

Plenary Article

Role of the spinal Na^+/H^+ exchanger in formalin-induced nociception

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ABSTRACT

This study assessed the role of the Na^+/H^+ exchanger (NHE) in the formalin-induced nociception as well as the expression of the NHE isoform 1 (NHE1) in the rat spinal cord by using immunohistochemistry. Rats received a 50 μl injection of diluted formalin (0.5%). Nociceptive behavior was quantified as the number of flinches of the injected paw. Intrathecal administration of the partially selective NHE1 inhibitors DMA, EIPA (0.3–30 $\mu\text{M}/\text{rat}$) and the selective NHE1 inhibitor zoniporide (0.03–3 $\mu\text{M}/\text{rat}$) significantly increased formalin-induced flinching behavior in a dose-dependent manner during both phases of the test. Immunohistochemical analysis of the rat lumbar spinal cord showed that NHE1 was mainly expressed in the lamina I of the dorsal horn of the spinal cord. Double immunofluorescence staining showed co-localization of NHE1 with the peptide-rich sensory nerve fiber markers, substance P and calcitonin gene-related peptide, but not with markers of neuronal cell bodies (NeuN), microglia (OX-42) or astroglia (GFAP). Collectively, these pharmacological and anatomical results suggest that spinal NHE1 plays a role in formalin-induced nociception acting as a protective protein extruding H^+ .

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Inflammatory pain is a complex phenomenon characterized by spontaneous pain and hypersensitivity, which is in part caused by altered plasticity in the spinal cord [9]. The molecular mechanisms underlying this nociceptive plasticity, however, are not fully understood.

Intra- and extracellular pH can rapidly and transiently change in response to neuronal activity. Neurons may become acidified in response to neurotransmitters and chemical compounds [7,12]. Transient changes in intracellular pH can modulate many physiological functions including neurotransmitter release and neuronal excitability [3,35]. It has been reported that the mechanisms responsible for the regulation of intracellular pH in neurons consist of Na^+/H^+ exchangers, $\text{Na}^+/\text{HCO}_3^-$ co-transporters and Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchangers [1,2,29]. The Na^+/H^+ exchanger (NHE) family consists of nine distinct isoforms, which share the same overall structure but differ in their cellular localization and sensitivity to amiloride and its derivatives [23,27]. The NHE1–NHE5 isoforms are expressed in the plasma membrane, whereas NHE6–NHE9 are localized in intracellular organelles [27]. Among them, NHE1 is the most abundant and ubiquitously expressed in mammalian cells including those found in the CNS

[21]. NHEs utilize the transmembrane Na^+ gradient to remove intracellular H^+ and thus they play a pivotal role in pH regulation of brain synaptosomes [18], and hippocampal [29] and trigeminal sensory neurons [15].

There is increasing evidence for a role of NHE in the modulation of pain transmission [15,30,32]. Electrophysiological studies performed on nociceptive C-fibers demonstrated that pharmacological inhibition of the exchanger leads to an increase in the frequency of discharge measured as the total number of spikes [32]. In support of this, behavioral studies showed that, at the peripheral level, the inhibition of NHE in three different models of acute inflammatory pain increased nociceptive behaviors [30]. However, there are no studies evaluating the involvement of NHE in pain processing at the spinal level as well as the localization of NHE1 in nociceptive neurons. The purpose of this study was to examine the role of NHE at the spinal level in formalin-induced flinching behavior and to determine the expression and localization of NHE1 in the spinal cord by using immunohistochemistry.

All behavioral experiments were performed on Wistar female rats (180–220 g). For immunohistochemistry studies, female Sprague–Dawley rats were used (Harlan, Indianapolis, IN, USA). All experiments followed the IASP Guidelines [40] and were approved by the Institutional Animal Care Committee (Cinvestav, México, D.F.).

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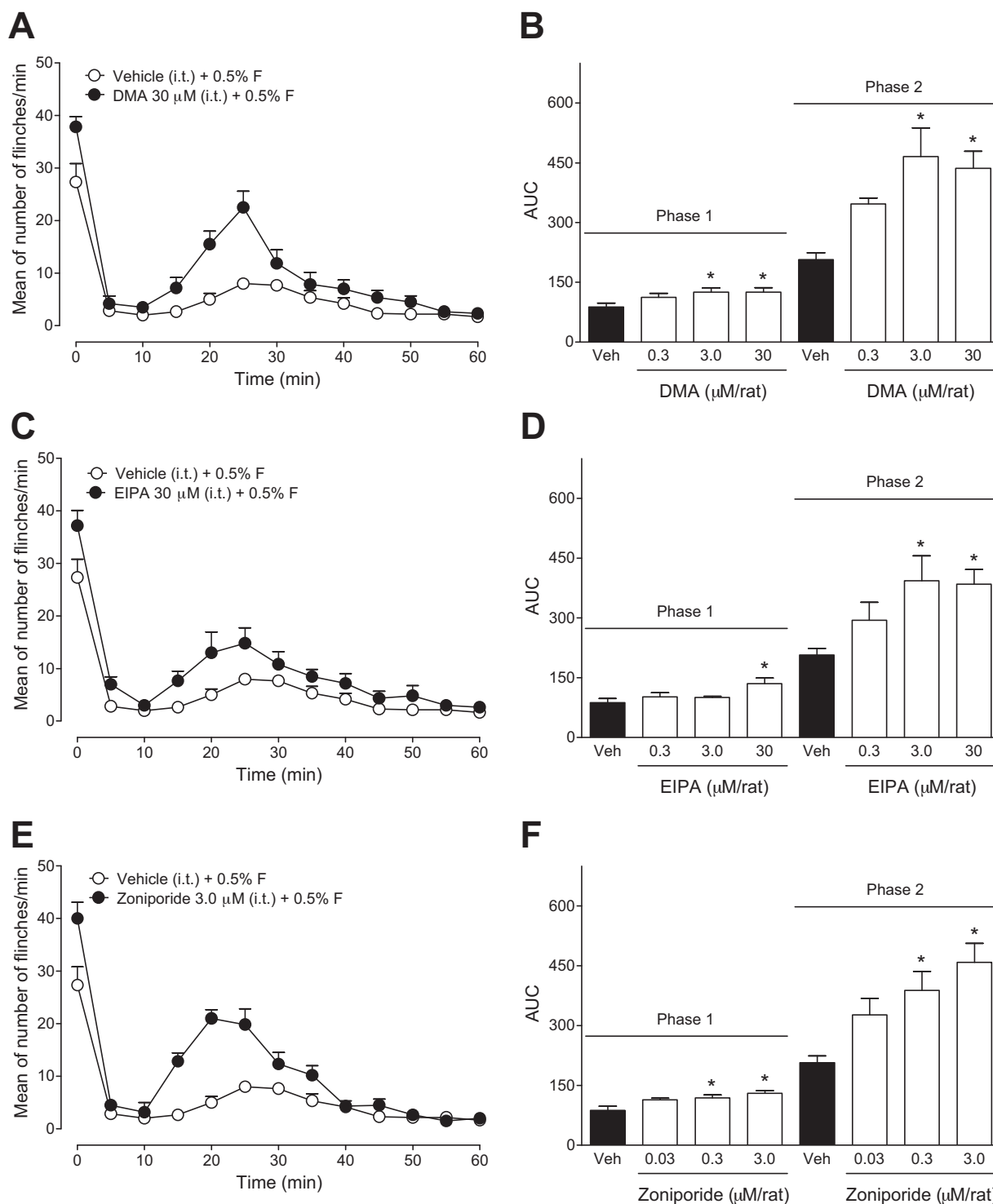


Fig. 1. Pro-nociceptive effect of selective NHE inhibitors in the formalin test. Left panels show the time course of the pronociceptive effect of DMA (30 μ M/rat, A), EIPA (30 μ M/rat, C) and zonisporide (3 μ M/rat, E) in rats receiving injection of 0.5% formalin into the dorsum of the hind paw. Right panels show the AUC of DMA (B), EIPA (D) and zonisporide (E) in both phases of the formalin test. Data are the mean \pm SEM; $n = 6$.

For measurement of nociception, rats received a spinal injection (10 μ l) of vehicle or increasing doses of 5-(N,N-dimethyl) amiloride hydrochloride (DMA, 0.3–30 μ M, Sigma–Aldrich), 5-(N-ethyl-N-isopropyl)amiloride (EIPA, 0.3–30 μ M, Sigma–Aldrich) and [1-(Quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl] guanidine dihydrochloride (zonisporide, 0.03–3 μ M, Trocis Bio-Science) before the 0.5% formalin injection. This concentration was used based on our previous study showing that NHE inhibitors

increased flinching behavior [30]. The NHE inhibitors were selected on the basis of their selectivity [10,23].

Expression of NHE1 protein in the lumbar spinal cord was assessed by immunohistochemistry. Double immunostaining of NHE1 with neuronal nuclear antigen (NeuN, neuronal marker), OX-42 (a microglial marker), glial fibrillary acidic protein (GFAP, an astrocytic marker), substance P and calcitonin gene-related peptide (SP and CGRP, peptide-rich nerve fiber markers) and purinergic

receptor P2X₃ (peptide-poor nerve fiber marker) was used to identify the cell type that expresses NHE1. A pre-absorption (Cat# NHE11-P, Alpha Diagnostic International Inc.) and omission of the primary antibody controls were included.

Chronic catheterization of the spinal subarachnoid space [39] and methods for assessing nociception [30] were performed as previously reported. For immunohistochemistry, the lumbar spinal cord, from naïve rats, was removed and post-fixed for 4 h. The L4–L6 segments of the lumbar spinal cord were cut into 30- μ m-thick-sections and thaw-mounted on gelatin-coated slides. Frozen sections were processed according to previously reported methods [19]. NHE1 was labeled using an anti-NHE1 antibody (rabbit; 1:100; Cat# NHE11A; Alpha Diagnostic International Inc.) [11]. Double immunostaining was carried out using the following antibodies: NeuN (mouse; 1:150; Cat# MAB377; Millipore), GFAP (mouse; 1:1000; Cat# G3893; Sigma–Aldrich), OX-42 (mouse; 1:150; Cat# MCA255G; Serotec), SP (guinea pig; 1:500; Cat# GP14110; Neuromics), CGRP (goat, 1:500; Cat# Ab36001; Abcam) and P2X₃ receptor (guinea pig; 1:10,000; Cat# GP10108; Neuromics).

All results are presented as mean \pm standard error of the mean (SEM) of six animals per group. Curves were constructed by plotting the number of flinches as a function of time. The area under the number of flinches against time curves (AUC) was calculated using the trapezoidal rule.

One-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare differences between treatments. Differences were considered significant when $P < 0.05$.

Subcutaneous injection of 0.5% formalin into the right hind paw of intrathecal vehicle pre-treated rats produced two distinct phases of nociceptive behavioral responses as previously reported [30,33]. Phase I of the nociceptive response began immediately after formalin administration and then declined gradually in approximately 10 min. Phase II began approximately 15 min after formalin administration and lasted approximately 1 h (Fig. 1).

Intrathecal administration of each NHE inhibitor alone did not produce nociceptive behavior (data not shown). However, pre-treatment (-10 min) with intrathecal DMA or EIPA significantly increased ($P < 0.05$) flinching behavior during both phases of the formalin test (Fig. 1A and C). The maximal effect of both NHE inhibitors was observed in the early phase II. NHE inhibitors augmented AUC, in a concentration-dependent manner (Fig. 1B and D). Thus, DMA or EIPA pre-treated rats displayed an approximately 90% increase in flinching behavior as compared to the vehicle group (Fig. 1B and D). Likewise, the selective NHE1 inhibitor zoniiporide [22] also increased flinching behavior in both phases of the test; however, the effect of zoniiporide was observed with lower concentrations than those used with DMA and EIPA (Fig. 1E and F). Altogether, these results suggest that the observed pro-nociceptive effect could be due to the inhibition of the spinal NHE1 isoform.

Fig. 2 illustrates the overall pattern of NHE1 like-immunoreactivity in the rat spinal cord. NHE1 was expressed in the gray matter of the spinal cord, but the densest labeling was in lamina I, which contained many heavily labeled NHE1 like-immunoreactive fibers. There was no positive staining in the dorsal horn from tissue sections in which pre-absorption with the corresponding blocking peptide was added or primary antibody was omitted (data not shown).

Double staining showed that in the dorsal horn, NHE1 colocalized preferentially with CGRP (Fig. 3D–F) and SP (Fig. 3G–I), but not with NeuN (Fig. 3A–C), OX-42 (Fig. 4A–C), GFAP (Fig. 4D–F), or P2X₃ (Fig. 4G–I). CGRP and SP like-immunoreactivity were prominent across lamina I and outer layer II (Ilo), which is in agreement with previous studies [5,26]. These results suggest that NHE1 is expressed primarily in peptide-rich sensory nerve fibers in the gray matter of the spinal cord.

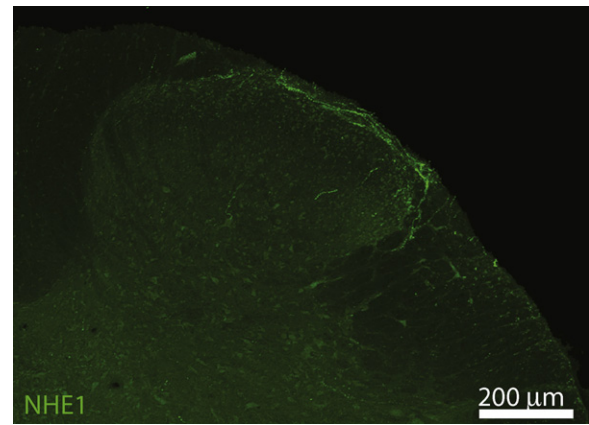


Fig. 2. Expression of NHE1 in the lumbar region of the spinal cord. confocal photomicrographs of the dorsal horn obtained from L4–L6 lumbar spinal cord sections that were stained with an antibody against NHE1. This image represents a montage of six separate images that were obtained from 30- μ m-thick tissue sections and acquired with a 20 \times lens.

The formalin test is a model of persistent pain that involves elements of peripheral and central sensitization and ongoing afferent activity [33]. Formalin (0.5%) injection produced two well defined phases of nociceptive behaviors. At this concentration, formalin seems to induce direct damage to peripheral terminals that express the transient receptor potential cation channel subfamily A member 1 (TRPA1) in a subset of TRPV1⁺ nociceptors [4,25,31]. This nerve injury produces a barrage of activity and an increase in the expression of activating transcription factor 3 (ATF3) [4], a cellular marker of nerve injury, and cFos [31], a marker of neuronal activation, that finally leads to central sensitization which is maintained by mechanisms within the dorsal horn and by ongoing peripheral inputs [6,31].

In this study, we demonstrated, for the first time, that NHE1 inhibition at the spinal level increases nociceptive behavior in the formalin test. Spinal administration of DMA, EIPA and zoniiporide augmented flinching behavior induced by formalin. Since DMA, EIPA and zoniiporide are selective and potent inhibitors of the NHE isoforms that preferentially block NHE1 [10,22,23], our data suggest that NHE1 plays an important role extruding H⁺ from the nociceptive neurons. In addition, there is evidence that these compounds inhibit the electroneutral exchange of Na⁺ and H⁺ with an IC₅₀ in the 10–100 μ M range [17,22,23]. In the present study, we have used concentrations in the same range that would be expected to block H⁺ extrusion via NHE1 without affecting the function of NHE2, NHE3 or NHE5 [17,23]. Thus, these results suggest that the pro-nociceptive effect observed in the formalin test may be due to changes in the intracellular pH induced by the inhibition of spinal NHE1.

Previous studies have shown that an increase of neuronal activity (such as that induced by formalin) may produce rapid fluctuations in intracellular pH, which may affect the functions of multiple enzymes, ion channels and other macromolecules, thus altering synaptic transmission and neuronal excitability [7,34,35]. For instance, repetitive stimulation of the dorsal root evokes transient acidification in the dorsal horn [8]. These data suggest that peripheral tissue injury leads to neuronal hyperactivity and acidification of the synaptic cleft [38]. This acidification is present in the extracellular and intracellular space. There is evidence for intracellular acidification in rat dorsal root ganglion neurons and a heterologous expression systems through transient receptor potential vanilloid 1 (TRPV1) [12] and acid-sensing ion channels (ASIC) [37] whereas other studies report an intracellular acidification of mammalian neurons associated with Ca²⁺ permeation

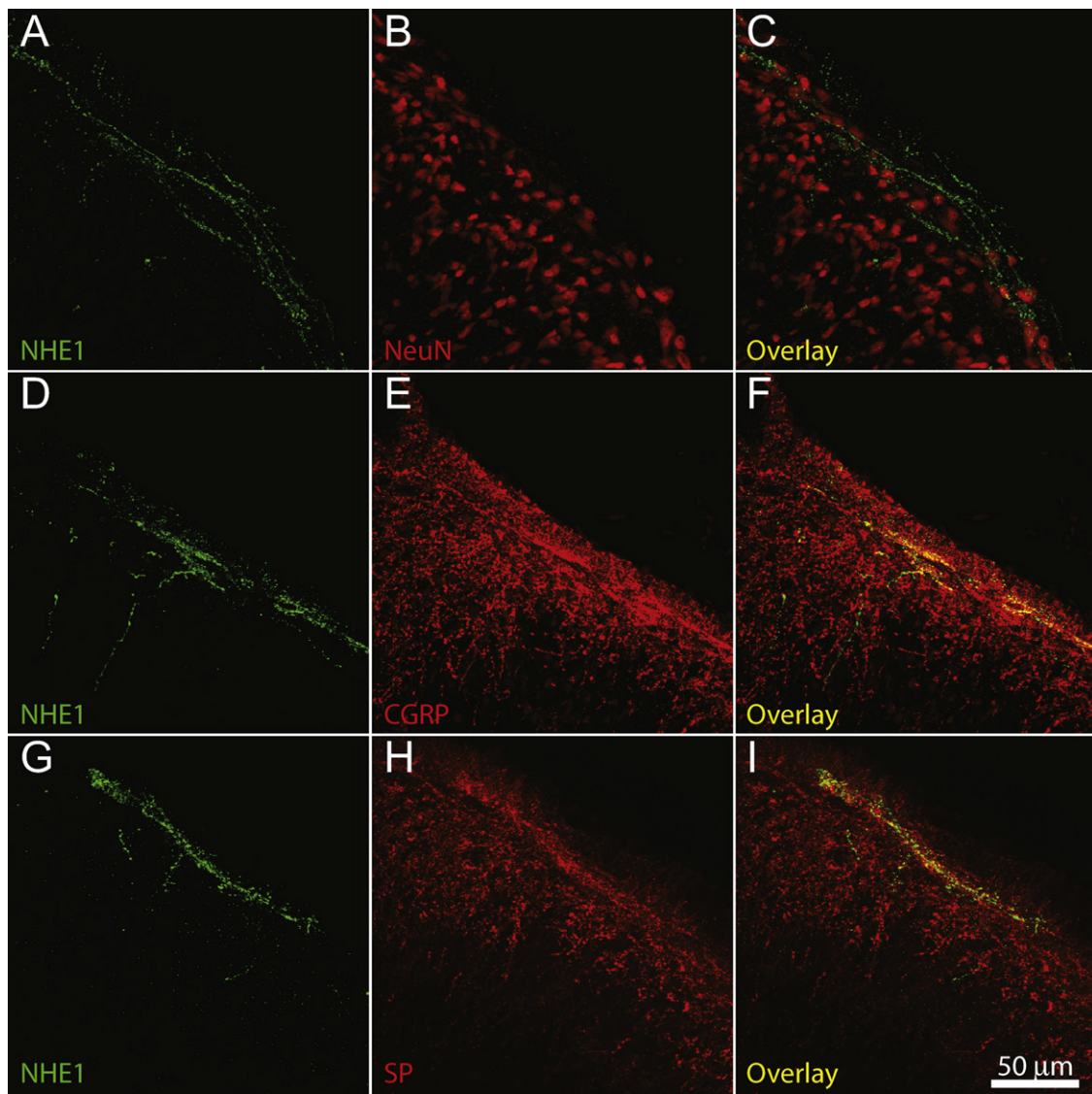


Fig. 3. Expression of NHE1 in sensory neurons of the spinal cord dorsal horn. Panels A–I are representative confocal photomicrographs of the dorsal horn obtained from L4–L6 lumbar spinal cord sections that were double-stained with antibodies against NHE1 (A)/NeuN (B), NHE1 (D)/CGRP (E) and NHE1 (G)/SP (H). When images D and E and G and H are merged in F and I, respectively, co-localization appears yellow. Images in A–C, D–F and G–I are from the same spinal cord section and were acquired at 0.5 μm z-plane intervals.

through NMDA receptors [16]. Accordingly, *in vitro* studies have shown that glutamate increases intracellular Ca^{2+} followed by a decrease in intracellular pH [15]. In the light of these findings, it is likely that during formalin-induced nociception, the increase of neuronal activity could produce changes in the intracellular pH in neurons of the spinal cord dorsal horn. Consequently, a mechanism involved in the regulation of intracellular pH should exist to protect neurons from excessive acidification. It is well known that NHE is an important H^+ extrusion mechanism, which contributes to the recovery from acidification in mammalian neurons [15,29] as well in non-neuronal cells (microglia and astrocytes) [20,28]. The fact that spinal DMA, EIPA and zonisporide increases formalin-induced nociception (this study), and that application of EIPA to trigeminal ganglion neurons in culture delays its intracellular pH recovery [15] strongly supports this suggestion.

Our pharmacological results suggest the presence of the NHE1 isoform at the lumbar levels of the spinal cord. Accordingly, previous data have demonstrated the presence of NHE1 mRNA and NHE1 protein in the spinal cord by RT-PCR and western blot, respectively [30]. However, the cellular location of NHE1 in the spinal cord was

unknown. In the present study, we detected, through immunohistochemistry, the protein of the NHE1 isoform in the spinal cord. Our results show that NHE1 is mainly expressed in axon nerve fibers of the lamina I of the dorsal horn spinal cord. Furthermore, these NHE1⁺ axons also express markers of peptide-rich sensory nerve fibers including SP and CGRP. However, NHE1-like immunoreactivity was not found in cell body neurons, microglial or astroglial cells. These results suggest that NHE1 is localized preferentially in axons and not in cell bodies of neurons or glial cells. Our data contrast with previous studies that have reported that the NHE1 isoform is functionally expressed in neurons, astrocytes and microglia [28,36]. The reason for this discrepancy is unclear, but could be related to: (1) differences in the tissue examined (brain versus spinal cord), (2) differences in the experimental approach (*in vitro* versus *in vivo*), or (3) differences in the antibody used. It is important to note that NHE1 was co-expressed with CGRP and SP, which are neurochemical markers widely used to label peptide-rich sensory nerve fibers, but not with P2X₃, a marker of peptide-poor sensory nerve fibers. The peptide-rich nerve fibers are known to project predominantly to lamina I and outer lamina II of the dorsal horn [26]. In the rat,

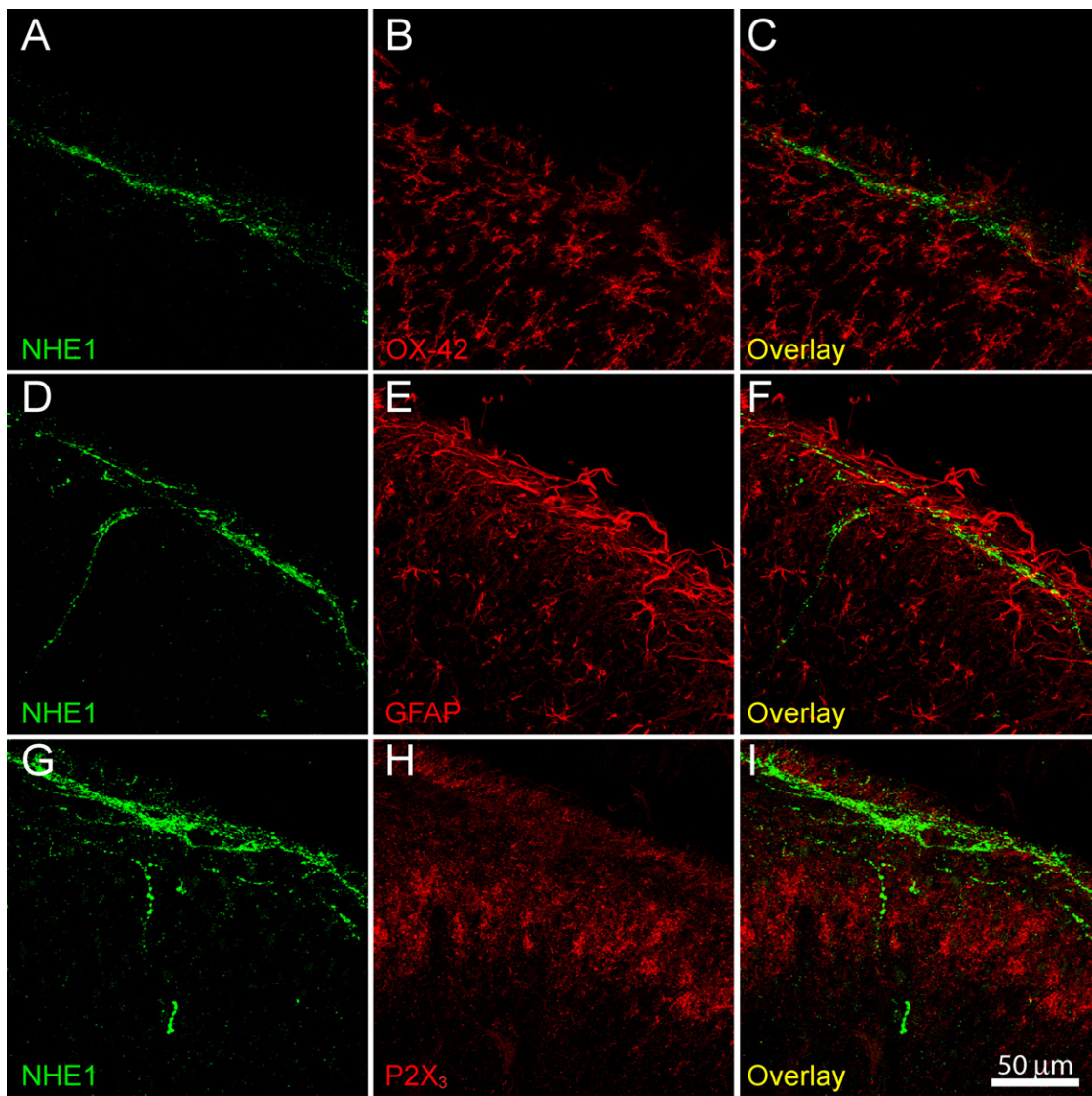


Fig. 4. Cellular localization of NHE1 in the spinal dorsal horn. Panels A–I are representative photomicrographs of the dorsal horn obtained from L4–L6 lumbar spinal cord sections that were double-stained with antibodies against NHE1 (A)/OX-42 (B), NHE1 (D)/GFAP (E) and NHE1 (G)/P2X₃ (H). C, F and I show no colocalization of NHE1 with the microglial marker OX-42, the astrocytic marker GFAP and the peptide-poor sensory nerve fiber marker P2X₃, respectively. Images were acquired at 0.5 μm z-plane intervals.

CGRP-like immunoreactivity is restricted to primary afferents [24], while SP⁺ nerve fibers in layers I and II may originate from three sources: (1) primary afferent neurons of the dorsal root ganglia, (2) axons derived from local circuit neurons and (3) from neurons from supraspinal sites like brainstem which project to the spinal cord [13,14]. Based on the present results and previous studies, it is likely that NHE1 is expressed preferentially in nociceptive nerve fibers; however, we cannot discard the possibility that NHE1 may also be expressed in projection neurons.

The spinal role of NHE1 in the formalin-induced nociception is supported by several lines of evidence. NHE1 is expressed on trigeminal [15] and dorsal root ganglion [30] neurons and is preferentially localized in peptide-rich nerve fibers in the dorsal horn (this study). NHE1 is an H⁺ extrusion mechanism that contributes to the recovery from acidification in neurons [15,29], and the inhibition of the spinal NHE1 enhances formalin-induced nociceptive behavior (this study). There are at least 4 possible explanations for the increase in nociception by NHE1 inhibitors: (1) to limit the extrusion of H⁺ and favoring intracellular acidification (our working hypothesis), (2) to limit H⁺ in extracellular space, (3) to delay the recovery of intracellular pH, or (4) a combination of the

aforementioned mechanisms. However, since we did not measure intracellular pH in neurons we cannot discard other options. Taken together, these data suggest an antinociceptive role for NHE1 in the dorsal horn of the spinal cord.

In conclusion, pharmacological and anatomical results suggest that NHE1 may be modulating pain transmission at the spinal level. Moreover these data suggest that the mechanisms involved in pH regulation could be a target for the development of a new class of analgesic drugs.

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References

- [1] M.O. Bevensee, M. Apkon, W.F. Boron, Intracellular pH regulation in cultured astrocytes from rat hippocampus. II. Electrogenic Na/HCO₃ cotransport, *J. Gen. Physiol.* 110 (1997) 467–483.

- [2] M.O. Bevensee, R.A. Weed, W.F. Boron, Intracellular pH regulation in cultured astrocytes from rat hippocampus. I. Role of HCO_3^- , *J. Gen. Physiol.* 110 (1997) 453–465.
- [3] U. Bonnet, D. Bingmann, M. Wiemann, Intracellular pH modulates spontaneous and epileptiform bioelectric activity of hippocampal CA3-neurons, *Eur. Neuropsychopharmacol.* 10 (2000) 97–103.
- [4] J.M. Braz, A.I. Basbaum, Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli, *Pain* 150 (2010) 290–301.
- [5] J.L. Brown, H. Liu, J.E. Maggio, S.R. Vigna, P.W. Mantyh, A.I. Basbaum, Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis, *J. Comp. Neurol.* 356 (1995) 327–344.
- [6] T.J. Coderre, K. Yashpal, J.L. Henry, Specific contribution of lumbar spinal mechanisms to persistent nociceptive responses in the formalin test, *Neuroreport* 5 (1994) 1337–1340.
- [7] M. Chesler, Regulation and modulation of pH in the brain, *Physiol. Rev.* 83 (2003) 1183–1221.
- [8] A. Chvatal, P. Jendelova, N. Kriz, E. Sykova, Stimulation-evoked changes in extracellular pH, calcium and potassium activity in the frog spinal cord, *Physiol. Bohemoslov.* 37 (1988) 203–212.
- [9] R. Dubner, M.A. Ruda, Activity-dependent neuronal plasticity following tissue injury and inflammation, *Trends Neurosci.* 15 (1992) 96–103.
- [10] C. Frelin, P. Barbry, P. Vigne, O. Chassande, E.J. Cragoe Jr., M. Lazdunski, Amiloride and its analogs as tools to inhibit Na^+ transport via the Na^+ channel, the Na^+/H^+ antiporter and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, *Biochimie* 70 (1988) 1285–1290.
- [11] C. Graham, I. Gatherer, I. Haslam, M. Glanville, N.L. Simmons, Expression and localization of monocarboxylate transporters and sodium/proton exchangers in bovine rumen epithelium, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 292 (2007) R997–R1007.
- [12] N. Hellwig, T.D. Plant, W. Janson, M. Schafer, G. Schultz, M. Schaefer, TRPV1 acts as proton channel to induce acidification in nociceptive neurons, *J. Biol. Chem.* 279 (2004) 34553–34561.
- [13] T. Hokfelt, J.O. Kellerth, G. Nilsson, B. Pernow, Substance p: localization in the central nervous system and in some primary sensory neurons, *Science* 190 (1975) 889–890.
- [14] S.P. Hunt, J.S. Kelly, P.C. Emson, J.R. Kimmel, R.J. Miller, J.Y. Wu, An immunohistochemical study of neuronal populations containing neuropeptides or gamma-aminobutyrate within the superficial layers of the rat dorsal horn, *Neuroscience* 6 (1981) 1883–1898.
- [15] S.M. Hwang, N.Y. Koo, M. Jin, A.J. Davies, G.S. Chun, S.Y. Choi, J.S. Kim, K. Park, Intracellular acidification is associated with changes in free cytosolic calcium and inhibition of action potentials in rat trigeminal ganglion, *J. Biol. Chem.* 286 (2011) 1719–1729.
- [16] R.P. Irwin, S.Z. Lin, R.T. Long, S.M. Paul, N-methyl-D-aspartate induces a rapid, reversible, and calcium-dependent intracellular acidosis in cultured fetal rat hippocampal neurons, *J. Neurosci.* 14 (1994) 1352–1357.
- [17] I.S. Jang, M.S. Brodwick, Z.M. Wang, H.J. Jeong, B.J. Choi, N. Akaike, The Na^+/H^+ exchanger is a major pH regulator in GABAergic presynaptic nerve terminals synapsing onto rat CA3 pyramidal neurons, *J. Neurochem.* 99 (2006) 1224–1236.
- [18] T. Jean, C. Frelin, P. Vigne, P. Barbry, M. Lazdunski, Biochemical properties of the Na^+/H^+ exchange system in rat brain synaptosomes. Interdependence of internal and external pH control of the exchange activity, *J. Biol. Chem.* 260 (1985) 9678–9684.
- [19] J.M. Jimenez-Andrade, W.G. Mantyh, A.P. Bloom, H. Xu, A.S. Ferng, G. Dussor, T.W. Vanderah, P.W. Mantyh, A phenotypically restricted set of primary afferent nerve fibers innervate the bone versus skin: therapeutic opportunity for treating skeletal pain, *Bone* 46 (2010) 306–313.
- [20] Y. Liu, D.B. Kintner, V. Chanana, J. Algharabli, X. Chen, Y. Gao, J. Chen, P. Ferrazzano, J.K. Olson, D. Sun, Activation of microglia depends on Na^+/H^+ exchange-mediated H^+ homeostasis, *J. Neurosci.* 30 (2010) 15210–15220.
- [21] E. Ma, G.G. Haddad, Expression and localization of Na^+/H^+ exchangers in rat central nervous system, *Neuroscience* 79 (1997) 591–603.
- [22] R.B. Marala, J.A. Brown, J.X. Kong, W.R. Tracey, D.R. Knight, R.T. Wester, D. Sun, S.P. Kennedy, E.S. Hamanaka, R.B. Ruggeri, R.J. Hill, Zoniporide: a potent and highly selective inhibitor of human Na^+/H^+ exchanger-1, *Eur. J. Pharmacol.* 451 (2002) 37–41.
- [23] B. Masereel, L. Pochet, D. Laeckmann, An overview of inhibitors of Na^+/H^+ exchanger, *Eur. J. Med. Chem.* 38 (2003) 547–554.
- [24] P.W. McCarthy, S.N. Lawson, Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene-related peptide-like immunoreactivity, *Neuroscience* 34 (1990) 623–632.
- [25] C.R. McNamara, J. Mandel-Brehm, D.M. Bautista, J. Siemens, K.L. Deranian, M. Zhao, N.J. Hayward, J.A. Chong, D. Julius, M.M. Moran, C.M. Fanger, TRPA1 mediates formalin-induced pain, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 13525–13530.
- [26] D.L. McNeill, K. Chung, S.M. Carlton, R.E. Coggeshall, Calcitonin gene-related peptide immunostained axons provide evidence for fine primary afferent fibers in the dorsal and dorsolateral funiculi of the rat spinal cord, *J. Comp. Neurol.* 272 (1988) 303–308.
- [27] J. Orłowski, S. Grinstein, Diversity of the mammalian sodium/proton exchanger SLC9 gene family, *Pflugers Arch.* 447 (2004) 549–565.
- [28] J.H. Pizzonia, B.R. Ransom, C.A. Pappas, Characterization of Na^+/H^+ exchange activity in cultured rat hippocampal astrocytes, *J. Neurosci. Res.* 44 (1996) 191–198.
- [29] K.M. Raley-Susman, E.J. Cragoe Jr., R.M. Sapolsky, R.R. Kopito, Regulation of intracellular pH in cultured hippocampal neurons by an amiloride-insensitive Na^+/H^+ exchanger, *J. Biol. Chem.* 266 (1991) 2739–2745.
- [30] H.I. Rocha-González, G. Castañeda-Corral, C.I. Araiza-Saldaña, M. Ambríz-Tututi, N.L. Caram-Salas, J.E. Torres-López, J. Murbartian, V. Granados-Soto, Identification of the Na^+/H^+ exchanger 1 in dorsal root ganglion and spinal cord: its possible role in inflammatory nociception, *Neuroscience* 160 (2009) 156–164.
- [31] S.D. Shields, D.J. Cavanaugh, H. Lee, D.J. Anderson, A.I. Basbaum, Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors, *Pain* 151 (2010) 422–429.
- [32] K.H. Steen, H. Wegner, P.W. Reeh, The pH response of rat cutaneous nociceptors correlates with extracellular $[\text{Na}^+]$ and is increased under amiloride, *Eur. J. Neurosci.* 11 (1999) 2783–2792.
- [33] A. Tjølsen, O.G. Berge, S. Hunskaar, J.H. Rosland, K. Hole, The formalin test: an evaluation of the method, *Pain* 51 (1992) 5–17.
- [34] G.C. Tombaugh, Intracellular pH buffering shapes activity-dependent Ca^{2+} dynamics in dendrites of CA1 interneurons, *J. Neurophysiol.* 80 (1998) 1702–1712.
- [35] L.E. Trudeau, V. Pargura, P.G. Haydon, Activation of neurotransmitter release in hippocampal nerve terminals during recovery from intracellular acidification, *J. Neurophysiol.* 81 (1999) 2627–2635.
- [36] L. Vitzthum, X. Chen, D.B. Kintner, Y. Huang, S.Y. Chiu, J. Williams, D. Sun, Study of Na^+/H^+ exchange-mediated pH regulations in neuronal soma and neurites in compartmentalized microfluidic devices, *Integr. Biol. (Camb.)* 2 (2010) 58–64.
- [37] R. Waldmann, G. Champigny, F. Bassilana, C. Heurteaux, M. Lazdunski, A proton-gated cation channel involved in acid-sensing, *Nature* 386 (1997) 173–177.
- [38] T.L. Xu, B. Duan, Calcium-permeable acid-sensing ion channel in nociceptive plasticity: a new target for pain control, *Prog. Neurobiol.* 87 (2009) 171–180.
- [39] T.L. Yaksh, T.A. Rudy, Chronic catheterization of the spinal subarachnoid space, *Physiol. Behav.* 17 (1976) 1031–1036.
- [40] M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals, *Pain* 16 (1983) 109–110.