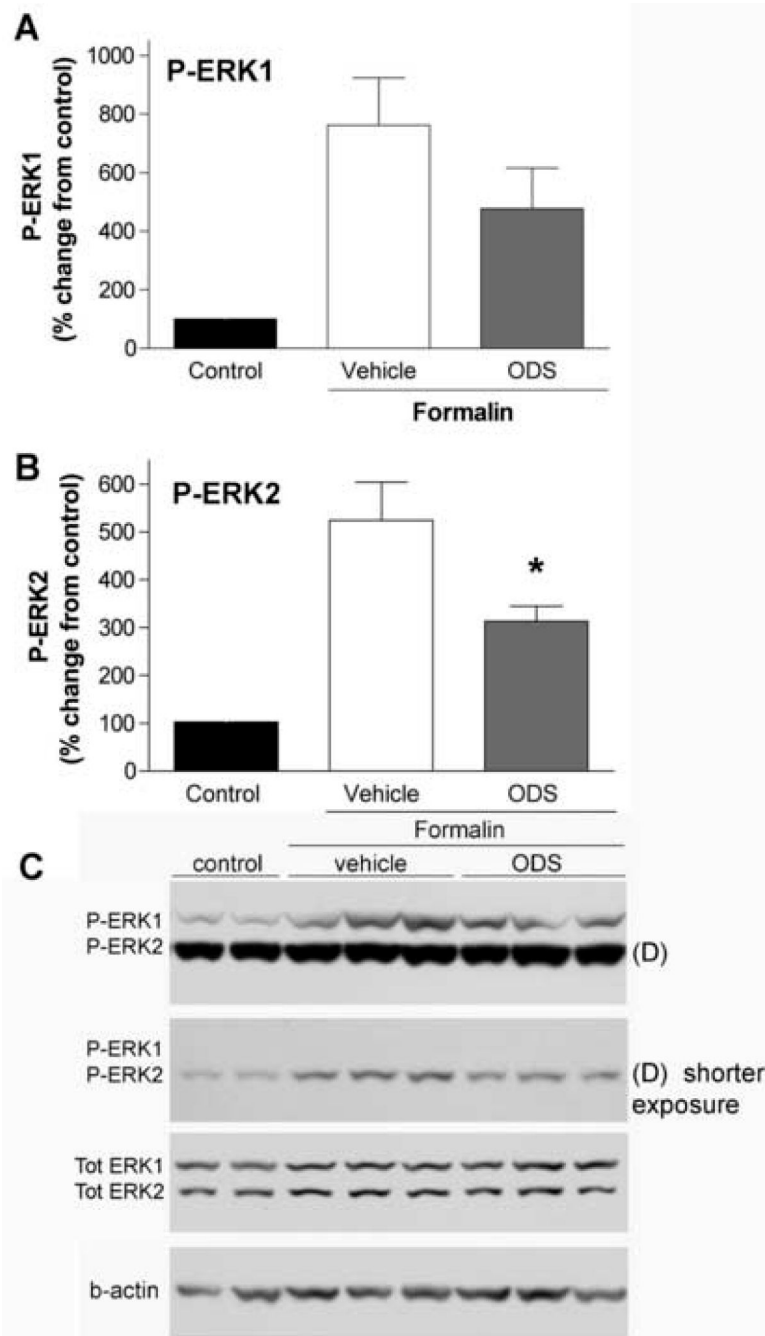


Figure 5.

Intrathecal injection of ondansetron reduces formalin-evoked phosphorylation of spinal ERK1/2. Percentage change of P-ERK1 (**A**) and P-ERK2 (**B**) in ipsilateral lumbar spinal cord 30 min after formalin injection in the control (no formalin injection), vehicle (saline, 10 μ l) and ondansetron (ODS, 1 μ g) group. The vehicle and ondansetron were delivered IT 15 min before the formalin injection. Reduction of formalin-evoked P-ERK1/2 by ondansetron was observed, however, only P-ERK2 reduction reached statistical significance (* $p < 0.05$, one-way ANOVA, $N=4-7$ /group). (**C**) Representative western blots showing the levels of P-ERK1/2, tot ERK1/2 and b-actin in ipsilateral spinal cords after formalin injections in rats with and without IT ondansetron. (**D**) P-ERK2 showed a higher level of phosphorylation in this

experiment and in order to avoid saturation during quantification two different exposure times was used for this membrane.

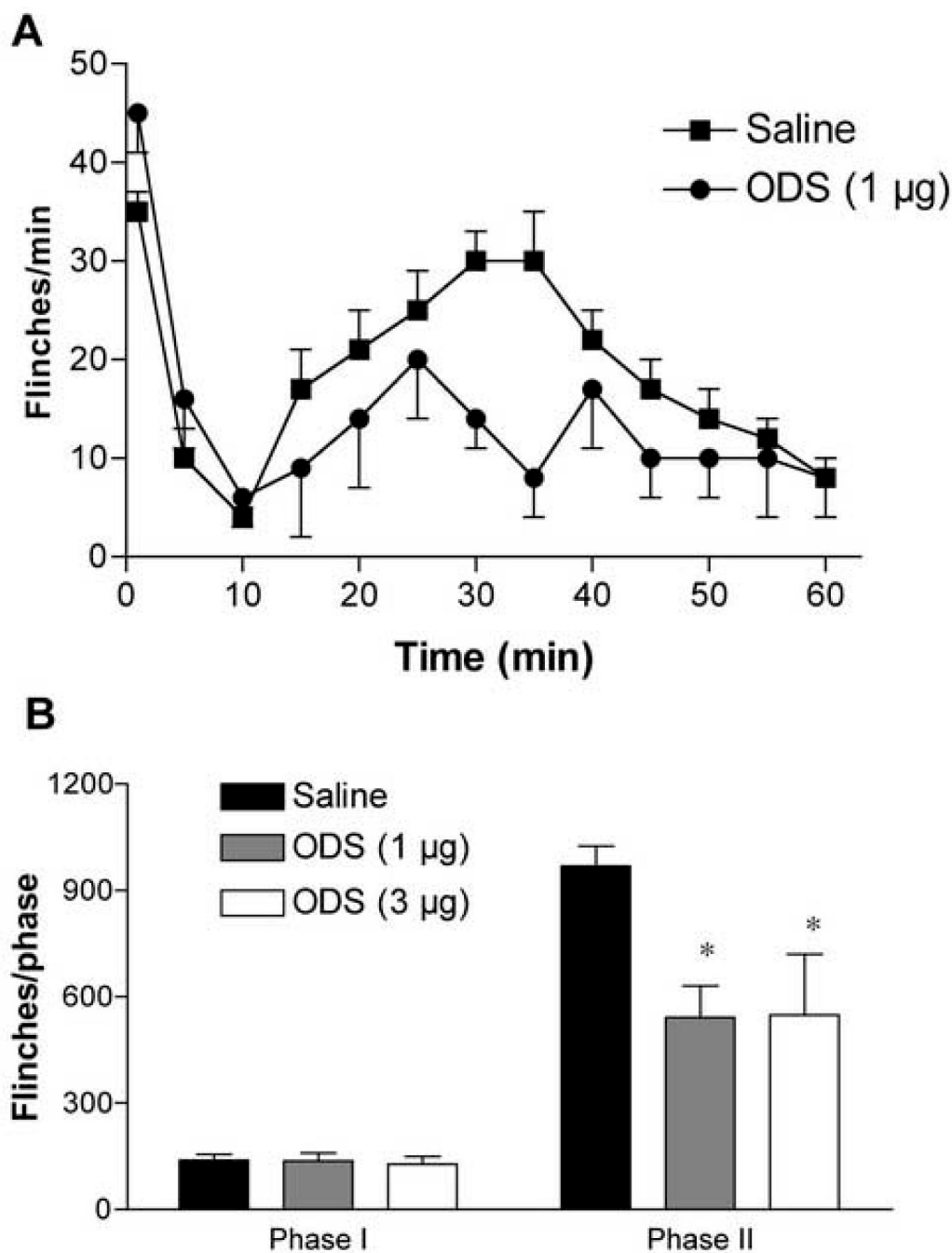


Figure 6. Blockade of spinal 5-HT₃ receptors attenuates formalin-evoked hyperalgesia. **(A)** Flinches/minute plotted versus time after injection of formalin in rats treated with IT injection of ondansetron (ODS, 1 µg), or vehicle (saline, 10 µl). Each point represents the number of flinches per min. **(B)** Histogram shows inhibitory effects of IT ondansetron at doses of 1 and 3 µg on phase I and phase II flinching behavior. The bars represent the total number of flinches in each phase. The data are presented as mean ± SEM of 4–11 rats/group. * $p < 0.05$, ODS vs. saline, one-way ANOVA.