Chloride dysregulation and inhibitory receptor blockade yield equivalent disinhibition of spinal neurons yet are differentially reversed by carbonic anhydrase blockade

Kwan Yeop Lee, Steven A. Prescott*

Abstract
Synaptic inhibition plays a key role in processing somatosensory information. Blocking inhibition at the spinal level is sufficient to produce mechanical allodynia, and many neuropathic pain conditions are associated with reduced inhibition. Disinhibition of spinal neurons can arise through decreased GABA$_A$/glycine receptor activation or through dysregulation of intracellular chloride. We hypothesized that these distinct disinhibitory mechanisms, despite all causing allodynia, are differentially susceptible to therapeutic intervention. Specifically, we predicted that reducing bicarbonate efflux by blocking carbonic anhydrase with acetazolamide (ACTZ) would counteract disinhibition caused by chloride dysregulation without affecting normal inhibition or disinhibition caused by GABA$_A$/glycine receptor blockade. To test this, responses to innocuous tactile stimulation were recorded in vivo from rat superficial dorsal horn neurons before and after different forms of pharmacological disinhibition and again after application of ACTZ. Blocking GABA$_A$ or glycine receptors caused hyperresponsiveness equivalent to that caused by blocking the potassium chloride cotransporter KCC2, but, consistent with our predictions, only disinhibition caused by KCC2 blockade was counteracted by ACTZ. ACTZ did not alter responses of neurons with intact inhibition. As pathological downregulation of KCC2 is triggered by brain-derived neurotrophic factor, we also confirmed that ACTZ was effective against brain-derived neurotrophic factor–induced hyperresponsiveness. Our results argue that intrathecal ACTZ has antiallodynic effects only if allodynia arises through chloride dysregulation; therefore, behavioral evidence that ACTZ is antiallodynic in nerve-injured animals affirms the contribution of chloride dysregulation as a key pathological mechanism. Although different disinhibitory mechanisms are not mutually exclusive, these results demonstrate that their relative contribution dictates which specific therapies will be effective.

Keywords: Neuropathic pain, Spinal cord, Disinhibition, KCC2, Chloride, BDNF, Acetazolamide, Bicarbonate

1. Introduction
Mechanical allodynia is a clinically important feature of neuropathic pain. Blocking synaptic inhibition at the spinal level is sufficient to cause mechanical allodynia, and, conversely, allodynia can be reversed by pharmacologically enhancing inhibition. Indeed, several studies have shown that inhibition of spinal neurons is reduced by nerve injury or other insults leading to neuropathic pain (for review, see Refs. 30, 48). In broad terms, disinhibition can result from reduced activation of GABA$_A$ and/or glycine receptors or from reduced current flow through activated receptors. The former has several possible causes such as reduced transmitter release and/or reduced receptor function, whereas the latter stems uniquely from dysregulation of intracellular chloride due to downregulation of the potassium chloride cotransporter KCC2. KCC2 hypofunction subverts ionotropic inhibition by allowing chloride to accumulate intracellularly, thus reducing chloride driving force.

While enhancing receptor activation will reverse disinhibition due to reduced receptor activation, GABA$_A$ receptor agonists have diminished analgesic effects when chloride is dysregulated. According to computer simulations, enhancing activation of postsynaptic receptors is beneficial only if chloride dysregulation is relatively modest such that residual inhibitory capacity can be enhanced. That said, increasing receptor activation increases intracellular chloride load, which, when chloride extrusion capacity is diminished, risks exacerbating chloride accumulation and causing paradoxical excitation once residual inhibition is exhausted. By comparison, enhancing KCC2 function only reverses disinhibition due to chloride dysregulation. The disinhibitory mechanism clearly affects to which treatments the resulting allodynia will respond.

Inhibition also operates presynaptically on the central terminals of primary afferent fibers, which, importantly, do not express glycine receptors or KCC2. Absence of KCC2 combined with expression of the chloride importer NKCC1 renders primary afferents with high intracellular chloride levels. Activation of presynaptic GABA$_A$ receptors thus causes depolarization but is nonetheless inhibitory because of shunting and sodium channel inactivation. Based on these expression patterns, reduced
The brain can be thought of as a complex network of interconnected neurons. In this study, we used in vivo multielectrode recordings of rat superficial dorsal horn neurons to test whether disinhibitory mechanisms can be pharmacologically distinguished. Specifically, because bicarbonate exits through activated GABA_A receptors and glycine receptors and exacerbates chloride accumulation, we predicted that reducing bicarbonate efflux by blocking carbonic anhydrase with acetazolamide (ACTZ) would restore inhibition compromised by chloride dysregulation, whereas ACTZ would be ineffective against disinhibition caused by receptor blockade. As predicted, disinhibition caused by KCC2 blockade or brain-derived neurotrophic factor (BDNF)-mediated downregulation of KCC2 was significantly counteracted by ACTZ, whereas disinhibition caused by GABA_A or glycine receptor blockade was not. These results caution against treating molecularly distinct disinhibitory mechanisms as functionally equivalent.

2. Materials and methods

All procedures were approved by the Animal Care Committee at The Hospital for Sick Children. Under urethane anesthesia (20% in normal saline; 1.2 g/kg, intraperitoneally), a laminectomy was performed to expose L4–S1 segments of the spinal cord of adult male Sprague-Dawley rats (350–450 g) obtained from Charles River, Montreal, Canada. The rat was then placed in a stereotactic frame, and its vertebrae were clamped above and below the recording site to immobilize the spinal cord. The hind paw was immobilized in plasticine with the plantar surface facing upwards for stimulation. Rectal temperature was kept at 37°C using a feedback-controlled heating pad (TR-200; Fine Science Tools, Taunton, MA) throughout the experiment.

2.1. Electrophysiology

A 4-electrode array with a total of 16 recording sites (A4 type, NeuroNexus) was implanted at the L5 spinal level. The array was oriented so that each electrode was at the same mediolateral position. Depth of electrode insertion below the dorsal surface was monitored. As recording sites are spaced at 50-µm intervals up each electrode, we determined on which site a unit was recorded and from this measured the depth of recorded neurons (see “Results” section). Brush stimulation was applied to the hind paw to identify neurons responsive to low-threshold mechanical input. Neurons that responded to limb displacement, indicating proprioceptive input, were excluded. The signal was amplified, band-pass filtered between 500 Hz and 10 kHz, digitized at 20 kHz with an OmniPlex Data Acquisition System (Plexon, Dallas, TX), and stored with stimulus markers on disk. Single units were isolated using Offline Sorter v3 software (Plexon) and were analyzed with NeuroExplorer 4 (Plexon).

2.2. Drug application

Using petroleum jelly, a well was built around the spinal cord to target drug delivery to the recording site. In each experiment, 200 µL of saline-containing drug was delivered into the well through a small tube (OD: 0.016 inches; Cole-Palmer, Montreal, Canada) inserted intratheically. For drug applications, R-(+)-[2R-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl]oxy] acetic acid (DIOA; Sigma-Aldrich, St Louis, MO) and VU 0240551 (VU; Tocris Bioscience, Bristol, United Kingdom) were diluted in a stock solution of dimethyl sulfoxide (DMSO) and then diluted in buffered saline for a final concentration of 100 µM for DIOA22 and 50 µM for VU.25 Bicuculline HCl (Tocris Bioscience) was dissolved in buffered saline to a concentration of 50 µM.42 Strychnine (Abcam Biomedicals, Cambridge, United Kingdom) was dissolved in buffered saline to a concentration of 50 µM.42 Acetazolamide (ACTZ; Sigma-Aldrich) was dissolved in buffered saline with pH 8.2; after dissolving ACTZ, pH was reduced to 7.4 and concentration was adjusted to 10 mM.2 Recombinant human BDNF (PeproTech, Rocky Hill, NJ) of 70 µg was prepared in saline.

2.3. Mechanical stimulation

Cutaneous receptive fields were identified on the basis of spiking evoked by mechanical stimuli applied by brush, blunt probes, or von Frey (VF) filaments to the glabrous skin of the left hind paw. By using weak search stimuli, we specifically targeted neurons receiving low-threshold input and avoided causing sensitization. Each stimulus comprised ten 1-second-long applications of the brush or VF filament (2, 4, 6, 8, and 10 g) repeated at 2-second intervals. Each stimulus was applied twice onto different locations within the receptive field for each test condition.

2.4. Data analysis

To quantify the spiking evoked by mechanical stimulation, we measured the mean firing rate during each stimulus, subtracted the spontaneous firing rate measured from the 10-second epoch preceding each stimulus, and then averaged across the 2 stimuli for each test condition. Data are reported as mean ± SEM. Because neurons were tested before and after each manipulation, all analysis was conducted using paired statistical tests identified in the “Results” section and in the figure legends.

3. Results

Given behavioral evidence that disinhibition at the spinal level produces mechanical allodynia, we used a mechanical search stimulus to identify spinal neurons sensitive to innocuous mechanical stimulation of the hind paw and then tested how their response to innocuous stimulation was altered by disinhibition. We recorded from a total of 161 neurons at a depth of 187 ± 76 (mean ± SD) below the dorsal surface of the spinal cord, which corresponds to lamina II.45 Lamina II contains interneurons that are crucial for somatosensory processing.46,47 Recent work indicates that disinhibition of those neurons ungages polysynaptic circuits that relay low-threshold input to lamina I projection neurons5,22,23,25,27,44 (for review, see Ref. 31). Understanding how those neurons are affected by disinhibition is, therefore, important for explaining the neural basis of allodynia and for designing more effective treatments against allodynia.

Figure 1A illustrates a typical neuron whose response to brush stimulation was dramatically increased by blockade of KCC2 with DIOA. Brushing evokes dynamic mechanical allodynia, which is clinically more troublesome than static allodynia;20,21 yet, most preclinical animal studies focus on quantifying static mechanical allodynia using VF hairs.49 Therefore, although we focused on dynamic brush stimulation, we also verified that disinhibition caused increased responsiveness to punctate stimulation with VF.
hairs. Figure 1B illustrates a typical sequence of responses to increasing VF stimuli before and after blockade of KCC2 with DIOA. The average input–output curve was significantly altered by disinhibition (P < 0.05; 2-way repeated-measures analysis of variance [ANOVA]) (Fig. 1C). For both brush and VF stimulation, all neurons exhibited increased responsiveness after KCC2 blockade, which suggests that all neurons express KCC2 and are susceptible to disinhibition by chloride dysregulation.

Given the bicarbonate efflux that occurs through activated GABA_A and glycine receptors, we predicted that reducing bicarbonate efflux by blocking carbonic anhydrase with ACTZ would counteract disinhibition caused by chloride dysregulation; in other words, net current is (at least partially) rebalanced by offsetting reduced hyperpolarizing current (chloride influx) with reduced depolarizing current (bicarbonate efflux) (Fig. 2A). To test this, we blocked KCC2 by intrathecal application of DIOA or VU and we subsequently applied ACTZ. Two different KCC2 antagonists were tested because they have different off-target effects.13 Figure 2B shows that both forms of KCC2 blockade significantly increased responses to brush stimulation (P < 0.001; one-way repeated-measures ANOVA and Student–Newman–Keuls tests) and, moreover, ACTZ significantly reversed that increase (P = 0.026 and P < 0.001 for DIOA and VU, respectively). Likewise, ACTZ significantly reversed the increased responsiveness to VF stimulation caused by KCC2 blockade (P < 0.05; 2-way repeated-measures ANOVA) (Fig. 1C). ACTZ appeared to have a uniform effect across the neurons tested. Application of vehicle had no effect on evoked spiking (P = 0.99; paired t test) (Fig. 2C).

To prevent the injection of ACTZ from washing out the antagonist, the second injection included the same antagonist as injection 1 plus ACTZ. To determine whether the first injection caused maximal disinhibition, we checked whether it occluded the effects of a second injection of antagonist. Indeed, a second injection of DIOA alone did not cause any further increase in spiking (P = 0.11, paired t test) (Fig. 2D), thus legitimizing our measurement of ACTZ effects in Figure 2B. Notably, ACTZ does not counteract disinhibition through a competitive mode of action at KCC2 (or at GABA_A or glycine receptors) and, therefore, the effects of ACTZ are unaffected by supersaturating levels of antagonist.

ACTZ applied in the absence of KCC2 antagonist did not significantly alter spiking (P = 0.33, paired t test) (Fig. 2E). This indicates that reducing bicarbonate efflux enhances inhibition only in the context of reduced chloride influx. This likely reflects the role of bicarbonate efflux in exacerbating chloride accumulation when KCC2 is downregulated,15 rather than chloride accumulation stopping once chloride reversal potential reaches resting membrane potential (because there is no more driving force), bicarbonate-mediated depolarization sustains an inward chloride driving force. Importantly, the bicarbonate gradient is continually replenished by carbonic anhydrase,21 meaning membrane potential (and chloride reversal potential) could rise as high as the bicarbonate reversal potential near −20 mV. The present data are consistent with behavioral evidence that intrathecal ACTZ, despite having antiallodynic effects in nerve-injured mice, had little or no effect on acute pain behaviors (see “Discussion” section). These results also support our prediction that ACTZ will not counteract
disinhibition caused by blockade of GABA<sub>A</sub> or glycine receptors. Specifically, when GABA<sub>A</sub> receptors are blocked, they will not pass any bicarbonate but bicarbonate can still pass through activated glycine receptors; however, as chloride influx through glycine receptors is normal, glycine receptor function will not be enhanced by ACTZ and net inhibition will not be restored (even if, at the network level, enhanced glycine receptor–mediated inhibition could rescue decreased GABA<sub>A</sub> receptor–mediated inhibition). The same logic applies to GABA<sub>A</sub> receptors during glycine receptor blockade.

**Figure 3A** summarizes our prediction that ACTZ will not counteract disinhibition caused by blockade of GABA<sub>A</sub> or glycine receptors. As explained above, this absence of effect does not rely on the blockade of both receptor types. Blockade of GABA<sub>A</sub> receptors (with bicuculline) or glycine receptors (with strychnine) caused a significant increase in evoked spiking (P = 0.007 and P < 0.001, respectively; one-way repeated-measures ANOVA and Student–Newman–Keuls tests) (**Fig. 3B**). Effects of the 4 different blocking drugs—DIOA, VU, bicuculline, and strychnine—did not differ significantly from one another (P > 0.5 for all comparisons; Student–Newman–Keuls tests). But, unlike the efficacy of ACTZ after KCC2 blockade, ACTZ did not significantly counteract the disinhibition caused by GABA<sub>A</sub> or glycine receptor blockade (P = 0.70 and P = 0.14, respectively; Student–Newman–Keuls tests). **Figure 3C** summarizes our key finding, namely that ACTZ had a significantly greater effect on disinhibition caused by KCC2 blockade than it did on disinhibition caused by receptor blockade (P < 0.001; one-way repeated-measures ANOVA and Student–Newman–Keuls tests).

Although the pharmacological manipulations we used produce allodynia when injected intrathecally into awake behaving animals and reduction of both KCC2 and GABA<sub>A</sub>/glycine receptor function has been observed after nerve injury (see "Introduction" section), our manipulations do not necessarily reproduce the precise pathological changes induced by nerve injury. But, as injury-induced changes develop over the course of several days, it is impossible to record from the same neurons before and after development of disinhibition in order to gauge the reversal of that disinhibition by ACTZ. Instead, we capitalized on the fact that BDNF released from activated microglia is both necessary and sufficient for development of neuropathic pain and can produce disinhibition in <1 hour when applied intrathecally. Brain-derived neurotrophic factor has been shown to downregulate KCC2 expression in spinal neurons, and hence we predicted that the resulting disinhibition would be counteracted by ACTZ (**Fig. 4A**). As summarized in **Figure 4B**, BDNF caused subtler disinhibition than KCC2 or receptor antagonists but its effect was nonetheless highly significant (P < 0.001; paired t test). Furthermore, the resulting disinhibition was significantly counteracted by subsequent application of ACTZ (P < 0.001; paired t test).
4. Discussion

The results of this study show that distinct manipulations designed to disrupt synaptic inhibition in the spinal dorsal horn can acutely increase the responsiveness of spinal neurons to low-threshold tactile stimulation. But, despite causing equivalent increases in evoked spiking (and similar allodynia according to previous behavioral experiments; see “Introduction” section), the different forms of disinhibition were differentially reversed by subsequent application of ACTZ. Specifically, ACTZ reversed the effects of chloride dysregulation caused by direct blockade or BDNF-induced downregulation of KCC2, but it did not reverse the effects of GABA$_A$ or glycine receptor blockade. The differential efficacy of ACTZ in counteracting disinhibition raises important issues for understanding the molecular basis for allodynia and, ultimately, for developing more effective therapies with which to treat allodynia.

First and foremost, the differential effects of ACTZ demonstrate that not all disinhibitory mechanisms are equivalent. This cautions against treating “disinhibition” as a mechanism in its own right when, in fact, disinhibition can arise from mechanisms that are very distinct in biophysical and molecular terms. Instead, one must identify the specific molecular mechanism to choose the most effective therapy by which to counteract the resulting disinhibition. That said, there may be no obvious signs or symptoms by which to differentiate between different molecular mechanisms, or at least preclinical animal testing does not provide any clues to what those differences might be. Even at the cellular level, neurons exhibited similar increases in responsiveness following different manipulations. The lack of overt clinically detectable differences highlights the importance of identifying biomarkers that could differentiate between underlying mechanisms. Using short therapeutic trials to test responsiveness to drugs targeting one or another molecular mechanism could be very informative in deciding which therapy to pursue over the longer term.

Past behavioral studies have shown that intrathecal ACTZ has antiallodynic effects in nerve-injured animals. By comparison, higher doses of ACTZ were required to produce even modest reduction of acute pain behaviors. Sun et al. observed that although intraperitoneal ACTZ could reduce pain triggered by capsaicin and formalin (but not thermal pain), intrathecal ACTZ was ineffective. By comparison, intrathecal ACTZ was observed to reduce heat hyperalgesia but not mechanical allodynia after muscle inflammation, but intramuscular injection of ACTZ...
had the same effect,\textsuperscript{36} which leaves the mechanism of action uncertain. One must of course recognize that carbonic anhydrase plays an important role in pH regulation, and pH changes could have a broad range of modulatory effects.\textsuperscript{36} Furthermore, intracellular bicarbonate levels are also influenced by transport mechanisms. Despite these many uncertainties as to mechanism, the success (albeit mixed) of various carbonic anhydrase inhibitors as anticonvulsants is promising, and progress in that field may be transferable to neuropathic pain. Based on the present results, one could reasonably hypothesize that the variable success of carbonic anhydrase blockade in mitigating seizure activity might reflect the degree to which chloride dysregulation contributes to network hyperexcitability.

The differential effects of ACTZ that we observed argue that intrathecal ACTZ would reduce allodynia only if that allodynia was due, at least in large part, to chloride dysregulation. Therefore, previous behavioral data showing that intrathecal ACTZ is antiallodynic argue that chloride dysregulation is indeed a major contributing pathological factor at least in rodent nerve injury models. This is consistent with recent data showing that KCC2 modulators reduce allodynia\textsuperscript{10} because enhancing KCC2 function in the absence of KCC2 downregulation is inconsequential for synaptic inhibition. But, it is certainly conceivable that not all forms of neuropathic pain involve the same degree of chloride dysregulation. The above discussion must not be taken to imply that molecularly distinct disinhibitory mechanisms are mutually exclusive or that therapies must target only one mechanism. On the contrary, neuropathic pain is associated with diverse molecular changes that can impact different aspects of synaptic inhibition, let alone other processes. Indeed, ACTZ has been shown to enhance the efficacy of positive allosteric modulators of GABA\(_A\) receptors.\textsuperscript{3} But, even this last observation does not mean that chloride dysregulation and decreased receptor function necessarily co-occur (although they might); instead, the synergy speaks to how mechanisms interact. Specifically, mechanisms that occur in series can cooperate; receptor activation gates a conductance that must be multiplied by a driving force to produce a current, in which case increasing conductance yields diminishing returns in terms of rescuing the synaptic current as driving force is compromised. Conversely, increasing the driving force even a little multiplicatively enhances the capacity of a small increase in conductance to increase synaptic current. By comparison, mechanisms organized in parallel, such as GABA\(_A\) and glycine receptors co-expressed postsynaptically in the same neuron, tend to yield additive effects. Polypharmacy can be used to capitalize on synergistic effects. A rigorous understanding of the contributing pathological mechanisms and how they interact can inform the choice of strategic drug combinations.

According to Figure 4B, ACTZ almost completely reversed the BDNF-mediated increase in spiking, although BDNF also has been shown to affect primary afferent potassium channels,\textsuperscript{8,9} presynaptic inhibition,\textsuperscript{10} and N-methyl-d-aspartate (NMDA) receptors.\textsuperscript{14,15} All of those effects may contribute to neuropathic pain, yet none should be counteracted by ACTZ. With that in mind, we interpret the effect of ACTZ reported in Figure 4B to demonstrate that postsynaptic disinhibition (even without considering its precise molecular mechanism) plays a particularly important role in mechanical allodynia. In contrast, enhanced NMDA receptor function will encourage long-term potentiation and central sensitization, but that plasticity is typically induced by the activation of C fibers and, therefore, presumably contributes more to hyperalgesia than to allodynia.\textsuperscript{17,18} Likewise, Chen et al. surmised that mechanical allodynia reflects postsynaptic disinhibition rather than presynaptic disinhibition.\textsuperscript{10}

To conclude, this study shows that distinct disinhibitory mechanisms are differentially susceptible to therapeutic interventions. This has important clinical implications for choosing an appropriate therapy with which to reverse disinhibition arising from one or the other mechanism. However, identifying which disinhibitory mechanisms are at play complicated by the similarity in their outward manifestations. Even so, simultaneously modulating mechanisms that interact cooperatively holds great promise. These practical insights speak to the importance of distinguishing between molecular mechanisms even if, on the surface, their consequences seem quite similar.

**Conflict of interest statement**

The authors have no conflicts of interest to declare.

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