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## Activity-dependent hyperpolarization of $E_{GABA}$ is absent in cutaneous DRG neurons from inflamed rats

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### Abstract

A shift in GABA<sub>A</sub> signaling from inhibition to excitation in primary afferent neurons appears to contribute to the inflammation-induced increase in afferent input to the central nervous system (CNS). An activity-dependent depolarization of the GABA equilibrium potential ( $E_{GABA}$ ) has been described in CNS neurons which drives a shift in GABA<sub>A</sub> signaling from inhibition to excitation. The purpose of the present study was to determine if such an activity-dependent depolarization of  $E_{GABA}$  occurs in primary afferents and whether the depolarization is amplified with persistent inflammation. Acutely dissociated retrogradely labeled cutaneous DRG neurons from naïve and inflamed rats were studied with gramicidin perforated patch recording. Rather than a depolarization, 200 action potentials delivered at 2 Hz resulted in a ~10 mV hyperpolarization of  $E_{GABA}$  in cutaneous neurons from naïve rats. No such hyperpolarization was observed in neurons from inflamed rats. The shift in  $E_{GABA}$  was not blocked by 10  $\mu$ M bumetanide. Furthermore, because activity-dependent hyperpolarization of  $E_{GABA}$  was fully manifest in the absence of  $HCO_3^-$  in the bath solution, this shift was not dependent on a change in  $HCO_3^-$ - $Cl^-$  exchanger activity, despite evidence of  $HCO_3^-$ - $Cl^-$  exchangers in DRG neurons that may contribute to the establishment of  $E_{GABA}$  in the presence of  $HCO_3^-$ . While the mechanism underlying the activity-dependent hyperpolarization of  $E_{GABA}$  has yet to be identified, because this mechanism appears to function as a form of feedback inhibition, facilitating GABA mediated inhibition of afferent activity, it may serve as a novel target for the treatment of inflammatory pain.

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## Keywords

Chloride equilibrium potential; inflammatory pain; nociceptor sensitization; sodium-potassium-chloride co-transporter (NKCC1); feedback-inhibition

Pain and hypersensitivity observed in the presence of inflammation are due, at least in part, to an increase in primary afferent input to the dorsal horn. Recent evidence suggests that a shift in GABA<sub>A</sub> signaling from inhibition to excitation in primary afferents may play a prominent role in this inflammation-induced increase in afferent input. This suggestion is based on several lines of evidence. First, in the absence of inflammation, spinal application of the GABA<sub>A</sub> receptor agonist muscimol is analgesic, while in the presence of inflammation, muscimol facilitates inflammatory hyperalgesia, at least at low doses (Anseloni and Gold, 2008). Second, GABA<sub>A</sub> receptors are present in all primary afferents (White, 1990, Paul et al., 2012, Zhu et al., 2012b) with evidence that in the absence of tissue injury, receptor activation results in the inhibition of glutamate release (Yuan et al., 2009). Third, spinal application of the GABA<sub>A</sub> receptor antagonists are antinociceptive (Yaksh, 1989, Anseloni and Gold, 2008) and inhibit afferent activity initiated within the dorsal horn (Rees et al., 1995, Lin et al., 1999) following tissue injury or inflammation.

A compelling explanation for the inflammation-induced shift in GABA<sub>A</sub> signaling is that it is due to a depolarization of the GABA<sub>A</sub> current equilibrium potential ( $E_{GABA}$ ) in primary afferents (Price et al., 2009). There are several lines of evidence in support of this hypothesis. First,  $E_{GABA}$  in primary afferents is 15~30 mV more positive to the afferent resting membrane potential (Alvarez-Leefmans et al., 1988, Rocha-Gonzalez et al., 2008, Zhu et al., 2012b). Second, this depolarized  $E_{GABA}$  is thought to reflect the distinct pattern of Cl<sup>-</sup> co-transporter expression in primary afferent neurons that underlie anion homeostasis: a high level of Na<sup>+</sup>- K<sup>+</sup>- Cl<sup>-</sup>-cotransporter1 (NKCC1), which accumulates intracellular Cl<sup>-</sup>, and a low level of K<sup>+</sup>- Cl<sup>-</sup>-cotransporter 2 (KCC2), which extrudes Cl<sup>-</sup> (Kanaka et al., 2001). However, GABA induced excitation would result from a depolarization of  $E_{GABA}$  above the action potential threshold. Third, tissue injury has been shown to result in the phosphorylation and membrane translocation of NKCC1 (Galan and Cervero, 2005), as well as an increase in total spinal protein levels of NKCC1 (Lagraize et al., 2010). Furthermore, inflammatory mediators are released in the spinal cord as well as the periphery following tissue injury and have also been shown to increase NKCC1 phosphorylation which was accompanied by a positive shift in  $E_{GABA}$  in an in vitro study of dorsal root ganglion (DRG) neurons, (Funk et al., 2008). And fourth, attenuation of NKCC1 activity with a genetic deletion (Sung et al., 2000) or the use of the relatively specific NKCC1 blocker, bumetanide, results in the attenuation of touch evoked allodynia and the sensitization of spinal nociceptive neurons associated with peripheral capsaicin (Pitcher et al., 2007).

The evidence in support of an increase in NKCC1 in primary afferents as a mechanism of the inflammation-induced shift in GABA<sub>A</sub> signaling is compelling. Nevertheless, the vast majority of the data are in support of this mechanism were collected with acute models of noxious stimulation (i.e., capsaicin administration), or within hours of the induction of more persistent inflammation (Lagraize et al., 2010). Furthermore, despite evidence of a shift in GABA<sub>A</sub> signaling in the presence of persistent inflammation (Anseloni and Gold, 2008), our recent results suggest that a steady-state depolarizing shift in  $E_{GABA}$  cannot account for the persistence of excitatory GABA<sub>A</sub> signaling (Zhu et al., 2012b). Rather, our results suggested that an increase in GABA<sub>A</sub> current density secondary to an increase in tyrosine kinase activity in combination with a decrease in low threshold K<sup>+</sup> current density was more likely

to account for the steady-state inflammation-induced shift in GABA<sub>A</sub> signaling (Zhu et al., 2012b).

Regardless of the steady-state mechanisms contributing to an inflammation-induced shift in GABA<sub>A</sub> signaling, further depolarization of E<sub>GABA</sub> in primary afferents will still result in an increase in the driving force on Cl<sup>-</sup> and therefore may still contribute to an increase in excitatory GABA<sub>A</sub> signaling. Importantly, there is evidence that the regulation of intracellular Cl<sup>-</sup> concentration is dynamic such that the impact of GABA can change in association with prolonged GABA application or ongoing neural activity. For example, prolonged GABA application can result in the emergence of an excitatory response in CNS neurons from immature animals because Cl<sup>-</sup> extrusion mechanisms are not sufficiently developed to handle large Cl<sup>-</sup> loads (Cordero-Erausquin et al., 2005). Similarly, and more relevant to afferent signaling, there is evidence of activity-dependent changes in NKCC1 (Brumback and Staley, 2008) and depolarization of E<sub>GABA</sub> (Khalilov et al., 2003, Brumback and Staley, 2008). Thus, while our data argue against a steady-state shift in E<sub>GABA</sub> as a mechanism underlying the inflammation-induced increase in GABA mediated excitation of isolated sensory neurons we hypothesized that an inflammation-induced change in the regulation of intracellular Cl<sup>-</sup> would enable a dynamic shift in E<sub>GABA</sub> to contribute to the inflammation-induced changes in GABA signaling observed in vivo. Specifically, we hypothesized that an increase in the accumulation capacity of NKCC1 would contribute to an activity-dependent depolarizing shift in E<sub>GABA</sub>. To test this hypothesis, perforated patch clamp techniques were used to study activity-dependent changes in E<sub>GABA</sub> in acutely isolated retrogradely labeled cutaneous DRG neurons from naïve and inflamed rats.

## Experimental Procedures

### Animals

Adult male Sprague Dawley rats (Harlan-Sprague Dawley, Indianapolis, IN) weighing between 250 and 350 g were used for all the experiments. Rats were housed two per cage in the University of Pittsburgh AAALAC approved animal facility on a 12:12 light: dark schedule with food and water available. All procedures involving animals were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Labeling and inflammation

DRG neurons that innervate the glabrous skin of rat hind paw were retrogradely labeled with 1,1'-Diiodo-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, 17 mg/ml in DMSO and saline), which was injected (3–5 sites at 2–3 µl/site) with a 30g needle, 14–17 days prior to electrophysiological recording. Complete Freud's adjuvant (CFA, Sigma-Aldrich, St Louis MO; mixed 1:1 with saline), was injected (100 µl) into the site previously injected with DiI. DRG neurons from inflamed rats were studied 72 hours after CFA injection. Both DiI and CFA were injected under isoflurane-induced anesthesia.

### Preparation of isolated DRG neurons

Prior to tissue harvest, rats were deeply anesthetized with a subcutaneous injection (1 ml/kg) of a cocktail containing ketamine (55 mg/ml), xylazine (20 mg/ml) and acepromazine (5.5 mg/ml). L4 and L5 DRG were harvested bilaterally and either pooled for non-inflamed animals or processed in parallel (for inflamed animals) so that neurons ipsilateral and contralateral to the site of inflammation could be studied. DRG were cleaned of connective tissue, enzymatically treated and mechanically dispersed as previously described (Lu et al.,

2006). Isolated neurons were plated on poly-lysine coated coverslips and electrophysiology experiments were performed 2–8 h after plating.

## Electrophysiology

Voltage clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, USA) controlled with pClamp software (Molecular Devices) via a Digidata 1320A A/D converter. Data were low-pass filtered at 10 kHz and digitally sampled at 2 kHz. Capacity transients were canceled via amplifier circuitry. Borosilicate glass (WPI, Sarasota Springs, FL) patch electrodes were pulled on a Sutter P-2000 horizontal puller (Sutter Instruments) and were 1.5–2.5 M $\Omega$  in resistance when filled with electrode solution of the following composition (in mM): K-Methansulphonate 140, MgCl<sub>2</sub>·6H<sub>2</sub>O 2, CaCl<sub>2</sub> 1, EGTA 11, HEPES 10 and NaCl 5; pH was adjusted to 7.2 with Tris Base, and osmolality was adjusted to 317 mOSm with sucrose.

The gramicidin perforated patch configuration was used to measure  $E_{GABA}$  (Akaike, 1996) as described previously (Zhu et al., 2012b). Briefly, gramicidin stock solution in DMSO (1.5 mg/100  $\mu$ l) was diluted with electrode solution in a 1:300 ratio. The tip of the electrode was loaded with a small volume of gramicidin free electrode solution and gramicidin containing electrode solution was back loaded. Experiments were not started until access resistance was less than 7 M $\Omega$ . Seal resistance was continuously monitored throughout experiments, and data were excluded from further analysis if there was any evidence of a change in stability throughout the experiment or there was a decrease in seal resistance over time greater than 10%. Furthermore, neurons were repeatedly stimulated throughout each experiment with the same voltage protocol in the presence and absence of GABA and if there was any evidence of instability in the currents evoked in the absence of GABA, data from that neuron were not included in subsequent analyses. Finally, to rule out the impact of movement artifacts assessed with the fast drug application system, we assessed the presence of “current” associated with a step in the perfusion system with normal bath flowing through both capillaries; data were excluded from further analysis if there was any evidence of current evoked with this protocol.

A hyperpolarizing ramp protocol was used to determine  $E_{GABA}$  (Figure 1A). The membrane potential was depolarized to  $\sim E_{GABA}$  for two seconds to inactivate transient outward and inward currents and then hyperpolarized from this potential to  $-80$  mV at a rate  $\sim 0.063$  mV/ms. The amplitude of the depolarizing voltage step used for the start of the hyperpolarizing ramp was adjusted to  $E_{GABA}$ . The depolarizing step to  $\sim E_{GABA}$  was used to minimize the possibility that flux of Cl<sup>-</sup> during the  $E_{GABA}$  measurement would change the intracellular Cl<sup>-</sup> concentration. As a result, the GABA current evoked during the two second pre-pulse was always less than 20 pA. The membrane potential was then returned to rest at  $-60$  mV. The same protocol was run once with the application of 60  $\mu$ M GABA and three times in the absence of GABA application. To minimize the possibility that GABA<sub>B</sub> receptor activation influenced estimates of  $E_{GABA}$ , the GABA<sub>B</sub> receptor antagonist CGP 55845 (1  $\mu$ M) was included in the GABA solution applied to all neurons. Our previous results indicate that CGP 55845 has no influence on  $E_{GABA}$  (Zhu et al., 2012b).  $E_{GABA}$  was determined from the difference between the current trace evoked in the presence of GABA and the average of the 3 traces obtained in its absence as the point on the voltage ramp where the corresponding GABA current equaled 0 pA (Figure 1B). If necessary, the linear phase of the GABA current was extrapolated to 0 pA to determine  $E_{GABA}$ . While the relatively small currents evoked with 60  $\mu$ M GABA resulted in a less than optimal signal to noise ratio for the determination of  $E_{GABA}$  from the GABA evoked currents, this low concentration was chosen for three important reasons. First, with smaller currents, we minimized the possibility of a GABA current-induced shift in  $E_{GABA}$  (Cordero-Erausquin et al., 2005). Second, we have previously demonstrated that the desensitization of GABA evoked current in DRG

neurons is concentration dependent such that there is a significant decay in current evoked with GABA concentrations greater than 100  $\mu\text{M}$  (Zhu et al., 2012a). Thus, we minimized the impact of channel desensitization across the ramp protocol on the estimate of  $E_{\text{GABA}}$ . To further ensure the stability of GABA current during the ramp, GABA was applied at least 100 ms prior to the start of the ramp. Third, full recovery from GABA-induced desensitization is also concentration dependent, such that longer intervals are needed between GABA applications to obtain stable currents. Importantly, preliminary data indicated that current evoked with 60  $\mu\text{M}$  GABA was stable with an inter-stimulus interval of two minutes.

The stability of  $E_{\text{GABA}}$  was determined with at least three measurements taken at an interval of two minutes. Neurons were then stimulated with a voltage clamp protocol based on a typical action potential wave form recorded from a DiI labeled, acutely dissociated DRG neuron from a naïve rat. While we have evidence of an inflammation-induced change in the action potential waveform of cutaneous neurons (Zhang et al., 2012), we chose to use the same stimulus for all neurons studied because we were primarily interested in changes in the dynamic regulation of  $E_{\text{GABA}}$  rather than an interaction between changes in the action potential waveform and changes in the regulation of  $E_{\text{GABA}}$ . The action potential waveform was applied for 200 pulses at a rate of 2 Hz. These parameters were chosen based on a series of preliminary studies on naïve neurons in which we determined the minimal number of pulses needed to produce a consistent change in  $E_{\text{GABA}}$  within 5 minutes of the last pulse. It should be noted, however, that there was variability between neurons with respect to the minimal number of pulses needed to produce a shift in  $E_{\text{GABA}}$  with a shift detected in one of the 14 neurons studied with as few as 50 pulses.  $E_{\text{GABA}}$  was determined within one minute of the last pulse and again every two minutes for 20 minutes. Changes in  $E_{\text{GABA}}$  following stimulation were compared to measurements obtained prior to stimulation. Two bath solutions were used in this study. One was a  $\text{HCO}_3^-$  buffered bath consisting of (in mM): NaCl 103, KCl 3,  $\text{NaHCO}_3$  26,  $\text{NaH}_2\text{PO}_4$  1.25,  $\text{CaCl}_2$  2.5,  $\text{MgCl}_2$  0.6 and Glucose 10, osmolality was adjusted to 325 mOsm with sucrose, bubbled with 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . The second was a HEPES buffered bath consisting of (in mM): NaCl 130, KCl 3,  $\text{CaCl}_2$  2.5,  $\text{MgCl}_2$  0.6, HEPES 10, Glucose 10; pH was adjusted to 7.4 with Tris-Base and osmolality was adjusted to 325 mOsm with sucrose. All salts were from Sigma-Aldrich (St Louis, MO).

## PCR

Rats from inflamed and naive groups were anesthetized, and L4 /L5 DRG were harvested. Total RNA was extracted from DRG with Trizol (Invitrogen, Carlsbad, CA). cDNA synthesis was carried out using 1  $\mu\text{g}$  of total RNA with Superscript II Reverse Transcriptase (Invitrogen), 0.1 M DTT, RNaseOUT™, dNTP Mix, 5 $\times$  First-Strand Buffer, random primer (Invitrogen) at 42°C for 50 min, as described by the manufacture protocol. cDNA was then kept at -20 °C for future use. For PCR, we used hot start Taq DNA Polymerases, all the reactions were denatured at 95 °C, annealed at 58 °C and extended at 72 °C. After 36 cycles the product was separated on a 2% agarose gel. Primers used for amplification of SLC4A family members are listed in Table 1. The gel was then stained with 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide and imaged with a LAS3000 (Fujifilm Inc, Japan).

## Test compounds

4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS),  $\gamma$ -aminobutyric acid (GABA), bumetanide and gramicidin were from Sigma-Aldrich. CGP 55845 was from Tocris Bioscience (Bristol, UK). GABA and bumetanide were dissolved in water, and CGP 55845 and DIDS were dissolved in DMSO to make stocks that would be diluted 1:1000. Stock solutions were then kept in a -20°C freezer and diluted with bath solution to their working concentration before use.



All drugs were applied with a SF-77B Perfusion Fast-Step system (Warner Inc.). The Fast-Step was controlled by the pClamp 10.0 software.

## Statistics

All pooled data are presented as mean  $\pm$  standard error of the mean. Student's T-test was used for two-group comparisons and repeated measure two-way ANOVA was used for multiple group comparisons (i.e.,  $E_{GABA}$  before and after stimulus in DRG neurons from naïve and inflamed animals). The Tukey test was used for post-hoc pair wise multiple comparisons.  $p < 0.05$  was considered statistically significant.

## Results

### Activity-dependent shift of $E_{GABA}$

Because we are primarily interested in mechanisms underlying inflammation-induced changes the GABA regulation of nociceptive signaling and there is evidence that nociceptive cutaneous afferents are enriched in the population of DRG neurons with a small to medium cell body diameter (Fang et al., 2005), small to medium diameter retrogradely labeled DRG neurons were the focus of the present study. The membrane capacitance ranged between 17.7 to 49 pF, with an average of  $32.8 \pm 8.8$  pF.

In the absence of stimulation,  $E_{GABA}$  was relatively stable in cutaneous neurons from both naïve and inflamed rats prior to stimulation during the determination of baseline  $E_{GABA}$ , as well as in control neurons studied over time in the absence of stimulation (Figure 1C). Of note, while there appears to be considerable heterogeneity in  $E_{GABA}$  among cutaneous neurons, this degree of heterogeneity is consistent with previous results from unlabeled DRG neurons (Rocha-Gonzalez et al., 2008), as well as our previous results from cutaneous DRG neurons (Zhu et al., 2012a). While the basis for this heterogeneity has yet to be determined, we suggest it is likely a reflection of heterogeneity in afferent function.

The action potential waveform used to stimulate cutaneous neurons (Figure 2A) resulted in the activation of primarily inward currents (Figure 2A inset). Stimulation of cutaneous neurons from naïve rats with the action potential waveform 200 times at 2 Hz resulted in a hyperpolarizing shift in  $E_{GABA}$  in 10 of 10 neurons studied of  $-9.9 \pm 0.9$  mV (Figure 2B, C). The hyperpolarization of  $E_{GABA}$  was fully manifest in minutes, and recovered to baseline within  $16 \pm 1.8$  min. Consistent with our previous analysis of the impact of inflammation on  $E_{GABA}$  in cutaneous neurons when recorded in  $HCO_3^-$  buffered bath (Zhu et al., 2012b),  $E_{GABA}$  in cutaneous neurons from inflamed rats ( $-32.6 \pm 2.3$ ,  $n = 9$ ) was significantly more depolarized than that in cutaneous neurons from naïve rats ( $-40.7 \pm 2.5$  mV,  $n = 10$ , Figure 2C,  $p = 0.02$ , Students t-test). However, in contrast to the hyperpolarization observed in cutaneous neurons from naïve rats, the average stimulation-induced shift in  $E_{GABA}$  in the 9 cutaneous neurons tested from inflamed rats was depolarization of  $1.1 \pm 1.4$  mV. The impact of inflammation on the activity-dependent shift in  $E_{GABA}$  was statistically significant (Two-way mixed design ANOVA,  $p < 0.01$ , Figure 2C).

### NKCC1 does not contribute to the activity-dependent shift in $E_{GABA}$

Because NKCC1 is suggested to contribute to the activity-dependent  $E_{GABA}$  changes in CNS neurons (Khalilov et al., 2003, Balena and Woodin, 2008), and is considered as an important determinant of  $E_{GABA}$  in primary afferent neurons we sought to determine whether a decrease in NKCC1 activity could contribute to the activity-dependent hyperpolarizing shift in  $E_{GABA}$  in cutaneous neurons from naïve rats. To test this possibility, we used the relatively selective NKCC1 blocker bumetanide ( $10 \mu M$ ) (Balena and Woodin,

2008) to determine whether block of NKCC1 would occlude the activity-dependent hyperpolarization of  $E_{GABA}$ . In a second group of neurons  $E_{GABA}$  was first determined, bumetanide was applied and  $E_{GABA}$  was monitored until it re-stabilized, and then the impact of the action potential protocol on  $E_{GABA}$  was determined. Consistent with the suggestion that NKCC1 contributes to the determination of  $E_{GABA}$ , bumetanide induced a hyperpolarizing shift of  $E_{GABA}$  from  $-42.3 \pm 3.3$  mV to  $-47.5 \pm 2.0$  mV ( $n = 6$ ). This change was manifest in 5 minutes and reached a plateau within 20 minutes. Interestingly, the presence of bumetanide had no influence on the activity-dependent shift in  $E_{GABA}$  which was  $-11.4 \pm 1.5$  mV (Figure 3). Furthermore, despite the continued presence of bumetanide,  $E_{GABA}$  returned to baseline levels within 20 minutes in all neurons tested. The activity-dependent shift in  $E_{GABA}$  in the presence of bumetanide is not significantly different than the shift observed in untreated neurons ( $p > 0.05$ , students t-test). These results suggested that NKCC1 does not contribute to the activity-dependent shift in  $E_{GABA}$  in cutaneous neurons from naïve rats.

### **A $HCO_3^-$ - $Cl^-$ exchanger(s) contributes to the determination of $E_{GABA}$ in cutaneous DRG neurons**

We recently reported that  $E_{GABA}$  determined in  $HCO_3^-$  buffered bath solution is more hyperpolarized than that determined in HEPES buffered solution (Zhu et al., 2012b). To confirm this observation, we compared  $E_{GABA}$  measured in  $HCO_3^-$ -buffered bath solution to that determined in HEPES buffered bath solution. Consistent with our previous results,  $E_{GABA}$  was significantly ( $p < 0.01$ , students t-test) more hyperpolarized when determined in  $HCO_3^-$ -buffered bath solution ( $-43.0 \pm 2.3$  mV,  $n = 16$ ) than that in HEPES-buffered bath solution ( $-26.4 \pm 3.2$ ,  $n = 7$ ). This observation suggests that a  $HCO_3^-$ -dependent  $Cl^-$  extrusion mechanism contributes to the determination of  $E_{GABA}$  in cutaneous neurons. As there was no difference in the holding current of neurons recorded in  $HCO_3^-$ - and HEPES-buffered bath solutions, we predicted that an electro-neutral  $HCO_3^-$ - $Cl^-$  exchanger may be the underlying mechanism. There are three neuronal electro-neutral  $HCO_3^-$ - $Cl^-$  exchangers in the SLC4A family:  $Na^+$ -dependent  $HCO_3^-$ - $Cl^-$  exchangers NDCBE, NCBE, and  $HCO_3^-$ - $Cl^-$  exchanger AE3. To determine which, if any of these family members are present in DRG and therefore potential candidates for the exchanger underlying the hyperpolarizing shift in  $E_{GABA}$  in the presence of  $HCO_3^-$ , conventional PCR was used to screen for the presence of mRNA encoding these three family members in adult rat DRG. The results of this analysis indicated that mRNA encoding all three family members were detectable in DRG (Figure 4A). To further confirm the contribution of SLC4A family members to the hyperpolarizing shift in  $E_{GABA}$  observed in  $HCO_3^-$  buffered bath solution, we assessed the impact of the non-selective  $Cl^-$  exchanger blocker, DIDS on  $E_{GABA}$  since all the  $HCO_3^-$  exchangers share a DIDS binding site and can all be inhibited by DIDS. Consistent with the contribution of a SLC4A family member to determination of  $E_{GABA}$  in cutaneous DRG neurons, 5 minute exposure to DIDS (50  $\mu$ M) resulted in a depolarizing shift in  $E_{GABA}$  from  $-46.5 \pm 3.2$  mV to  $-38.0 \pm 2.2$  mV (Figure 4B). This shift is statistically significant ( $p < 0.05$ ,  $n=6$ , paired t-test).

### **A $HCO_3^-$ exchanger is not necessary for the activity-dependent shift in $E_{GABA}$**

The presence of a  $HCO_3^-$ - $Cl^-$  exchanger in cutaneous DRG neurons raised the possibility that the activity-dependent shift in  $E_{GABA}$  is due to an increase in exchanger activity. To test this possibility, we measured the  $E_{GABA}$  in cutaneous neurons from naïve rats before and after stimulation in HEPES buffered solution. The results of this experiment indicated that the activity-dependent shift in  $E_{GABA}$  in HEPES buffered bath solution was comparable to that observed in  $HCO_3^-$  buffered bath solution (Figure 5A). Interestingly, while the magnitude of the activity-dependent shift in  $E_{GABA}$  was comparable in  $HCO_3^-$ - and HEPES-buffered bath solutions, the magnitude of the change in intracellular  $Cl^-$  associated

with the activity-dependent shift in  $E_{GABA}$  should be different under these recording conditions. This is because of the significant difference in baseline  $E_{GABA}$  under these recording conditions. To confirm this prediction, we used  $E_{GABA}$  to estimate the concentration of intracellular  $Cl^-$  ( $[Cl^-]_i$ ) before and after stimulation, with the Goldman Hodgkin-Katz equation (with extracellular  $Cl^-$  ( $[Cl^-]_o$ ) = 140 mM,  $[HCO_3^-]_o$  = 26 mM,  $[HCO_3^-]_i$  = 10 mM and the  $Cl^-:HCO_3^-$  permeability ratio of the  $GABA_A$  receptor as 4:1 (Kaila et al., 1997). The result of this analysis indicated that the change in intracellular  $Cl^-$  needed to produce a 10 mV hyperpolarizing shift in  $E_{GABA}$  in a HEPES buffered bath solution was significantly greater than that in  $HCO_3^-$  buffered bath solution (Figure 5B). While the mechanism underlying the activity-dependent shift in  $E_{GABA}$  remains to be determined, the observation that the shift appears to be dependent on the driving force on  $Cl^-$  suggests that the mechanism may be a channel enabling diffusion of  $Cl^-$  down its electro-concentration gradient.

## Discussion

We hypothesized that an increase in NKCC1 activity would contribute to an activity-dependent depolarizing shift in  $E_{GABA}$  that would be larger in cutaneous neurons from inflamed rats than that in neurons from naïve rats. In contrast to our hypothesis, we observed an activity-dependent hyperpolarizing shift in  $E_{GABA}$  in neurons from naïve rats. Interestingly, no activity-dependent shift in  $E_{GABA}$  was detectable in cutaneous neurons from inflamed rats. Additional analysis of the mechanisms underlying the activity-dependent hyperpolarization of  $E_{GABA}$  indicated that the shift was still fully manifest in the presence of bumetanide. Furthermore, despite evidence for the presence of a  $HCO_3^-:Cl^-$  exchanger in cutaneous neurons that contributes to the steady-state determination of  $E_{GABA}$ , the activity-dependent hyperpolarization of  $E_{GABA}$  was still fully manifest in HEPES buffered bath solution.

Additional experiments will ultimately be needed to determine whether an activity-dependent hyperpolarization of  $E_{GABA}$  is inhibitory or excitatory. In CNS neurons, the available evidence suggests a depolarizing shift in  $E_{GABA}$  is associated with a decrease in inhibition (Prescott et al., 2006). However, in primary afferent neurons where GABA mediated membrane depolarization is the norm because of the persistent expression of NKCC1,  $GABA_A$  receptor activation, and consequently membrane depolarization, is still inhibitory. Thus, the consequences of a hyperpolarizing shift in  $E_{GABA}$  are not necessarily as straightforward as in the CNS. Nevertheless, we suggest that the available evidence indicates that even in primary afferent neurons, a hyperpolarizing shift in  $E_{GABA}$  will facilitate GABA mediated inhibition. This suggestion is based on the observation that there is a hyperpolarizing shift in  $E_{GABA}$  in NKCC1 knock out mice (Sung et al., 2000) and in wild type animals following application of the NKCC1 blocker, bumetanide (Pitcher et al., 2007). These hyperpolarizing shifts in  $E_{GABA}$  are associated with an increase in nociceptive threshold (Sung et al., 2000) and the attenuation of injury-induced decreases in nociceptive threshold. Importantly, if the activity-dependent hyperpolarization of  $E_{GABA}$  contributes to the normal inhibitory actions of GABA, the inflammation-induced decrease in the activity-dependent hyperpolarization of  $E_{GABA}$  may contribute to the inflammation-induced pro-nociceptive shift in spinal  $GABA_A$  signaling observed in the presence of inflammation (Anseloni and Gold, 2008).

Although our results suggested that NKCC1 is not responsible for the activity-dependent hyperpolarization of  $E_{GABA}$  in DRG neurons from naïve animals, a conclusion that a decrease in NKCC1 activity does not contribute to the activity-dependent hyperpolarizing shift in  $E_{GABA}$  should be made with caution. This is because there is evidence that activity is sufficient to down-regulate NKCC1, resulting in the hyperpolarization of  $E_{GABA}$  (Balena



and Woodin, 2008). Furthermore, despite evidence that the concentration of bumetanide used was sufficient to hyperpolarize  $E_{GABA}$ , this concentration may have been insufficient to block enough NKCC1 activity to occlude an activity-dependent decrease in NKCC1 activity. Consistent with this suggestion,  $E_{GABA}$  in NKCC1 knockout mice is  $-53.6$  mV while that in DRG of wildtype with  $10 \mu\text{M}$  bumetanide block is  $-37.2$  mV (Sung et al., 2000). Unfortunately, the concentration range over which bumetanide has selectivity for NKCC1 over the other  $\text{Cl}^-$  co-transporters is limited, making the interpretation of results with higher concentrations of this compound difficult. Other strategies may ultimately be needed to rule out the contribution of NKCC1 to the activity-dependent shift in  $E_{GABA}$  in cutaneous neurons.

Our results with HEPES buffered bath argue against a role for an increase in  $\text{HCO}_3^-$ -dependent  $\text{Cl}^-$  transporter activity as a mechanism for the activity-dependent hyperpolarization of  $E_{GABA}$ . Nevertheless, the presence of  $\text{HCO}_3^-$  exchangers in the primary afferent neurons and their contribution to the maintenance of steady-state  $E_{GABA}$  has at least two important implications. First, since most of the  $E_{GABA}$  data in primary afferents available in the literature were collected with HEPES buffered solutions (Alvarez-Leefmans et al., 1988, Sung et al., 2000, Gilbert et al., 2007, Rocha-Gonzalez et al., 2008), the estimates of  $E_{GABA}$  in these studies may be artificially depolarized. Second, a more hyperpolarized  $E_{GABA}$  under physiological conditions may contribute to the dramatic shifts in  $E_{GABA}$  signaling observed in vivo (Anseloni and Gold, 2008). That is, if  $E_{GABA}$  is already above action potential threshold in the absence of tissue injury as suggested by results obtained in HEPES buffered bath solution in this and previous studies, then a further depolarization of  $E_{GABA}$  as should occur following inflammatory mediator-induced increases in NKCC1 activity (Funk et al., 2008), should have a relatively minor influence on nociceptive signaling. In contrast, if  $E_{GABA}$  is hyperpolarized to action potential threshold as suggested by the results of the present study, an increase in NKCC1 activity sufficient to drive  $E_{GABA}$  above the action potential threshold should have a considerably larger impact on nociceptive afferent input to the CNS.

While it is possible that a secondary active transport mechanism contributes to the activity-dependent hyperpolarization of  $E_{GABA}$ , a more likely mechanism is an anion channel that enables the passive redistribution of  $\text{Cl}^-$  in response to potential difference between  $E_{GABA}$  and resting membrane potential. In this case, there will be  $\text{Cl}^-$  efflux as long as the resting membrane potential is more negative than  $E_{GABA}$ . As suggested, such a mechanism would account for the larger efflux observed in the HEPES buffered bath solution because of the greater potential difference between  $E_{GABA}$  and resting membrane potential under these conditions. A  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channel would be ideally suited to mediate the activity-dependent hyperpolarizing shift in  $E_{GABA}$ , given that activity will result in an increase in intracellular  $\text{Ca}^{2+}$  concentration that can persist long after activity has been terminated (Lu et al., 2006). Several of these channels have been described in sensory neurons (Mayer, 1985) including TMEM16A (AKA ANO1). While it remains to be determined how TMEM16A and the other  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  channels are regulated in the presence of persistent inflammation, the decrease in high threshold voltage-gated  $\text{Ca}^{2+}$  current observed in cutaneous neurons 3 days after CFA injection (Lu et al., 2010), could account for the loss of the hyperpolarizing shift in  $E_{GABA}$  observed in the presence of persistent inflammation.

In summary, while we have been forced to reject our hypothesis regarding the contribution of the dynamic regulation of  $E_{GABA}$  to the inflammation-induced shift in  $E_{GABA}$  signaling, we have described a novel feedback inhibitory mechanism that should serve to attenuate nociceptive input into the CNS in the absence injury. Furthermore, rather than a further depolarization of  $E_{GABA}$  as a means to enable the emergence of excitatory actions of GABA, inflammation is associated with a decrease in this feedback inhibitory mechanism.

Identification of this mechanism may provide a novel target for the treatment of inflammatory hypersensitivity.

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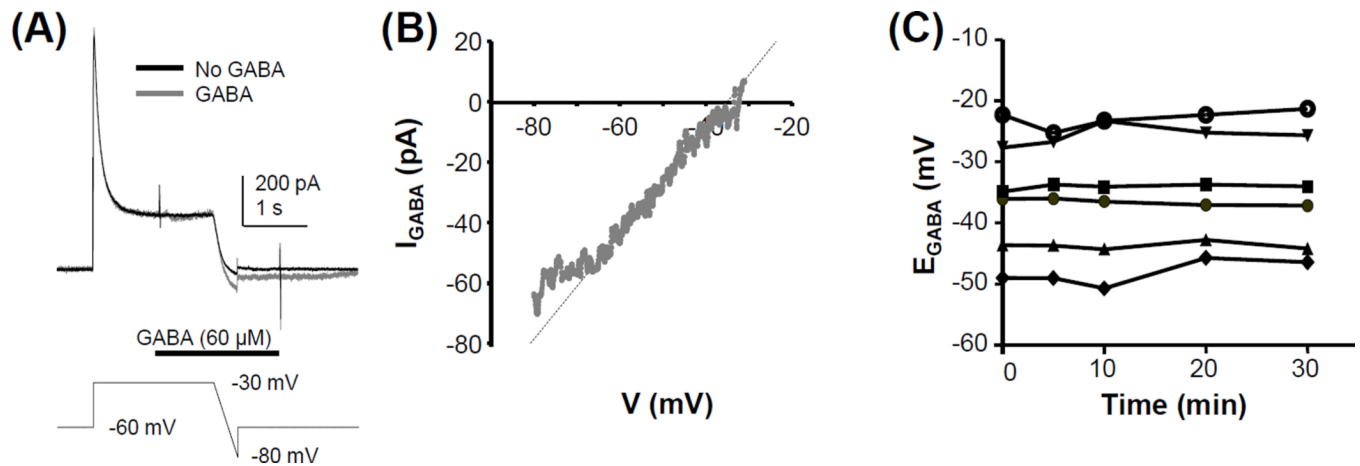
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### Highlights

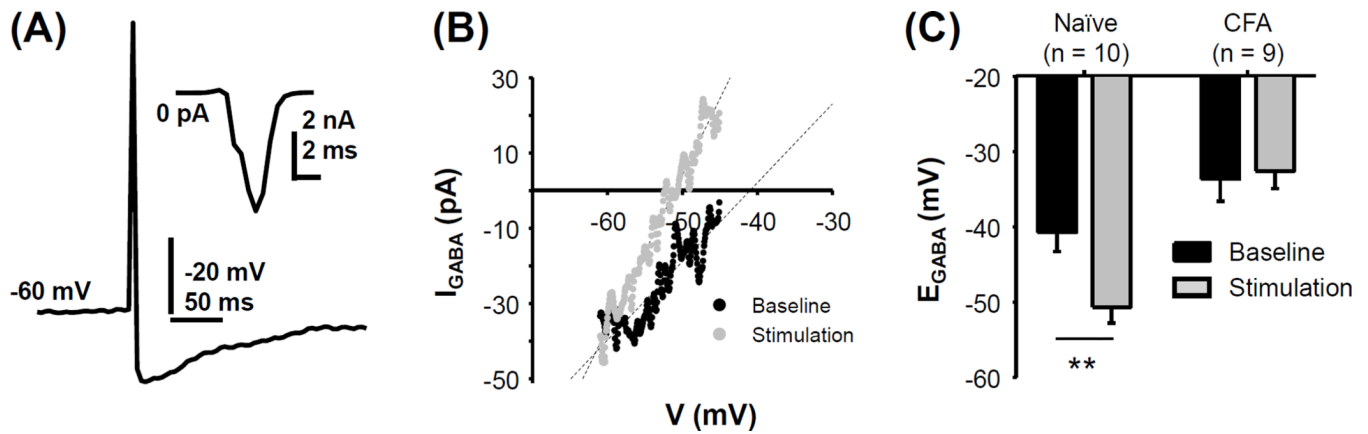
- There is a persistent inflammation-induced shift in spinal GABAA signaling
- There is evidence for activity dependent depolarization of EGABA in CNS neurons
- Our results indicate activity drives a hyperpolarization of EGABA in cutaneous DRG neurons.
- This shift is due to neither an increase in NKCC1 or a decrease in HCO<sub>3</sub>--Cl – exchanger activity.
- Persistent inflammation is associated with a loss of the activity-dependent hyperpolarization of E<sub>GABA</sub>



**Figure 1.**

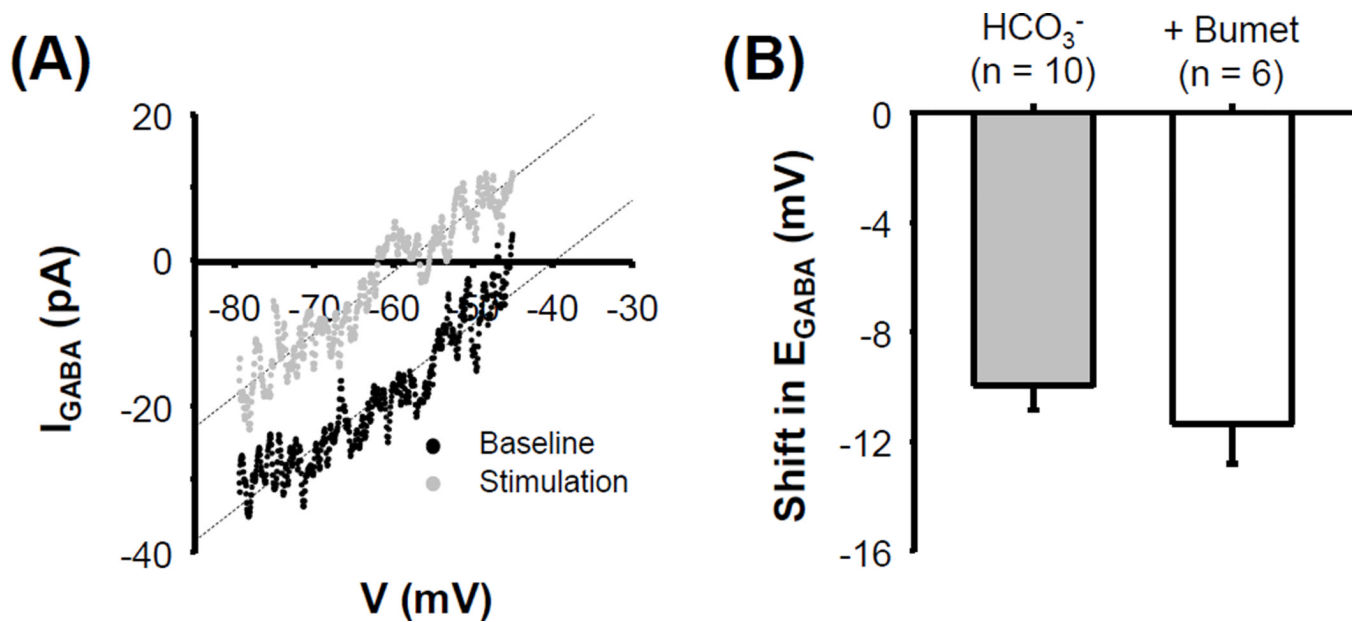
Determination of  $E_{GABA}$ . (A) Current evoked in response to a ramp protocol used to determine  $E_{GABA}$ . The protocol was run before (black trace) and after (gray trace) the application of GABA (60  $\mu$ M). GABA application was initiated during the sustained depolarizing step used to inactivate transient inward and outward currents prior to the ramp. The voltage protocol is shown beneath the current traces. Data were from a cutaneous neuron from a naïve rat (B) The difference between the current evoked during the ramp, in the presence and absence of GABA in A is plotted as function of the change in voltage. The voltage at which the linear component of the difference current is 0 pA is  $E_{GABA}$ . (C)  $E_{GABA}$  monitored for 30 minutes at a 5 to 10 minute interval was relatively stable in the 6 control neurons tested.





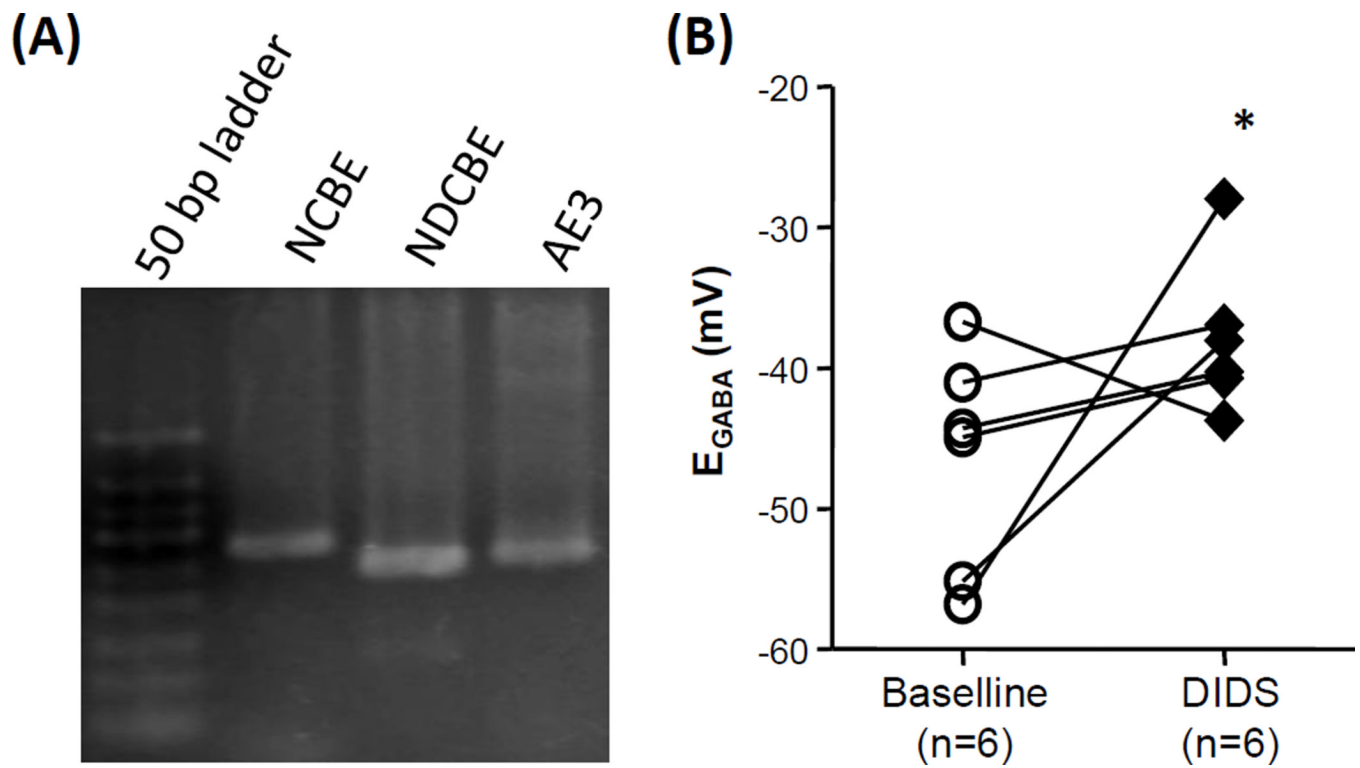
**Figure 2.**

The activity-dependent hyperpolarization of  $E_{GABA}$  is absent in cutaneous neurons from inflamed rats. (A) Action potential waveform used to drive “activity-dependent” changes in  $E_{GABA}$  was elicited in a small diameter cutaneous neuron from a naïve rat with direct current injection under current clamp configuration with gramicidin patch. *Inset*: current evoked in response to the action potential waveform used as a “stimulus” in voltage clamp. (B) Stimulation of a small diameter cutaneous neuron from a naïve rat with the waveform shown in A, 200 times at a frequency of 2 Hz resulted in a hyperpolarization of  $E_{GABA}$  (black trace: before stimulus; grey trace: after stimulus). (C) Pooled  $E_{GABA}$  data obtained before and after 200 pulse stimulation of cutaneous neurons from naïve and inflamed (CFA) rats. There was a significant interaction between group (naïve vs inflamed) and the impact of stimulation (before vs after) due to the hyperpolarizing shift in  $E_{GABA}$  present in neurons from naïve but not inflamed rats. Number of neurons recorded is indicated in the parenthesis. \*\*  $p < 0.01$ .

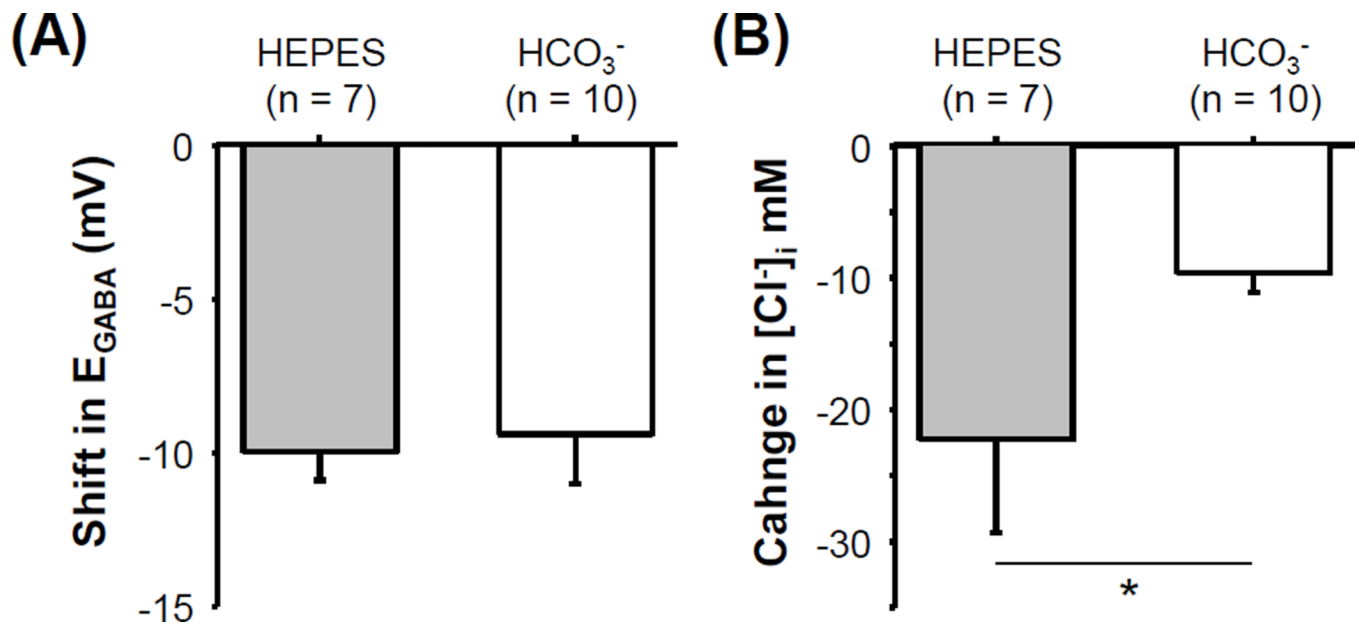


**Figure 3.**

Activity-dependent hyperpolarization of  $E_{GABA}$  was fully manifest in the presence of the NKCC1 antagonist bumetanide. (A)  $E_{GABA}$  was assessed in a cutaneous neuron from a naïve rat before (black trace) and after (gray trace) stimulation with the action potential waveform 200 times at 2 Hz, 30 minutes after applying bumetanide (10  $\mu$ M). (B) Pooled data indicate that the activity-dependent hyperpolarization of  $E_{GABA}$  in neurons from naïve rats was comparable ( $p > 0.05$ ) in the presence and absence of bumetanide. Pooled data from the HCO<sub>3</sub><sup>-</sup> group were from the naïve group in Figure 2C and are plotted again to facilitate the comparison between groups.



**Figure 4.**  $\text{HCO}_3^-$ - $\text{Cl}^-$  exchangers are present in DRG and contribute to the determination of  $E_{\text{GABA}}$  in cutaneous neurons. **(A)** An ethidium bromide stained agarose gel loaded with PCR products generated with primers specific to each of the electro-neutral members of the SLC4A family of  $\text{HCO}_3^-$ - $\text{Cl}^-$  exchanger. mRNA was harvested from whole ganglia obtained from a naïve rats. Comparable results were obtained in ganglia from three different rats. **(B)**  $E_{\text{GABA}}$  was determined in cutaneous neurons from naïve rats before and after the application of the  $\text{HCO}_3^-$ - $\text{Cl}^-$  exchanger blocker, DIDS (50  $\mu\text{M}$ ). The depolarization of  $E_{\text{GABA}}$  in the presence of DIDS was significant ( $p < 0.05$ ).



**Figure 5.**

No evidence for an increase in HCO<sub>3</sub><sup>-</sup>-Cl<sup>-</sup> exchanger activity in the activity-dependent hyperpolarization of E<sub>GABA</sub>. **(A)** E<sub>GABA</sub> was determined before and after action potential stimulation of a cutaneous neurons from naïve rats in HCO<sub>3</sub><sup>-</sup>- and HEPES-buffered bath solution as described in Figure 2C. Pooled data of the activity-dependent shift in E<sub>GABA</sub> are plotted. Data for the HCO<sub>3</sub><sup>-</sup> group were from Figure 2C and are plotted again to facilitate the comparison between groups. **(B)** The decrease in the concentration of intracellular Cl<sup>-</sup> ([Cl<sup>-</sup>]<sub>i</sub>) associated with the activity-dependent hyperpolarization of E<sub>GABA</sub> was estimated based on the composition of the bath solution and measurements of E<sub>GABA</sub> before and after stimulation. \* is p>0.05.

**Table 1**Primers for HCO<sub>3</sub><sup>-</sup> exchangers

Gene	Sequence	Start→END	SIZE
NCBE_F	5'-GGTGTGGCACTGGTATTTGTGAG-3'	3091-3362	272
NCBE_R	5'-TGTCAGCAGTGACTAGAAGGTTCC-3'		
NDCBE_F	5'-CGTGCTCTTCTGGTCCTGTATTCT-3'	2122-2358	237
NDCBE_R	5'-ATGAACCATCCTCGATCATCCCTC-3'		
AE3_F	5'-TCCCAACGATGACAAGGACAGT-3'	1312-1556	245
AE3_R	5'-TCTTCTCCAGCAGTTTCAGGCT-3'		