

## Acetazolamide and midazolam act synergistically to inhibit neuropathic pain

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### ABSTRACT

Treatment of neuropathic pain is a major clinical challenge that has been met with minimal success. After peripheral nerve injury, a decrease in the expression of the K–Cl cotransporter KCC2, a major neuronal Cl<sup>−</sup> extruder, leads to pathologic alterations in GABA<sub>A</sub> and glycine receptor function in the spinal cord. The down-regulation of KCC2 is expected to cause a reduction in Cl<sup>−</sup> extrusion capacity in dorsal horn neurons, which, together with the depolarizing efflux of HCO<sub>3</sub><sup>−</sup> anions via GABA<sub>A</sub> channels, would result in a decrease in the efficacy of GABA<sub>A</sub>-mediated inhibition. Carbonic anhydrases (CA) facilitate intracellular HCO<sub>3</sub><sup>−</sup> generation and hence, we hypothesized that inhibition of CAs would enhance the efficacy of GABAergic inhibition in the context of neuropathic pain. Despite the decrease in KCC2 expression, spinal administration of benzodiazepines has been shown to be anti-allodynic in neuropathic conditions. Thus, we also hypothesized that spinal inhibition of CAs might enhance the anti-allodynic effects of spinally administered benzodiazepines. Here, we show that inhibition of spinal CA activity with acetazolamide (ACT) reduces neuropathic allodynia. Moreover, we demonstrate that spinal co-administration of ACT and midazolam (MZL) act synergistically to reduce neuropathic allodynia after peripheral nerve injury. These findings indicate that the combined use of CA inhibitors and benzodiazepines may be effective in the clinical management of neuropathic pain in humans.

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### 1. Introduction

Neuropathic pain is a major clinical problem that, despite increasing basic science understanding of the disorder, is still poorly managed by available therapeutics. Recent preclinical work in neuropathic pain models suggests that peripheral nerve injury (PNI) results in a pathology of the spinal GABAergic system. This may include decreases in spinal dorsal horn GABAergic interneurons [24], decreases in GABA release [24] (but see [27,34]) or alterations in GABA<sub>A</sub> and glycine receptor reversal potentials [9,10,28] attributed to a decrease in K<sup>+</sup>–Cl<sup>−</sup> cotransporter type 2 (KCC2) expression in spinal dorsal horn neurons [10]. A possible mechanism to reverse these effects is through treatment with benzodiazepines [20,21,41] which augment spinal GABAergic neurotransmission postsynaptically. This notion is supported by the efficacy of spinally applied benzodiazepines for chronic pain conditions in humans (for review see [12]); and their efficacy in preclinical pain models [20,21].

While spinally administered benzodiazepines are clearly anti-allodynic in neuropathic conditions [20] it is also true that reduced KCC2 expression after peripheral nerve injury causes a decrease in

the efficacy of inhibition mediated by GABA<sub>A</sub> and glycine receptors in a subset of dorsal horn neurons [10,28]. This reduction of KCC2 expression would reduce the Cl<sup>−</sup> extrusion capacity of dorsal horn neurons leading to a reduction of Cl<sup>−</sup> mediated hyperpolarization through GABA<sub>A</sub> and glycine channels. Under physiological conditions, GABA<sub>A</sub> receptor channels mediate substantial currents carried not only by Cl<sup>−</sup> but also by HCO<sub>3</sub><sup>−</sup> [15,16] as do glycine receptors [4]. Hence, a reduced Cl<sup>−</sup> extrusion capacity will favor the depolarizing channel-mediated efflux of HCO<sub>3</sub><sup>−</sup>, especially in neurons equipped with cytoplasmic carbonic anhydrase (CA). Because this enzyme is able to quickly replenish intracellular HCO<sub>3</sub><sup>−</sup> during the channel-mediated net efflux, the HCO<sub>3</sub><sup>−</sup>-dependent depolarization can become large enough to result in GABAergic or glycinergic excitation during ongoing neuronal network activity, as previously demonstrated in the adult rat hippocampus [33,38]. Notably, synchronous network activity in the dorsal horn of the spinal cord is enhanced after PNI [35]. Therefore, inhibition of CA after PNI may be expected to reduce HCO<sub>3</sub><sup>−</sup>-mediated excitatory GABAergic and/or glycinergic currents in the context of neuropathic pain.

Using a model of PNI based on spinal nerve ligation (SNL) [19], we have addressed two questions: (1) does inhibition of spinal CA activity reduce neuropathic allodynia? and (2) can inhibition of spinal CA augment the anti-allodynic activity of spinally administered benzodiazepines? Our results show that acetazolamide (ACT), a widely used CA inhibitor [37], reduces neuropathic

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allodynia, and that spinally administered ACT and midazolam (MZL) act synergistically to reduce allodynia as a result of PNI. These findings suggest that these two clinically used drugs may be utilized in combination to achieve relief of neuropathic pain in patients.

## 2. Materials and methods

### 2.1. Animals

Male, Sprague–Dawley rats (Harlan, Indianapolis, IN), 250–275 g, approximately 2 months old, at the time of surgery, were maintained in a climate-controlled room on a 12-h light/dark cycle (lights on at 6 AM), and food and water were available ad libitum. All testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health guidelines for the handling and use of laboratory animals. Prior approval for the project was received from the Institutional Animal Care and Use Committee of the University of Arizona.

### 2.2. Solutions and drugs

Acetazolamide (ACT) and midazolam (MZL) were purchased from Sigma–Aldrich (St. Louis, MO). ACT was made fresh as described below and 100 mM stock solutions of MZL was made in ddH<sub>2</sub>O. For all experiments the ACT was diluted into physiological buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 0.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM NaHCO<sub>3</sub> and 5 mM glucose) at a concentration of 10 mM with a starting pH of 8.2 to allow for ACT solubility. The pH was then adjusted down to 7.4 prior to use. The physiological buffer was used at pH 7.4 for MZL experiments and as a vehicle to control for any possible effects due to the alkalinity of the vehicle. Doses of ACT and MZL were based on [1,30].

### 2.3. SNL surgery

Prior to surgery all animals were assessed for mechanical withdrawal thresholds. SNL was done by tight ligation of the L<sub>5</sub> and L<sub>6</sub> spinal nerves as described by Kim and Chung [19]. Anesthesia was induced using ketamine/xylazine at 80 mg/kg. The dorsal vertebral column from L<sub>4</sub> to S<sub>2</sub> was exposed and the L<sub>5</sub> and L<sub>6</sub> spinal nerves of the left hindpaw were identified and carefully isolated. The L<sub>5</sub> and L<sub>6</sub> 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. An intrathecal catheter was placed (as described previously [40]) at the time of SNL surgery. 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. Any animals showing motor impairment from the intrathecal catheter placement were immediately euthanized.

### 2.4. Behavioral testing

#### 2.4.1. Mechanical thresholds

Animals were placed in acrylic boxes with wire mesh floors and allowed to habituate for 1 h. After baseline or predrug mechanical thresholds were taken animals received intrathecal injections in a volume of 10  $\mu$ l followed by flushing of the catheter with 6  $\mu$ l sterile saline solution. 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 calcu-

lated using the up-down method [5]. Stimulation frequency was 0.1 Hz or lower.

#### 2.4.2. Rotarod

Animals were tested for their ability to balance on a slowly rotating rod (Rotamex 4/8, 6.5 cm diameter for rats, constant rate of rotation of 8 revolutions per min, Columbus Instruments, Columbus, OH) after intrathecal administration of compound. Prior to drug administration, rats were trained in four consecutive sessions to stay on the rod and reach a cut off time of 180 s. Once trained, the animals were given drugs and tested at time points matching mechanical threshold measures for the appropriate route of administration. Only doses that achieved peak effects in reversal of SNL-induced allodynia were used.

#### 2.4.3. Statistics

All data are presented as means  $\pm$  SEM unless otherwise noted. Percentile data are presented as withdrawal frequency for a given time point minus predrug withdrawal threshold divided by presurgery baseline. Significant differences between groups for area under curve measures were assessed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc test. Measures of dose by time and rotarod were done by two-way ANOVA with Bonferroni post hoc test. Dose–response curves were analyzed by variable slope nonlinear regression using GraphPad Prism 5.0 for Mac OS X (GraphPad, San Diego, CA). For dose–response curves with MZL, where an inverted U curve was observed,  $E_{\max}$  was set to the most efficacious dose to avoid producing a right-ward shift in the EC<sub>50</sub> calculation; however, all doses are shown for a full representation of the data obtained. The same strategy was also used for A<sub>50</sub> analysis with MZL. Isobologram and A<sub>50</sub> analysis were done with JFlashCalc [26], which is freely available for use at <http://www.u.arizona.edu/~michaelo>.

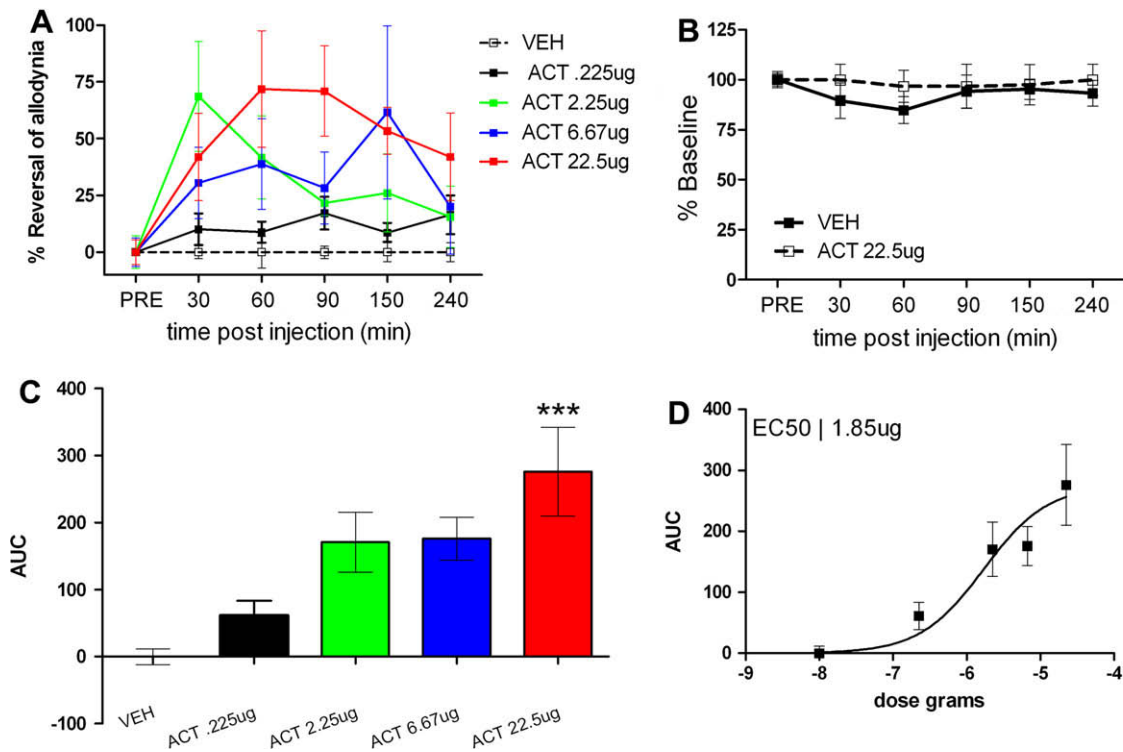
## 3. Results

### 3.1. Effects of intrathecal ACT on neuropathic allodynia

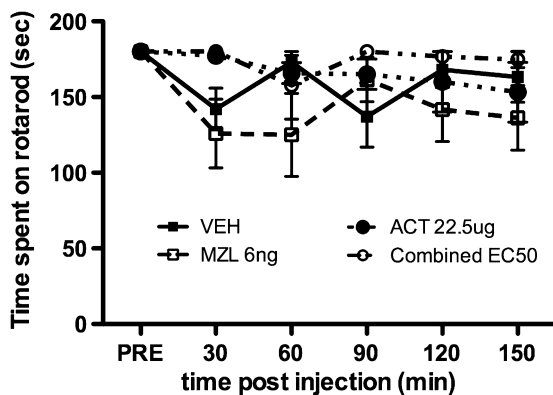
We first tested whether intrathecally administered ACT was capable of reducing neuropathic allodynia in the SNL model in rats. ACT was given in doses of 0.225 ( $n = 4$ ), 2.25 ( $n = 6$ ), 6.67 ( $n = 6$ ) and 22.5  $\mu$ g ( $n = 8$ ) and mechanical withdrawal thresholds were measured at 30, 60, 90, 150 and 240 min post-injection of ACT or vehicle ( $n = 6$ ). A significant main effect of ACT was observed (Fig. 1A,  $F(4, 144) = 6.95, p < 0.0001$ ). ACT dose-dependently inhibited neuropathic allodynia compared to vehicle controls (Fig. 1C and D) with a peak effect at the highest dose (Fig. 1C) at 60 min post-injection (Fig. 1A). ACT, at the highest dose tested ( $n = 6$ ), had no effect on mechanical withdrawal thresholds in sham animals compared to vehicle ( $n = 6$ , Fig. 1B). This dose of ACT did not, either, impair motor performance in the rotarod test compared to vehicle treatment ( $n = 7$ ) at any of the time points tested ( $n = 5$ , Fig. 2). The EC<sub>50</sub> for ACT was calculated as  $1.85 \pm 3.3 \mu$ g (Fig. 1D). Hence, intrathecal injection of ACT inhibits allodynia resulting from PNI indicating that spinal CA activity contributes to neuropathic mechanical allodynia.

### 3.2. Effects of intrathecal MZL on neuropathic allodynia

We then tested whether intrathecally administered MZL was capable of reducing neuropathic allodynia. MZL was given in doses of 0.4 ( $n = 9$ ), 2 ( $n = 7$ ), 6 ( $n = 8$ ), 20 ( $n = 9$ ) and 60 ng ( $n = 6$ ) and mechanical withdrawal thresholds were measured at 30, 60, 90, 150 and 240 min post-injection of MZL or vehicle ( $n = 8$ ). A significant main effect of MZL was observed (Fig. 3A,  $F(5, 246) = 6.31$ ,



**Fig. 1.** Intrathecal acetazolamide (ACT) inhibits neuropathic allodynia in the spinal nerve ligation (SNL) model. (A) Predrug mechanical withdrawal thresholds were measured and ACT was injected intrathecally and mechanical withdrawal thresholds were reassessed at the indicated time points. A significant main effect of ACT was observed ( $F(4, 144) = 6.95, p < 0.0001$ ). (B) ACT, at the highest dose given, did not alter mechanical withdrawal thresholds in sham animals. (C) ACT dose-dependently inhibited neuropathic allodynia with an  $EC_{50}$  (D) of  $1.85 \pm 3.3 \mu\text{g}$ . \*\*\* $p < 0.001$  with one-way ANOVA and Dunnett's post hoc test.



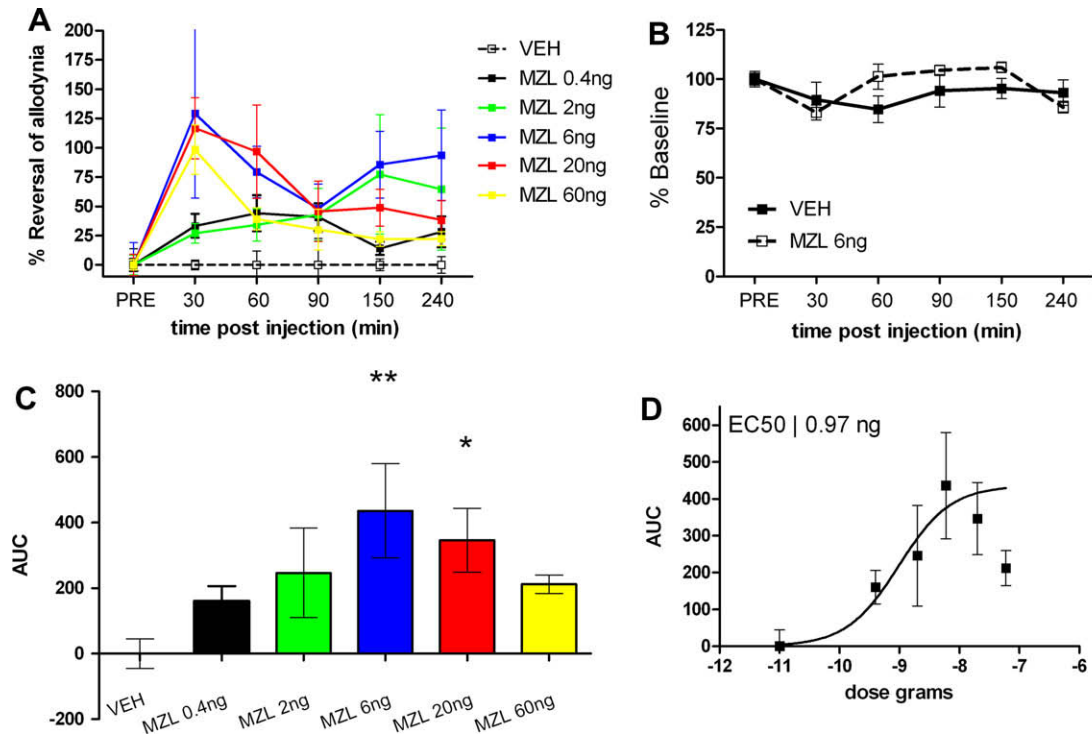
**Fig. 2.** Acetazolamide (ACT), midazolam (MZL) and ACT and MZL in combination do not impair motor performance. The rotarod test was used to assess whether maximum effective doses of ACT or MZL impaired motor performance. Rats with intrathecal catheters were trained as described in the text and VEH, ACT, MZL or ACT + MZL (combined  $EC_{50}$ ) were administered and rotarod performance was assessed at the indicated time points. Neither ACT, MZL nor ACT (0.97 ng) + MZL (1.85  $\mu\text{g}$ ) impaired motor performance as compared to vehicle injection.

$p < 0.0001$ ). MZL, as expected, also dose-dependently inhibited neuropathic allodynia compared to vehicle controls (Fig. 3C and D) with a peak effect between the 6 and 20 ng doses (Fig. 3C) at 30–60 min post-injection (Fig. 3A). Area under the curve analysis indicated that the MZL dose-response curve shows an inverted U-shape suggesting the effectiveness of MZL may be dose limiting. MZL, at the highest effective dose observed with area under the curve analysis, 6 ng ( $n = 8$ ), had no effect on mechanical withdrawal thresholds in sham animals compared to vehicle ( $n = 8$ , Fig. 3B). This dose of MZL did not, either, impair motor performance in the rotarod test at any of the time points tested ( $n = 7$ , Fig. 2). The  $EC_{50}$  for MZL was calculated as  $0.97 \pm 0.72 \text{ ng}$  (Fig. 3D). Hence,

intrathecal injection of MZL inhibits allodynia resulting from PNI but, apparently, in a dose-limiting fashion.

### 3.3. Effects of intrathecal ACT combined with MZL on neuropathic allodynia

We next tested the possibility that ACT and MZL might act synergistically to inhibit neuropathic allodynia. We used combined doses of ACT and MZL with a fixed dose ratio based on the  $EC_{50}$ s of both compounds (in ng) given separately. Hence, animals received intrathecal injections of ACT 1850 ng + MZL 0.97 ng ( $n = 7$ ), ACT 616.7 ng + MZL 0.32 ng ( $n = 6$ ) or ACT 185 ng + MZL 0.097 ng ( $n = 5$ ) representing  $EC_{50}$  values for each compound divided by 1, 3 and 10, respectively. At the highest combined dose motor performance was not affected ( $n = 5$ , Fig. 2). Mechanical withdrawal thresholds were measured at 30, 60, 90, 150 and 240 min post-injection of ACT + MZL or vehicle ( $n = 5$ ). A significant main effect of ACT + MZL was observed (Fig. 4A,  $F(3, 126) = 6.29, p = 0.0005$ ). Area under curve measures for ACT + MZL versus vehicle treatment are shown in Fig. 4B. The effect of the compounds administered together peaked at 60 min (Fig. 4A) and these values were used to construct a dose-response curve for ACT + MZL where the dose is shown as the combined amount of drug given (Fig. 4C). This was done in order to control for possible artefacts due to an expansion of the area under the curve measure from a prolonged action of the drugs given together, as was observed (Fig. 4A). The  $EC_{50}$  of ACT + MZL at this fixed dose ratio was  $190 \pm 141 \text{ ng}$ .  $EC_{50}$ s for ACT or MZL alone were also calculated under this paradigm using peak effects (60 min) for the compounds individually. The  $EC_{50}$  for ACT alone was  $2.16 \pm 1.75 \mu\text{g}$  and the  $EC_{50}$  for MZL was  $1.13 \pm 0.68 \text{ ng}$  (Fig. 4C). These observed  $EC_{50}$ s were consistent with  $EC_{50}$ s calculated using the area under the curve measurements (Fig. 1D and Fig. 3D).



**Fig. 3.** Intrathecal midazolam (MZL) inhibits neuropathic allodynia in the spinal nerve ligation (SNL) model. (A) Predrug mechanical withdrawal thresholds were measured and MZL was injected intrathecally and mechanical withdrawal thresholds were reassessed at the indicated time points. A significant main effect of MZL was observed ( $F(5, 246) = 6.31, p < 0.0001$ ). (B) MZL, at the peak effect dose, did not alter mechanical withdrawal thresholds in sham animals. (C) MZL dose-dependently inhibited neuropathic allodynia with an  $EC_{50}$  (D) of  $0.97 \pm 0.72$  ng. \* $p < 0.05$ , \*\* $p < 0.01$  with one-way ANOVA and Dunnett's post hoc test.

Isobolographic analysis was used to determine whether ACT and MZL act synergistically to inhibit neuropathic allodynia. For this analysis we calculated  $A_{50}$ s for ACT or MZL alone; a theoretical combined  $A_{50}$  if the drugs acted in an additive fashion; and an actual  $A_{50}$  from the observed effects of the drugs given together in the fixed dose ratio of 1850–0.97. As in the  $EC_{50}$  measurements shown in Fig. 4C, we used peak effects at 60 min for the drugs alone and in combination for these calculations. In this case the  $A_{50}$  for ACT given alone was found to be  $2.19 \pm 0.75$   $\mu$ g (Fig. 4D), again consistent with  $EC_{50}$ s calculated by area under the curve or peak effect methods. The  $A_{50}$  of MZL was  $1.07 \pm 0.88$  ng (Fig. 4D), also consistent with  $EC_{50}$ s obtained by the methods described above. The theoretical additive  $A_{50}$  was found to be a combined dose of  $1058 \pm 480$  ng whereas the actual  $A_{50}$  observed with ACT and MZL administered together was  $182 \pm 65$  ng (Fig. 4D, closely matching the  $EC_{50}$  calculated from regression analysis of  $190 \pm 141$  ng). The theoretical additive  $A_{50}$  differed from the observed  $A_{50}$  significantly ( $p < 0.001$ ) indicating that ACT and MZL act synergistically to inhibit neuropathic allodynia (Fig. 4D).

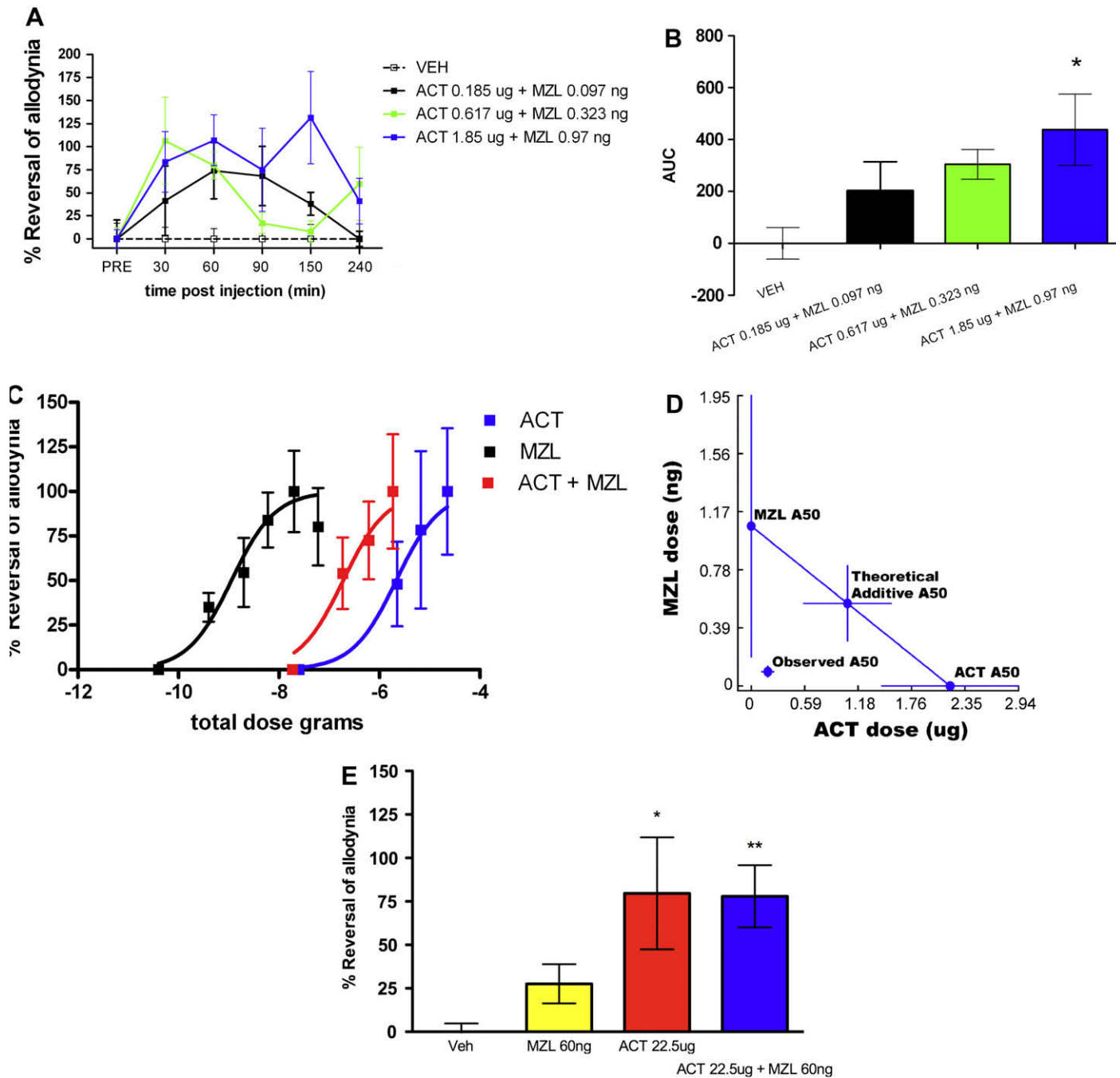
Finally, our findings indicated that the efficacy of MZL may be limited at high doses. Hence, we tested if co-administration of ACT with MZL could reduce this effect. We administered MZL at 60 ng ( $n = 6$ ), ACT at 22.5  $\mu$ g ( $n = 7$ ) and MZL at 60 ng + ACT at 22.5  $\mu$ g ( $n = 8$ ) or vehicle ( $n = 12$ ) intrathecally to SNL rats and measured reversal of allodynia at 60 min post-drug injection, consistent with the peak time point for efficacy of these drugs given alone or in combination. We did not use the fixed dose ratio dosage schedule used for synergism studies in this experiment because that dose of ACT would have exceeded the maximum dose of ACT given in any other experiments. ACT or ACT + MZL significantly reversed neuropathic allodynia while MZL given alone did not show a significant reversal, consistent with a loss of efficacy for MZL at this dose (Fig. 4E). Hence, this finding suggests that a

dose-limiting loss of efficacy for MZL at high doses in PNI animals can be avoided with co-administration of ACT.

#### 4. Discussion

Our work shows that ACT reduces mechanic neuropathic allodynia which, together with previous observations on the alleviating actions of CA inhibition on thermal hyperalgesia [30], suggests that spinal CA is a promising target for novel drugs in the treatment of several types of chronic pain. Even more importantly, the present data show that ACT and MZL act synergistically to reduce allodynia as a result of PNI. Therefore, the combined use of benzodiazepines with CA inhibitors may represent a new therapeutic approach for treatment of neuropathic pain.

Previous experiments have shown that intrathecal ACT (at a 22.5  $\mu$ g dose) inhibits thermal hyperalgesia after muscle inflammation [30]. Our present results indicate that ACT dose-dependently inhibits PNI-induced neuropathic allodynia and that ACT and MZL, when given in combination, act synergistically to alleviate the allodynia. Moreover, our findings suggest that the combined use of ACT and MZL may avoid possible dose-limiting effects of MZL which are illustrated by the inverted U shape of the MZL dose–response curve. These results lend support to a mechanistic scheme, described in detail below, wherein behavioral manifestations (allodynia) of the positive shift in reversal potential ( $E_r$ ) of  $GABA_A$  that results from KCC2 down-regulation after PNI [10,28] can be blocked by reducing  $HCO_3^-$  availability by CA inhibition. A potential anti-neuropathic pain role for CA inhibitors has been postulated previously [6] based largely on the CA inhibitor activity of topiramate, which alleviates PNI-induced allodynia in rats [2], shows synergism with tramadol after PNI [7] and reduces daily pain scores in humans with lumbar radicular pain [18]. Although topiramate has several molecular targets and inhibits



**Fig. 4.** Intrathecal acetazolamide (ACT) combined with midazolam (MZL) acts synergistically to inhibit neuropathic allodynia in the spinal nerve ligation (SNL) model. (A) Predrug mechanical withdrawal thresholds were measured and ACT + MZL, at a fixed dose ratio of 1850–0.97 ng (see text for explanation) was injected intrathecally and mechanical withdrawal thresholds were reassessed at the indicated time points. A significant main effect of ACT + MZL was observed ( $F(3, 126) = 6.29, p = 0.0005$ ). (B) ACT + MZL dose-dependently inhibited neuropathic allodynia with a combined dose  $EC_{50}$  (C) of  $190 \pm 141$  ng, curves for ACT and MZL alone are shown for comparison. (D) Isobolographic analysis revealed that ACT and MZL act synergistically to inhibit neuropathic allodynia. The  $A_{50}$  for ACT given alone was  $2.19 \pm 0.75$   $\mu$ g. The  $A_{50}$  of MZL was  $1.07 \pm 0.88$  ng. The theoretical additive  $A_{50}$  for a combined dose was  $1058 \pm 480$  ng while the observed  $A_{50}$  for ACT and MZL administered together was  $182 \pm 65$  ng. The theoretical additive mixture  $A_{50}$  differed from the observed  $A_{50}$  significantly ( $p < 0.001$ ) indicating that ACT and MZL act synergistically to inhibit neuropathic allodynia. (E) MZL, ACT or MZL + ACT were given intrathecally and paw withdrawal thresholds were measured at 60 min post-drug administration to test whether ACT would restore efficacy of MZL at a dose where efficacy was lost. ACT alone and ACT + MZL significantly reversed neuropathic allodynia while MZL alone did not. \* $p < 0.05$ , \*\* $p < 0.01$  with one-way ANOVA and Dunnett's post hoc test.

some CAs, topiramate is a potent inhibitor of CAVII [39], an isozyme important for  $HCO_3^-$ -mediated excitatory GABA actions in the hippocampus [31,33]. Our findings with ACT support the notion that CA inhibition is effective for the treatment of neuropathic allodynia. Further work is warranted to establish whether CA inhibition underlies the anti-neuropathic pain effects of topiramate.

Our work also shows that MZL alone dose-dependently inhibits neuropathic allodynia in the SNL model. Benzodiazepines require the presence of a  $\gamma 2$  subunit and different  $\alpha$  subunit compositions can lead to different behavioral effects. Recent studies have elucidated that spinal GABA<sub>A</sub> receptors are composed of  $\alpha 2, \alpha 3$  and  $\alpha 5$  subunits [20] and that  $\alpha 2$ - and  $\alpha 3$ -containing subunits are crit-

ical for benzodiazepine anti-allodynic effects in inflammatory, PNI [20] and anti-nociception in chemical pain models [21]. Critically, benzodiazepines that “spare”  $\alpha 1$ -containing subunits do not induce sedation yet achieve analgesia in all of these models [20,21,25]. Our results with intrathecal MZL are consistent with previous electrophysiological experiments in the SNL model [22]; with behavioral and biochemical experiments in the chronic constriction injury model of PNI [23]; with experiments in rats with chronic inflammation of the hindpaw from complete Freund’s adjuvant (CFA) injection [1]; and with reports of alleviation of pain in humans with spinal benzodiazepines [12]. Hence, these results provide further support for a spinal site of action for anti-allodynic effects of benzodiazepines after PNI.

A major question raised by these findings is: what are the mechanisms via which CA inhibitors and benzodiazepines act synergistically to inhibit neuropathic allodynia after PNI? PNI produces pathological changes in the spinal GABAergic and glycinergic network such that a decrease in the efficacy of inhibition is observed and this process may underlie the allodynic state that follows the injury [9,10,24,28]. PNI induces a positive shift in the reversal potential ( $E_r$ ) of GABA<sub>A</sub> receptors or glycine receptor-mediated currents in a subset of dorsal horn neurons [9,10,17,28]. The decrease in KCC2 expression after PNI would be expected to result in a decreased Cl<sup>-</sup> extrusion capacity in dorsal horn neurons; hence, the positive shift in  $E_r$  for GABA<sub>A</sub> and glycine receptors may reflect a reduced or collapsed Cl<sup>-</sup> gradient with Cl<sup>-</sup> reversal potential ( $E_{Cl}$ ) close to the resting membrane potential ( $V_{rest}$ ) [10,28]. Under these conditions, HCO<sub>3</sub><sup>-</sup>-mediated currents would be expected to lead to a further positive shift in  $E_r$  and, consequently, to strongly depolarizing and even excitatory GABA<sub>A</sub> or glycine channel responses that would be sensitive to CA inhibition.

While it is clear that PNI produces changes in the spinal GABAergic and glycinergic system that reduce the efficacy of GABA<sub>A</sub>- and glycine receptor-mediated hyperpolarizing inhibition, it is also true that spinally applied benzodiazepines are effective for reducing neuropathic allodynia and/or hyperalgesia [20,22,23,36]. According to the findings discussed above this would appear to be paradoxical; however, there are several scenarios that can resolve this potential paradox. The degree of positive shift in  $E_r$  after PNI is paramount to whether GABA<sub>A</sub> activity would be expected to produce gross excitation or shunting inhibition [3,13,28,29], and with smaller positive shifts in  $E_r$ , benzodiazepine-mediated augmentation of GABA<sub>A</sub> currents would be expected to produce a larger shunting inhibition. However, this would clearly be dependent on the intensity and duration of GABAergic network activity (see [35]), as prolonged activity would move  $E_r$  progressively more positive as a result of Cl<sup>-</sup> accumulation driven by the HCO<sub>3</sub><sup>-</sup>-mediated depolarization [14]. Under this scenario, CA inhibition is expected to augment the anti-allodynic activity of benzodiazepines because it reduces the HCO<sub>3</sub><sup>-</sup>-dependent progressive positive shift in  $E_r$ . Moreover, modeling studies have shown that an increase in shunting conductance can lead to an increase in excitability [28]. Such findings may explain the inverted U shape of the MZL dose–response curve found here and, according to the model postulated above, and our findings, this would be reversed by inhibition of CA activity.

A further point of importance in the present context is that phasic GABA<sub>A</sub> receptors are found preferentially in the postsynaptic membrane whereas tonic GABA<sub>A</sub> subunits, which possess high affinity for GABA, are found extrasynaptically in CNS neurons [11]. Phasic, synaptic GABA<sub>A</sub> receptors preferentially contain  $\alpha 1$ ,  $\alpha 2$  or  $\alpha 3$  and  $\gamma 2$  subunits and are benzodiazepine sensitive while tonic, extrasynaptic channels contain a  $\delta$  subunit and, with the exception of those that also possess  $\alpha 5$  and  $\gamma 2$  subunits, are benzodiazepine-insensitive [11]. Tonic receptors generate persistent currents as observed in dorsal horn neurons with prolonged GABA application [8]. On the other hand, after PNI, benzodiazepines gen-

erate anti-allodynia on the spinal level largely via  $\alpha 2$ - and  $\alpha 3$ -containing subunits suggesting that synaptic, or phasic, receptors are responsible for this effect [20]. Hence, during network activity, which can be generated in the spinal cord either by the convulsant 4-aminopyridine [32] or PNI [35], the large increase in conductance caused by the benzodiazepine sensitive, phasic postsynaptic events might well lead to a shunting inhibition despite a substantial depolarization of the target cell, while the low-conductance, tonic depolarization mediated by the extrasynaptic GABA<sub>A</sub> receptors is expected to provide a tonic excitatory drive that is sensitive to CA blockade [3,31]. Consistent with this, we observed striking synergism between benzodiazepines and CA inhibitors after PNI suggesting that CA inhibition augments the inhibitory effects of benzodiazepine action by suppressing HCO<sub>3</sub><sup>-</sup>-dependent excitatory events.

In conclusion, the major finding of the present work is that ACT and MZL act synergistically to inhibit neuropathic allodynia. In light of the available in vitro data reviewed above, a parsimonious way to explain this synergism is that CA inhibition blocks an HCO<sub>3</sub><sup>-</sup>-dependent positive shift in the  $E_r$  of GABA and/or glycine-mediated currents and the consequent tonic excitatory drive mediated by extrasynaptic GABA<sub>A</sub> receptors, while preserving shunting inhibition that is augmented by benzodiazepine actions at postsynaptic GABA<sub>A</sub> receptors. Obviously, further work is needed at the in vitro level in order to directly examine the cellular and synaptic basis of the ACT-MZL synergism and clinical studies are required to determine the safety of intrathecally applied CA inhibitors in humans. Since MZL and ACT, as well as several other inhibitors of CA [37], are clinically approved, we propose that their use in combination opens up a novel approach for the treatment of chronic neuropathic pain.

### Conflict of interest

The authors declare that they have no conflicts of interest.

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