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Inhibition of carbonic anhydrase augments GABA_A receptor-mediated analgesia via a spinal mechanism of action

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Abstract

Peripheral nerve injury negatively influences spinal GABAergic networks via a reduction in the neuron-specific K⁺-Cl⁻ cotransporter KCC2. This process has been linked to the emergence of neuropathic allodynia. In vivo pharmacological and modeling studies show that a loss of KCC2 function results in a decrease in the efficacy of GABA_A-mediated spinal inhibition. One potential strategy to mitigate this effect entails inhibition of carbonic anhydrase activity to reduce HCO₃⁻-dependent depolarization via GABA_A receptors when KCC2 function is compromised. We have tested this hypothesis here. Our results show that, similarly to when KCC2 is pharmacologically blocked, peripheral nerve injury causes a loss of analgesic effect for neurosteroid GABA_A allosteric modulators at maximally effective doses in naïve mice in the tail flick test. Remarkably, inhibition of carbonic anhydrase activity with intrathecal acetazolamide rapidly restores an analgesic effect for these compounds suggesting an important role of carbonic anhydrase activity in regulating GABA_A-mediated analgesia after peripheral nerve injury. Moreover, spinal acetazolamide administration leads to a profound reduction in the mouse formalin pain test indicating that spinal carbonic anhydrase inhibition produces analgesia when primary afferent activity is driven by chemical mediators. Finally, we demonstrate that systemic administration of acetazolamide to rats with peripheral nerve injury produces an anti-allodynia effect by itself and an enhancement of the peak analgesic effect with a change in the shape of the dose response curve of the α 1-sparing benzodiazepine L-838,417. Thus, carbonic anhydrase inhibition mitigates the negative effects of loss of KCC2 function after nerve injury in multiple species and through multiple administration routes resulting in an enhancement of analgesic effects for several

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GABA_A allosteric modulators. We suggest that carbonic anhydrase inhibitors, many of which are clinically available, might be advantageously employed for the treatment of pathological pain states.

Introduction

The Institute of Medicine report on “Pain in America” published in 2011 highlights the urgent need for a better understanding of mechanisms that drive chronic pain and the development of therapeutics that target these mechanisms for the improved management of clinical pain disorders [13]. It has long been recognized that pharmacological manipulation of spinal GABAergic circuits can achieve analgesia [37; 49]. However, it has recently become clear that following peripheral nerve injury (PNI) there are changes in GABAergic function that limit the analgesic effect of spinally applied GABA_A receptor agonists and allosteric modulators and that spinal GABAergic circuits may even promote pathological pain resulting from PNI [9; 10; 12; 25; 36]. The strongest evidence for this latter point comes from multiple lines of evidence demonstrating that the neuron specific K⁺-Cl⁻ cotransporter, KCC2, is downregulated contributing to a loss of Cl⁻-dependent fast inhibitory neurotransmission and potentially to the generation of GABA_A receptor-mediated excitation [10]. While this has been shown to occur following PNI in outer lamina dorsal horn neurons, and in several other pain models [32; 33; 51], it is also true that GABA_A agonists and positive allosteric modulators retain anti-allodynic effects [2; 27; 28] and grafting of GABAergic neurons into the spinal cord following PNI alleviates symptoms of neuropathic pain [6].

While brief GABA_A receptor activation leads to Cl⁻-influx-dependent hyperpolarization, prolonged receptor engagement leads to a strong HCO₃⁻-efflux dependent depolarization [23; 24; 43] that has been linked to several neurological disorders [2; 4; 15; 35]. This situation might be exacerbated when KCC2 expression is decreased therefore compromising Cl⁻ gradients in GABA responsive neurons [12]. The influence of this HCO₃⁻-dependent depolarization can be mitigated by carbonic anhydrase (CA) inhibition [29; 41; 45]. We have shown previously that spinal inhibition of CA with acetazolamide (ACT) reduces neuropathic allodynia in rats and that ACT and benzodiazepines have synergistic spinal effects following PNI [2]. Importantly, we have also shown that in the presence of KCC2 blockade, certain GABA_A agonists and positive allosteric modulators lose analgesic efficacy in the tail flick test [3]. This suggests that loss of Cl⁻ extrusion capacity impairs the ability of GABA_A receptor engagement to achieve inhibition of spinal network activity. This notion is supported by modeling experiments demonstrating an activity-dependent decrease in GABA_A-mediated inhibition in the presence of decreased KCC2 expression [12]. A potential strategy to mitigate this effect, and therefore restore full analgesic efficacy of GABA_A agonists and allosteric modulators, is via inhibition of CAs.

Here we hypothesized that inhibition of CA activity should mitigate the effects of decreased Cl⁻ extrusion capacity following PNI resulting in augmentation of GABA_A-mediated analgesia and/or anti-allodynia. Our results demonstrate that CA inhibition increases the analgesic effect of spinally applied GABA_A agonists and allosteric modulators following PNI and that this effect is especially profound for neurosteroid site agonists and for a $\alpha 1$ -sparing benzodiazepine. Moreover, demonstrating an analgesic effect for this mechanism of action with a different behavioral endpoint, spinal CA inhibition reduces 1st and 2nd phase responses in the formalin test. Based on these findings, we propose that combining CA inhibition with GABA_A-directed therapeutics can be advantageously employed for the treatment of pathological pain [2].

Methods

Animals

Male ICR mice (18-22g) and male Sprague-Dawley rats (250-300g) were purchased from Harlan. Animals were housed in a climate-controlled room on a 12-12hr light/dark cycle where food and water were available ad libitum. All experiments were performed under an approved protocol in accordance with the policies and recommendations from IASP, NIH and IACUC of the University of Arizona. CAVII^{-/-} mice have been described previously [39]. These mice and their heterozygous (CAVII^{+/-}) wild-type (CAVII^{+/+}) littermates were bred at The University of Arizona Animal Care Facility and were used at 8-12 weeks of age for behavioral assessment. Both male and female mice were used in these studies. No sex differences were noted in any test.

Solutions and Drugs

THIP, ganaxolone, THDOC and clonidine hydrochloride were purchased from Tocris (Ellisville, MO). DIOA and Acetazolamide (ACT) were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam was purchased from USP (Rockville, MD). L-838417 was a kind gift from Johnson and Johnson Pharmaceuticals. Stock solutions of clonidine, and midazolam were made in distilled H₂O. DIOA, THIP, THDOC and ganaxolone were made in 100% dimethyl sulfoxide (DMSO). DIOA was diluted in Ringer's solution (145mM NaCl, 5mM KCl, 1mM MgCl₂, 0.4mM Na₂HPO₄, 25mM NaHCO₃ and 5mM glucose) containing 10% DMSO. L-838417 was made in 20% β -cyclodextrin with 10% DMSO. ACT was made in Ringer's solution with a starting pH of 8.2 for ACT solubility. The pH was adjusted to 7.8 prior to use. All other drugs were diluted to final doses in Ringer's solution for injection. Final concentrations of DMSO in solutions used for intrathecal injection in all tests were 10%. In experiments where β -cyclodextrin was used in the solvent, the final concentration of β -cyclodextrin was held constant across all experimental conditions (e.g. in vehicle controls). Intrathecal injection of 10% DMSO vehicle showed no behavioral effect in previous experiments [3].

Surgical procedures

Prior to surgery, all animals were assessed for mechanical withdrawal thresholds.

Spinal Nerve Ligation (SNL) surgery in rats—SNL was performed by tight ligation of the L₅ and L₆ spinal nerves as described by Kim and Chung [26]. Anesthesia was induced using ketamine/xylazine at 80 mg/kg. The dorsal vertebral column from L₄ to S₂ was exposed and the L₅ and L₆ spinal nerves of the left hindpaw were identified and carefully isolated. The L₅ and L₆ spinal nerves were tightly ligated distal to the dorsal root ganglion with a 4-0 silk suture and the incision was closed. Sham control rats underwent the same surgery and handling as the experimental animals but without the SNL. All animals were allowed to recover for at least 14 days and all testing was performed between 14 and 28 days post-SNL or sham. Following SNL, only animals that developed paw withdrawal thresholds less than 4.7 g by day 14 post-surgery were used.

Spared Nerve Injury (SNI) surgery in mice—Spared nerve injury (SNI) was performed as previously described [11] by a lesion of 2 of the 3 terminal branches of the sciatic nerve (tibial and common peroneal nerves), leaving the remaining sural nerve intact. Under isoflurane anesthesia (induction = 5%, maintenance = 1.7–2% isoflurane in room air), an incision was made on the lateral surface of the thigh and a section was made directly through the biceps femoris muscle exposing the sciatic nerve and its 3 terminal branches: the sural, common peroneal, and tibial nerves. The tibial and common peroneal nerves were tightly ligated with 5-0 silk and cut distal to the ligation, removing D2 mm of the distal

nerve stump. The skin incision was closed with staples. Mice were monitored daily following surgery and were tested for development of neuropathic allodynia 7 and 10 days following surgery. Testing with administration of intrathecal drugs was done between days 10 and 28 after surgery. The neuropathic pain state in these mice lasts for at least 28 days and is isolated to the ipsilateral paw.

Injections

The intrathecal (i.t.) injection was done as previously described by Hylden and Wilcox [21] using a 25- μ L Hamilton syringe with a 30 gauge, 1/2 inch needle. The injection was performed under brief (less than 3 minutes) isoflurane anesthesia (induction = 5%, maintenance = 1.7–2% isoflurane in room air) in a volume of 5 μ L. With the pelvic girdle held to keep the spinal cord in place, the needle was inserted into the intervertebral space between the L5 and L6 spinal cord level. A reflexive flick of the tail was used as an indicator of accurate needle placement and also as a confirmation of drug injection. For intraperitoneal injections in rats the rat was restrained and its head was held down at an angle to allow the abdominal contents to move away from the injection site. The needle was placed parallel to the linea alba and inserted at a 30–45 degree angle in one of the two lower quadrants of the abdomen. Proper placement of the needle was verified prior to injection by withdrawing the syringe plunger. A lack of intestinal or bladder content in the syringe confirmed a good placement of the needle. The injection was then administered. A volume of 0.5 mL was used.

Behavioral Tests

In all experiments observers and lab personnel conducting the injections were blinded to the experimental conditions.

Tail Flick—Analgesia was measured using the tail immersion method [22]. The mice were restrained with the tail extending out. The distal portion of the tail (2–3 cm) was immersed in a water bath thermostatically controlled at $52^{\circ}\text{C} \pm .5$. The tail withdrawal reaction time (in seconds) was initially recorded as the tail flick latency before drug administration and then recorded at 10, 20, 30, 45, and 60 minutes after the administration of the test drug. A cutoff latency of 10 seconds was maintained to prevent tissue damage. Percent maximum possible effect (%MPE) was calculated as

$$\%MPE = [(\text{Test latency} - \text{Baseline latency}) / (\text{Cutoff latency} - \text{Baseline latency})] * 100$$

Doses, when full dose-responses were not generated in this report, were chosen based on our previous work where full dose-response curves were reported [3].

Mechanical Thresholds—Animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for 1 hour followed by predrug mechanical thresholds recording. Using the up-down method [7], calibrated von Frey filaments (Stoelting, Wood Dale, IL) were used for mechanical stimulation of the plantar surface of the left hindpaw and withdrawal thresholds were calculated. Stimulation frequency was 0.1 Hz or lower.

Formalin test—Formalin (5% in saline) was injected into the hindpaw of ICR mice in a volume of 25 μ L and nociceptive behaviors (licking, biting or shaking in the affected paw) were measured for 45 min. The first phase was defined as 0 – 10 min and the second phase as 15– 45 minutes.

Rotarod—Mice were tested for motor coordination and balance on a rotarod (Rotamex 4/8, 3.8 cm rod diameter, Columbus Instruments, Columbus, OH). Training consisted of a 3 min trial in which the speed of the rod was set to 7 rpm. Upon falling, mice were continuously placed back on for the duration of the training. Mice received 3 training trials over 3 consecutive days with a cutoff time of 180 s. Mice were allowed to rest for 30 min between trials. For testing, the speed of the rod was set to 12 rpm. For the testing, the average of 3 consecutive runs per mouse was used for statistical analysis.

Hargreaves thermal test—Noxious thermal response was measured using the Hargreaves method [20]. CAVII^{-/-} [39], CAVII^{+/-} and wildtype (WT) mice were placed in plastic boxes on a clear glass plate and allowed to habituate for 45 min. A radiant heat source was focused under the left hindpaw of the animal. When turned on, the latency to paw withdrawal was measured by a motion sensor that halts both the lamp and timer when the paw was withdrawn. The machine was set to 30% intensity giving a base-line thermal latency of about 10 s. A cutoff time of 25 s was set to avoid potential tissue injury.

Statistics

All statistical analysis was done using Graph Pad Prism for Mac OS X Version 6.0c. Curve fitting for dose-response curves was done using non-linear regression analysis for variable slope. Comparisons between groups were made by two-way anova with Bonferroni's multiple comparison tests unless otherwise noted. Statistical details are given in figure legends as are the number of animals in each experimental group. Data are presented as mean $\pm$ SEM in all cases.

Results

We have shown previously that i.t. ganaxolone loses analgesic efficacy in the tail-flick test in mice co-treated with a KCC2 blocker, DIOA [3]. To test if this is a general principle for neurosteroid site binding ligands we evaluated the classical neurosteroid allosteric modulator, THDOC. I.t. administration of THDOC led to a dose-dependent ($ED_{50} = 5.1$ nmoles, 1.3 – 20.2 nmoles 95% CI) induction of analgesia in the mouse tail flick test (Fig 1A). When the peak effective dose of THDOC (30 nmol) was co-injected with DIOA, at a dose that we have used previously in similar experiments (5 nmol, [3]) the analgesic response of THDOC was significantly reduced over the complete time course (Fig 1B). Additionally, over a full dose range DIOA effectively right-shifted the dose-response curve for THDOC (Fig 1C). Hence, similar to observations with ganaxolone [3], blockade of KCC2 with DIOA leads to a reduction in the analgesic effect of THDOC.

Our previous findings showed that inhibition of KCC2 with DIOA disrupted the analgesic efficacy of some GABA_A agonists (e.g. muscimol) and allosteric modulators (e.g. ganaxolone) but not others (e.g. midazolam, [3]). Peripheral nerve injury is known to alter KCC2 expression in the dorsal horn such that a subset of neurons demonstrate reduced Cl⁻ extrusion capacity [9; 10; 25]. We therefore asked if the peak analgesic effect (defined with the maximally effective dose in naïve mice) of GABA_A receptor agonists and allosteric modulators would similarly be lost after PNI in the tail flick test and the SNI model. The α -subunit preferring GABA_A agonist THIP [16] produced significant analgesia for 30 min, consistent with previous studies [3; 5], in naïve mice whereas it was effective for 10 min in SNI mice (Fig 2A). On the other hand, the classical benzodiazepine, midazolam, produced analgesia of equal duration and magnitude in SNI and naïve mice (Fig 2B). The analgesic effects of benzodiazepines in the spinal cord are mediated largely by α 2- and α 5-containing receptors [27] wherein the α 2-containing receptors important for analgesia are localized largely presynaptically on primary afferents and presumably contribute to primary afferent

depolarization [47]. The sedating and addicting properties of benzodiazepines are mediated by $\alpha 1$ -containing receptors [49; 50]. The use of an $\alpha 1$ -sparing benzodiazepine bypasses negative effects of benzodiazepines produced by activation of $\alpha 1$ -containing receptors in brain. The $\alpha 1$ -sparing benzodiazepine, L-838,417, with largely $\alpha 2$ and $\alpha 3$ activity [31], produced analgesia at 10 and 20 min time points in naïve mice but only at the 10 min time point in SNI mice (Fig 2C). In contrast, both ganaxolone (Fig 2D) and THDOC (Fig 2E) produced robust analgesia in naïve mice but were ineffective in SNI mice at doses that produced maximal analgesia in naïve mice. The non- $GABA_A$ receptor ligand, clonidine, which is a selective $\alpha 2$ agonist, produced analgesia in naïve mice at 10, 20 and 30 min post injection but was effective only at 10 min in SNI mice (Fig 2F). Hence, while we observed a shorter duration of analgesic effects for all the $GABA_A$ receptor compounds in the tail flick test in mice with SNI, there was a remarkable reduction in the analgesic effect for neurosteroid site allosteric modulators in mice with PNI. It is unclear why the duration of analgesia produced by these compounds is reduced in mice with PNI but it is likely related to global changes related to PNI because the effect was observed across receptor classes tested here.

The CA inhibitor ACT can enhance anti-allodynic effects of benzodiazepines in rats with PNI through a central site of action [2]. Others have shown, through modeling experiments, that inhibition of CA might enhance the effective dose range of other $GABA_A$ receptor agonists and allosteric modulators especially when Cl^- extrusion capacity is reduced [12]. We therefore sought to determine if ACT could restore the analgesic effect of neurosteroid site allosteric modulators in mice with PNI. However, to do this we first had to determine the effects of ACT in the tail-flick test alone or in combination with PNI. In naïve mice, i.t. injection of ACT produced analgesia only at the highest dose tested (225 nmol, Fig 3A). Using a dose that was not effective in naïve mice, 22.5 nmol ACT did produce significant analgesia in SNI mice at 10 min after injection (Fig 3B). We focused on this concentration in subsequent studies. To examine whether ACT co-administration would alter the tail flick response in mice with SNI, we co-administered ACT with test compounds i.t. and compared the responses to vehicle or individual drugs alone. ACT co-treatment with THIP led to a prolongation of the analgesic response to THIP in SNI mice (Fig 4A). On the other hand, co-administration of ACT with either midazolam or L-838,417 did not significantly change the analgesic response of the drug when compared to the analgesic response of the drugs alone after PNI (Fig 4B and C, respectively). The co-administration of ACT with ganaxolone (Fig 4D) or THDOC (Fig 4E) led to a marked restoration of the analgesic effect comparable to the effects of these compounds when administered alone at the same dose to naïve mice. ACT co-administered with clonidine did not result in a significant analgesic response compared to vehicle controls. It is not clear why ACT apparently interferes with the analgesic effect of clonidine in this experiment (Fig 4F). Hence, we conclude that co-administration of ACT with neurosteroid site allosteric modulators leads to a rapid restoration of analgesia in mice with PNI.

Why did we not observe an ACT-mediated enhancement of benzodiazepine action in the tail flick test in mice when we have previously reported a synergistic interaction of midazolam with ACT [2]? One possible explanation lies in the inverted U shaped dose response curves of $GABA_A$ receptor agonists and allosteric modulators that we and others have observed when these drugs are applied spinally [2; 12]. Because we only tested L-838,417 in a narrow dose range we decided to construct a full dose-response curve for this compound in the tail-flick test. Similar to other benzodiazepines [2], L-838,417 showed a steep dose-response curve with an inverted U shape (Fig 5A), indicating a narrow effective dose range of the compound in the tail-flick test. We and others have suggested (including the present findings) that inhibition of carbonic anhydrase might be able to alter the inverted U shape of the dose response curve [2; 12], therefore we tested concentrations of L-838,417 (30 nmol)

at which the compound lacks analgesic effect at high doses in the tail flick test in the presence of ACT (22.5 nmoles). We did this in naïve mice because this dose of ACT has no effect in the tail-flick test in naïve mice, as opposed to SNI mice. ACT significantly enhanced the analgesic effect of L-838,417 at this dose for up to 30 min (Fig 5A and B), effectively creating the appearance of a plateau in the dose-response curve (Fig 5A).

Having determined that the inverted U shape of the L-838,417 dose response curve can be reversed by ACT co-treatment in the tail-flick test, we then sought to determine, in more detail, the effects of systemically administered L838,417 and ACT in a rat model of neuropathic pain. We used a rat model because L-838,417 has very limited systemic bioavailability in mice [27]. As we have shown previously with IT injection in rats, systemic ACT caused an anti-allodynia effect that was dose dependent (Fig 6A and B, $ED_{50} = 27$ mg/kg (121mmol/kg); 15.8 – 46.1 mg/kg 95% CI). Similarly, L-838,417 showed a dose-dependent anti-allodynia effect (Fig 6C and D, $ED_{50} = 1.9$ mg/kg (4.75 μ mol/kg); 0.56 – 6.3 mg/kg 95% CI) although it was less efficacious than ACT and completely lost efficacy at 30 mg/kg. We then tested L-838,417 and ACT administered together in a fixed dose ratio (1:10) in SNL rats. Co-administration of ACT with L-838,417 (Fig 6E and F, $ED_{50} = 5.3$ mg/kg (21.1 μ mol/kg); 1.7 – 17.0 mg/kg 95% CI) produced a dose-dependent reversal of mechanical allodynia and clearly eliminated the inverted U shaped dose response properties of L-838,417 given alone (Fig 6F), consistent with what we have previously observed in rats with i.t. injection of ACT and midazolam [2].

This and other previous findings strongly suggest that CA inhibition can lead to analgesia, antihyperalgesia [38] and anti-allodynia [2]. To ascertain if CA inhibition can also suppress ongoing pain responses through a spinal mechanism of action we utilized ACT in the formalin test in mice. I.t. ACT inhibited, in a dose dependent fashion, both peak 1st phase responses and 2nd phase responses in the formalin test in mice (Fig 7A). In the 2nd phase the ED_{50} was 56 nmol (14.6 – 216 nmol 95% CI, Fig 7B). Hence, i.t. ACT produces analgesia in mice via a spinal mechanism of action.

Previous studies have shown that the neuron-specific CA isoform 7 (CAVII) plays a role in ionic regulation of GABAergic signaling in hippocampus [39; 40]. We found that CAVII is expressed in mouse and rat spinal cord by Western blot (data not shown), therefore, we assessed whether CAVII might play a role in pain neurotransmission utilizing CAVII^{-/-} mice. CAVII^{-/-} and CAVII^{+/-} mice had normal mechanical (Fig 8A) and thermal thresholds in the 52°C hotplate (Fig 8B) and Hargreaves' (Fig 8C) tests when compared to wildtype littermates. They also showed no deficits in the rotorod test (Fig 8D). Likewise, CAVII^{-/-} mice displayed no difference compared to their wildtype and CAVII^{+/-} littermates, either in the SNI model of neuropathic pain (Fig 8E) or in the formalin test (Fig 8F). Hence, although our pharmacological data indicates that CAs play a key role in the ionic regulation of GABAergic responses in a variety of pain models and pharmacological circumstances, CAVII is not an obvious candidate for isoform specific intervention.

Discussion

We reach several major conclusions based on the results presented here. 1) Neurosteroid site positive allosteric modulators show a decreased peak analgesic effect in the tail flick test when KCC2 activity is blocked and following PNI. Remarkably, inhibition of CA activity with ACT rapidly and completely restores the analgesic effects of these compounds. 2) The α 1-sparing benzodiazepine, L-838,417, has a narrow efficacious dose window, reflected by an inverted U shaped dose response curve, in the tail flick test and in PNI-induced mechanical allodynia models. Combining this drug with CA inhibition changes the dose response curve such that there is now a plateau of analgesic or anti-allodynia effects at

higher doses potentially increasing opportunities for analgesic efficacy. 3) CA inhibition at the spinal level effectively reduces ongoing pain responses in the mouse formalin test. Interestingly, this effect is likely not mediated by CAVII, an important neuronal CA [39], because PNI-induced allodynia and formalin responses are completely unchanged in mice lacking CAVII. This result is most likely attributable to CAII, a ubiquitous CA isoform which is also expressed in neurons and shows a high sensitivity to ACT [39; 40]. Based on these findings, we propose that CA inhibition creates several exciting opportunities for the generation of novel therapeutics for pain.

There are several possible avenues for the further development of CA inhibitor-based therapeutics for pain. An obvious route would be systemic administration of CA inhibitors, many of which are clinically available. In that regard it is important to note that a commonly used pain medication, topiramate, has potent CA inhibitory activity [8; 46]. However, many CA inhibitors, almost all of which lack isoform specificity, have strong diuretic properties and may not be appropriate for certain patient populations. Another possible approach would be intrathecal application but this would require extensive safety testing because prolonged use at high local concentrations could disturb acid/base balance in the spinal cord. A possibility that we favor is the combined use of CA inhibitors with $\alpha 1$ -sparing benzodiazepines. This approach is appealing for several reasons. There is strong evidence that spinal benzodiazepines are effective analgesics at appropriate doses in animals [2; 3; 14; 19; 27] and in humans [42; 44; 48]. Moreover, this analgesic effect is mediated largely by $\alpha 2$ - and $\alpha 5$ -containing receptors [27] wherein the $\alpha 2$ -containing receptors important for analgesia are localized largely presynaptically on primary afferents and presumably contribute to primary afferent depolarization [47]. The receptor subtype distribution is important because the sedating and addicting properties of benzodiazepines such as midazolam are mediated by $\alpha 1$ -containing receptors in brain [49; 50]. We have shown here that combined use of a prominent $\alpha 1$ -sparing benzodiazepine, L-838,417, with largely $\alpha 2$ and $\alpha 3$ activity [31], with ACT leads to an enhancement of analgesia and anti-allodynia effects. Therefore, it is tempting to speculate that the combined use of an $\alpha 1$ -sparing benzodiazepine with a CA inhibitor could lead to powerful analgesia across a broad dose range in humans via a systemic route of administration.

We have consistently observed as inverted U shaped dose-response curves for GABA_A agonists and allosteric modulators in tests for anti-allodynia effects in PNI models and in the tail flick test [2; 3]. Interestingly, modeling experiments have demonstrated similar effects for positive allosteric modulators that are attributable to an inability of Cl⁻ extrusion capacity, which is mediated by KCC2, to keep up with Cl⁻ loading resulting from strong stimulation of GABAergic circuits [12]. It has long been understood that GABA_A receptors are permeable to both Cl⁻ and HCO₃⁻ [23] but these anions have opposing effects on membrane polarization at physiological resting membrane potentials. With prolonged activation of GABA_A receptors the Cl⁻ gradient rapidly collapses resulting in a strong HCO₃⁻-dependent depolarization [24; 43]. Hence, a possible prediction based on these modeling and electrophysiological studies is that the inverted U shape of dose response curves of analgesia for these compounds arises due to HCO₃⁻-dependent effects. Because HCO₃⁻-dependent effects are ultimately dependent on intracellular CAs, a test of this hypothesis can be done with CA inhibitors. We have previously shown that this is the case for i.t. midazolam [2] and we have shown here that CA inhibitors also mitigate the narrow effective dose range arising from an inverted U shaped dose response curve for L-838,417 in two independent assays via two independent routes of administration. We therefore conclude that it is likely that this pharmacological problem arises due to an inability of Cl⁻ extrusion capacity to compensate for the increased GABA_A receptor activity arising from the positive allosteric modulator leading to a prominent HCO₃⁻ efflux, an effect which is blocked by CA inhibition. An interesting corollary of this notion is the possibility that

positive modulators of KCC2 [18] might have a similar effect because they would be capable of compensating for the inability of the neuron to handle the Cl⁻ loading. Importantly, this effect would be exacerbated in situations where KCC2 expression or function is impaired such as following PNI or even in morphine-induced hyperalgesia [17] and may explain why ACT produced effective analgesia in lower doses in PNI mice than in naïve animals. This idea further strengthens the rationale for dual therapy with a positive allosteric modulator (e.g. α 1-sparing benzodiazepine) and a CA inhibitor.

An important question raised by our studies pertains to why there is a substantial loss of neurosteroid analgesic effect in the tail flick test when KCC2 is blocked or in mice that have undergone PNI? One possibility is a loss of receptor sites, however, the demonstration that simply including ACT with a neurosteroid immediately restores the peak analgesic effect rules out this possibility. Another explanation could include the distribution of neurosteroid site containing receptors in the spinal dorsal horn and/or on primary afferent terminals. Both THIP and neurosteroids are thought to act preferentially on γ -containing GABA_A receptors. In fact, the effects of THIP and THDOC on outer lamina spinal neuron GABA_A tonic currents appear to be mediated by γ subunits as they are lost in Gabrd^{-/-} mice, however, THIP retains an analgesic effect in the formalin test in mice lacking this subunit [5]. Unfortunately data is not available on THDOC in these mice. THIP has long been known to produce a strong effect on primary afferent fibers [1] although there is little data on the receptor subunit composition mediating this effect. Because benzodiazepines also have a strong effect on primary afferent fibers [47] and both THIP and benzodiazepines retain at least a portion of their analgesic potency in the presence of DIOA [3] or following PNI, one possibility is that primary afferents lack neurosteroid sensitivity at GABA_A receptors and these agonists are therefore more susceptible to changes induced by disruption of KCC2 activity in spinal dorsal horn neurons. The effect of neurosteroids on GABA_A receptors in DRG neurons has not been studied extensively but THDOC does enhance a tonic current in DRG neurons [30]. Interestingly, this presumably γ -subunit-dependent current is very strongly induced by inflammation in DRG neurons [30]. Significantly, neurosteroids can produce peripherally-mediated analgesia in naïve animals but this effect is mediated by effects on T-type Ca²⁺ channels and not by GABA_A receptors [34]. Hence, a THDOC- or ganaxolone-mediated effect on DRG neurons may only arise under certain conditions. More work is needed to add clarity to this issue, however, our data showing a restoration of THDOC and ganaxolone analgesia in SNI animals via CA inhibition is consistent with this idea.

We failed to demonstrate a role of CAVII in basal nociception, ongoing pain or neuropathic mechanical allodynia despite clear effects of CA inhibitors in both of the latter two tests. This is in line with the observations that the “house-keeping” CA isoform, CAII, is also expressed in neurons and plays a role in GABAergic actions and epileptiform activity in the hippocampus, albeit at a later stage of development due to its delayed developmental upregulation [39]. The lack of an observed effect in the pain models employed here likely excludes it from consideration as a spinal pain target. Thus, it seems that CAII may play a redundant role in regulation of CA activity at spinal synapses. In that regard, it would be interesting to test mice null for both CAVII and CAII.

In conclusion, we have shown an important role for CAs in regulation of acute thermal pain, formalin-evoked pain and neuropathic pain responses. Moreover, we have shown that CA activity plays a crucial role in shaping spinal GABAergic pharmacology. Because CA inhibition enhances or restores analgesic effects of GABA_A receptor allosteric modulators following injury and changes the inverted U shape of dose response curves of these compounds to plateaus of effective analgesia at high doses there is a strong rationale for

development of CA inhibitor combinations with positive allosteric modulators of GABA_A receptors for the treatment of pathological pain states.

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Glossary

Ganaxolone	neurosteroid allosteric modulator of GABA _A receptor
THIP	-subunit preferring agonist of the GABA _A receptor
THDOC	classic neurosteroid allosteric modulator of GABA _A receptor
Midazolam	classic benzodiazepine and GABA _A receptor allosteric modulator

Clonidine	α 2 adrenergic receptor agonist
DIOA	KCC2 inhibitor
Acetazolamide	general carbonic anhydrase inhibitor
L-838,417	α 1-sparing benzodiazepine targeting primarily α 2 and 3 containing GABA _A receptors

Perspective

Using behavioral pharmacology techniques, we show that spinal GABA_A-mediated analgesia can be augmented, especially following nerve injury, via inhibition of carbonic anhydrases. Carbonic anhydrase inhibition alone also produces analgesia suggesting these enzymes might be targeted for the treatment of pain.

Figure 1.

Evaluation of THDOC in the mouse tail flick test.

A) THDOC dose-dependently produces analgesia in the mouse tail flick test with an ED_{50} of 5.1 nmoles (1.3 – 20.2 nmoles 95% CI; $n = 5 - 8$ per dose). B) DIOA co-administration reduces the analgesic effect of THDOC in the mouse tail flick test over the entire time course (two way anova: $p = 0.0233$, 5.5 (1,54); $n = 5 - 6$ per group). C) DIOA significantly shifts the dose-response curve for THDOC to the right (curve fit comparison: $p = 0.0022$, 6.804 (2,58); $n = 6 - 8$ per dose). * $p < 0.05$. %MPE = Percent maximum possible effect.

Figure 2. Changes in analgesic effects for GABA_A site agonists and allosteric modulators following PNI.

(A) THIP produced significant analgesia vs. vehicle treatment at 10, 20 and 30 min post injection in naïve animals whereas THIP was only effective at 10 min time point in SNI mice ($n = 6 - 9$ per group). (B) Midazolam produced significant analgesia vs. vehicle treated mice at 10 and 20 min post injection in both naïve and SNI mice ($n = 5 - 9$ per group). (C) L-838,417 produced significant analgesia vs. vehicle treated mice at 10 and 20 min post injection in naïve mice but only at 10 min in SNI mice ($n = 7 - 13$ per group). (D) Ganaxolone produced significant analgesia vs. vehicle treatment at 10, 20 and 30 min post injection in naïve animals whereas it was ineffective in SNI mice ($n = 5 - 9$ per group). Likewise, THDOC (E) produced significant analgesia vs. vehicle treatment at 10, 20 and 30 min post injection in naïve animals whereas it was ineffective in SNI mice ($n = 5 - 9$ per group). The non-GABAergic, clonidine (F), produced significant analgesia vs. vehicle treatment at 10, 20 and 30 min post injection in naïve animals and was effective at the 10 min time point in SNI mice ($n = 8 - 9$ per group). Colored stars denote significant

differences between vehicle and treatment groups. Number signs denote significant differences between treatment groups. * or # $p < 0.05$, ** $p < 0.01$, *** or ### $p < 0.001$; two way anova with Bonferroni's multiple comparisons test. %MPE = Percent maximum possible effect.

Figure 3.

Effect of acetazolamide (ACT) in the tail-flick test in naïve and SNI mice.

A) ACT only produced significant analgesia in naïve mice at 225 nmol dose ($n = 9 - 11$ per group). B) A dose of 22.5 nmol ACT produced significant analgesia in SNI mice ($n = 7 - 9$ per group). Colored stars denote significant differences between vehicle and treatment groups. ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test. %MPE = Percent maximum possible effect.

Figure 4.

ACT modulates the analgesic effect of GABA_A agonists and allosteric modulators after PNI. (A) While THIP produced analgesia in SNI mice only at the 10 min time point, THIP plus ACT led to significant analgesia for 30 min ($n = 5 - 9$). On the other hand, the effect of midazolam (MZL, B, $n = 5 - 9$ per group) was equivalent with or without ACT treatment as was L-838,417 at a 1 nmol dose (C, $n = 7 - 10$ per group) in SNI mice. Ganaxolone- (D, $n = 5 - 10$) and THDOC- (E, $n = 7 - 9$) induced analgesia was restored at 10, 20 and 30 min time points by ACT cotreatment in SNI mice. (F) Clonidine produced analgesia in SNI mice at 10 and 20 min time points but failed to produce significant analgesia vs. vehicle when coadministered with ACT ($n = 5 - 9$). Colored stars denote significant differences between

vehicle and treatment groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two way anova with Bonferroni's multiple comparisons test. %MPE = Percent maximum possible effect.

Figure 5.

Effects of L838,417 in the tail flick test are enhanced by ACT.

A) L-838,417 produces dose-dependent analgesia in the tail flick test in naïve mice but the raising part of the curve is steep and shows an inverted U shape. Coadministration of ACT with the highest dose of L-838,417 produces a significant analgesic effect (student t-test, $n = 8 - 12$). (B) Time course of mouse tail flick test response of L-838,417 alone at 30 nmol (rightmost point in (A)) or in combination with ACT in naïve mice. Inclusion of ACT produces significant analgesia at 10 – 30 min post injection (two way anova with Bonferroni's multiple comparisons test, $n = 8 - 12$). Colored stars denote significant differences between vehicle and treatment groups. ** $p < 0.01$, *** $p < 0.001$. %MPE = Percent maximum possible effect.

Figure 6.

Anti-allodynia effects of systemic ACT and L838,417 in the rat SNL model.

A) Systemically administered ACT ($n = 10 - 12$ per dose) led to a dose-dependent reversal of allodynia in SNL rats when given IP with an ED₅₀ of 27 mg/kg (B, 15.8 – 46.1 mg/kg 95% CI). Similarly, L-838,417 ($n = 6 - 8$ per dose) led to a dose-dependent reversal of neuropathic allodynia (C) with an ED₅₀ of 1.9 mg/kg (D, 0.56 – 6.3 mg/kg 95% CI) but with an inverted U shaped curve. When ACT and L-838,417 were administered together in a mixed dose ratio ($n = 6 - 14$ per dose) to SNL rats the drug combination led to a dose-dependent reversal of neuropathic allodynia (E) with a combined dose ED₅₀ of 5.3 mg/kg (F, 1.7 – 17.0 mg/kg 95% CI) and clear plateau of anti-allodynia effects at high L-838,417 concentrations.

Figure 7.

Spinal ACT inhibits the 1st and 2nd phases of the formalin test in mice.

A) Formalin-induced responses are shown in 5 min blocks with increasing doses of ACT given intrathecally 15 min prior to formalin injection. ACT inhibited 1st and 2nd phase responses dose-dependently (two way anova with Bonferroni's multiple comparisons test, $n = 14 - 24$). B) Curve fitting for the dose-response curve for effect of intrathecal ACT in the 2nd phase of the formalin test reveals an ED₅₀ of 56 nmol (14.6 – 216 nmol 95% CI). Colored stars denote significant differences between vehicle and treatment groups. * $p < 0.05$, ** $p < 0.01$.

Figure 8.

No evidence for CAVII regulation of pain responses in mice.

Naïve CAVII^{-/-}, CAVII^{+/-} and wildtype (WT) mice were assessed in the von Frey test (A, n = 10), 52°C tail-flick test (B, n = 10), Hargreaves thermal test (C, n = 10), the rotarod test (D, n = 10), and for neuropathic allodynia following SNI (E, n = 7 - 8) and in the formalin test (F, n = 11 - 15). No significant differences were observed between genotypes in any test. Data were analyzed by one way anova (A – D) or two way anova (E and F).