# Impact of human CA8 on thermal antinociception in relation to morphine equivalence in mice

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**Recently, we showed that murine dorsal root ganglion (DRG) *Car8* expression is a *cis*-regulated eQTL that determines analgesic responses. In this report, we show that transduction through sciatic nerve injection of DRG with human wild-type carbonic anhydrase-8 using adeno-associated virus viral particles (*AAV8-V5-CA8WT*) produces analgesia in naive male C57BL/6J mice and antihyperalgesia after carrageenan treatment. A peak mean increase of about 4 s in thermal hindpaw withdrawal latency equaled increases in thermal withdrawal latency produced by 10 mg/kg intraperitoneal morphine in these mice. Allometric conversion of this intraperitoneal morphine dose in mice equals an oral morphine dose of about 146 mg in a 60-kg adult. Our work quantifies for the first time analgesia and antihyperalgesia in an inflammatory pain model after DRG transduction by CA8 gene therapy. *NeuroReport* 28:1215–1220 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.**

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## Introduction

According to the IOM Report (2011) on Chronic Pain in America, chronic pain affects at least 116 million US adults, accounting for over 30% of Americans [1,2]. Systemic analgesics, including opioids, can be limited by side effects and are inadequate in relieving chronic pain. Almost 250 million opioid prescriptions are dispensed annually, indicative of the 'opioid epidemic', which has led to a major rise in drug overdose deaths [2,3]. This has prompted the need to seek alternatives that selectively and safely 'silence' pain-transducing nociceptors, with the potential for prolonged effects (e.g. lasting weeks, months, or longer) [4,5].

Previously, we used complementation in homozygous null mutant (MT) mice, lacking Car8 protein because of an exon 8 deletion, to produce prolonged elevations in thermal pain thresholds after *AAV8-V5-Car8* intervention [6]. This approach demonstrated successful transduction of dorsal root ganglion (DRG) through sciatic nerve (SN) injections of adeno-associated virus (AAV) virus containing the expression vector encoding *Car8* wild-type (WT) cDNA to produce antinociception. However, this finding is 'paradoxical' with respect to the mechanism of analgesia seen with morphine. The Car8 protein is known to inhibit inositol 1,4,5-triphosphate receptor-1 (ITPR1) to decrease calcium release [6], whereas morphine depends on increased ITPR1-dependent calcium release to produce analgesia [7]. In another study, we addressed the morphine 'paradox' in which we found that murine DRG *Car8* gene expression is highly variable across inbred strains of mice and genetically regulates the analgesic effects of morphine in an antagonistic manner [8]. We surmised that the antagonism between Car8 expression and morphine may be related to their opposing effects on ITPR1-mediated calcium release [8].

In this report, we extended our previous findings [6] to evaluate the potential of the human form of carbonic anhydrase-8 (*CA8*) to produce thermal antinociception in naive C57BL/6J male mice. To quantify any resultant analgesia, we performed SN injections of AAV8 containing a vector encoding *V5-CA8WT* compared with injections containing vector encoding *V5-CA8MT* with the S100P null MT [9]. We report herein that *AAV8-V5-CA8WT* significantly increased thermal baseline withdrawal latencies before carrageenan inflammation, indicating analgesia. *AAV8-V5-CA8WT*

also attenuated decreases in withdrawal latencies after carrageenan inflammation, indicating antihyperalgesia. We further showed quantification of these analgesic and antihyperalgesic responses in morphine equivalents.

## Materials and methods

### Animals

All experiments and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and the current guidelines for investigation of experimental pain in conscious animals [10,11]. This protocol was approved by the Animal Care and Use Committee of the University of Miami. Male adult C57BL/6J mice, 12–14 weeks of age and weighing 30–35 g, were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Mice were kept in a home cage environment with access to food and water *ad libitum*. Animals were allowed to acclimatize for at least 7 days and were housed in a 12–12 h light–dark cycle in a virus/antigen-free facility with controlled humidity and temperature.

### Generation and SN injection of *AAV8-V5-CA8* viral particles

Generation and SN injection of AAV8 viral particles is described in our previous work [6]. These viral constructs express human *CA8 WT* or *MT CA8* vectors with V5 tag. The S100P mutation in the MT vector is associated with proteasome-mediated degradation that represents a null mutation comparable to the causal Car8 deletion mutation of the waddles mouse [6,9]. After SN exposure, 1.5 µl of about 2.0E14 genome copies/ml viral particles containing *V5-CA8WT* and *V5-CA8MT* were injected into the SN. The negative control group received saline injections through SN under anesthesia.

### Mouse model of inflammatory pain in relation to neurobehavioral testing

A volume of 15 µl of 1% λ carrageenan (Sigma; Sigma-Aldrich Corp., St. Louis, Missouri, USA) in saline was subcutaneously injected into the plantar surface of left hindpaw. Thermal sensitivity was measured using heat according to the Hargreaves' test, to obtain threshold calculations, respectively. Tests were performed in a quiet room with daylight-like illumination. Animals were habituated to the behavioral room and apparatus for at least 60 min for 1 week before a blinded investigator collected baseline data. The thermal sensitivity test was performed using an IITC Plantar Antinociception Meter apparatus (IITC Life Sciences, Los Angeles, California, USA) with a plastic box placed on a glass plate of constant temperature (30°C). The mouse plantar surface was exposed to a beam of radiant heat to induce paw withdrawal. The intensity of the Hargreaves' device was adjusted to obtain average values of 5–9 s in control mice, with a maximum of 20 s as cutoff to prevent potential injury. The latency time (in seconds) from the onset of the intense light beam to paw withdrawal was defined as the withdrawal latency of the paw. Two consecutive tests were averaged to establish the paw withdrawal latency.

### Morphine dosing regimen in relation to neurobehavioral testing

For the dose–response assessment, we chose to study doses of saline (vehicle), 1, 3, 10, 30, and 60 mg/kg. The thermal responses of male naive C57BL/6J mice were measured at 30–60 min after a single dose of intraperitoneal saline or morphine administration. This time frame was chosen based on the morphine response findings from Dogrul and Seyrak [12]. On a further note, the 30–60 min time frame was required to measure thermal responses after saline or morphine administration, and reflects the acclimation time needed in the behavioral device. Moreover, we show that the maximal response to morphine resides within this window. These morphine doses were selected based on the findings by Wilson *et al.* [13] in which they found that maximal thermal antinociception occurred at doses less than 100 mg/kg in C57BL/6J mice using a tail immersion assay.

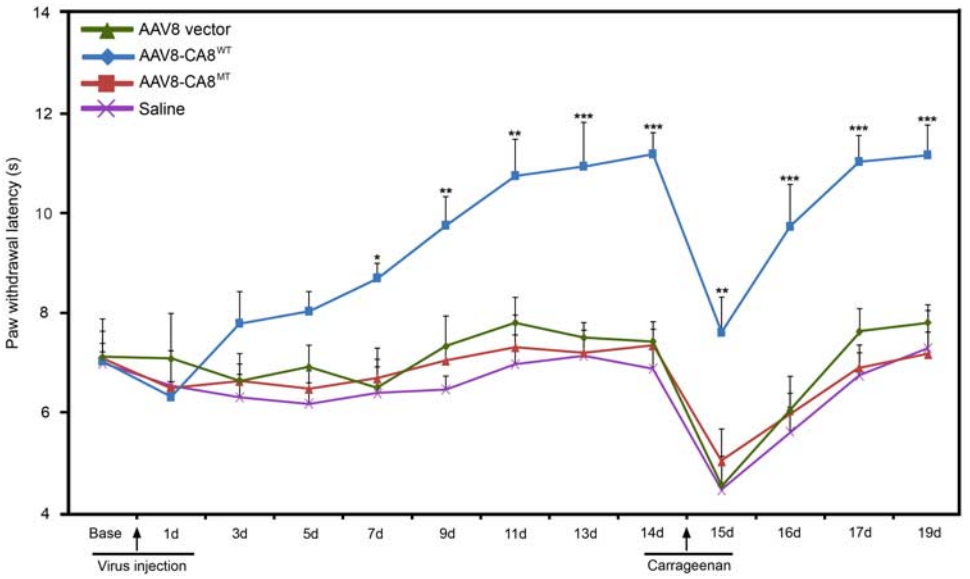
Thermal sensitivity was measured in these mice using the Hargreaves' assay, as previously described. The thermal latency data in the morphine dose–response assessment are presented after normalization to vehicle (saline). Normalization of the data entailed subtraction of the saline response to adjust for environmental effects due to intraperitoneal injections in nonanesthetized mice.

### Statistical analysis

The sample size was $n = 8$ per group for all experiments and the total number of mice studied was $N = 72$. For data presented in Fig. 1, a Tukey's multiple-comparison post-hoc test of a repeated two-way analysis of variance (treatment × time) was used to analyze these data (IBM SPSS Statistics 24, Armonk, New York, USA). Time was used as the repeated measure factor to determine main effects of treatment, time, and the interaction. Tests of between-subject effects were performed to show an observed power of 1.000 (computed using $\alpha = 0.05$), which include assumption of sphericity, Greenhouse–Geisser correction, and Huynh–Feldt correction. IBM SPSS Statistics 24 was modified to directly calculate the significance between groups at each time point incorporating a Bonferroni's correction.

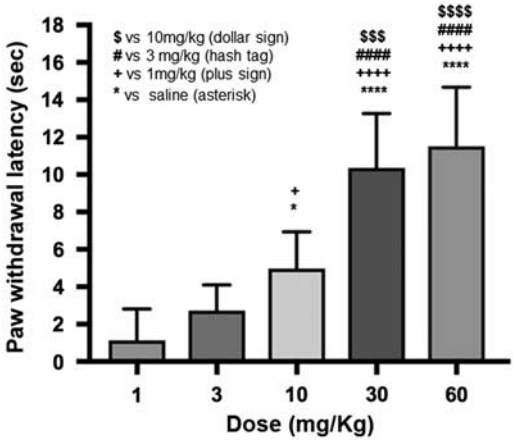
Power was estimated using a two-side two-sample *t*-test using GraphPad StatMate 2.00 software (La Jolla, California, USA) to determine sample size for data presented in Fig. 2. These calculations were in agreement with other studies [14,15]. This power analysis estimates that a group sample size of $n = 8$ is expected to achieve 95% power with a significant level ($\alpha$) of 0.05. The morphine dosing data are expressed as mean ± SEM. All data were normalized to saline response. Normalized data

**Fig. 1**




Gene transfer of *AAV8-V5-CA8WT* vector through sciatic nerve (SN) injection in a carrageenan inflammatory pain model produces analgesia and antihyperalgesia. Thermal latencies were measured at baseline and after intrasciatic saline or viral particle injection. Mice receiving SN injections of *AAV8-V5-CA8WT* (wild type) vector (1.5 µl, 2.65E14 genome copies/ml) had increased basal thermal latencies after day 7 compared with saline-treated mice. After carrageenan injections on day 14, mice in the AAV vector and *AAV8-V5-CA8MT* (mutant) vector groups had reductions in latency values markedly below baseline on day 15 and day 16, indicating failure to protect from carrageenan-related hyperalgesia. In contrast, after carrageenan injections on day 14, *AAV8-V5-CA8WT*-treated mice showed an attenuated reduction in thermal latencies on day 15 and day 16, in which these latency values did not differ from baseline values. *AAV8-V5-CA8WT* therapy restored thermal latencies to above baseline on day 17 and day 19, indicating a protective effect in response to inflammatory hyperalgesia ($N = 8$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with saline-treated mice).

**Fig. 2**




Morphine dose–response in naive mice for thermal withdrawal latencies. Eight 10-week-old naive male C57BL/6J mice were injected intraperitoneally in each group with morphine at each dose diluted in saline. Saline was used as vehicle control and all results were normalized to saline response. Each bar denotes mean ± SEM group response ($N = 8$; $^{****}P < 0.0001$, $^{*}P < 0.05$ vs. saline controls, $^{++++}P < 0.0001$, $^{+}P < 0.05$ vs. 1 mg/kg group; $^{\#\#\#\#}P < 0.0001$ vs. 3 mg/kg group; $^{\$\$\$\$}P < 0.0001$, $^{\$\$\$}P < 0.001$ vs. 10 mg/kg group).

were analyzed for statistical significance by one-way analysis of variance followed by Tukey's multiple-comparison post-hoc test for multiple comparisons (three or more groups) with one variance.

## Results

### DRG *CA8* overexpression alters thermal nociception before and after carrageenan inflammation

We constructed vectors containing the *WT CA8* cDNA with a V5 tag (*V5-CA8WT*) and *CA8 MT* cDNA sequence (S100P), which served as a negative control (*V5-CA8MT*) [8,9]. We assessed the effects of V5-CA8 overexpression on thermal nociceptive thresholds for 19 days after SN injection and DRG transduction before and after carrageenan injection and inflammation in the hindpaw of naive C57BL/6J mice (Fig. 1).

Before saline or viral SN injections, baseline thermal latency in naive mice was ~7 s (mean ± SD: 7.34 ± 1.09 s). As shown in Fig. 1, thermal withdrawal latencies were measured in saline-treated and vector-treated mice. Differences in thermal withdrawal latencies were found between groups [$F(3, 28) = 21.48$, $P < 0.001$] and across the 13 time points [$F(12, 336) = 20.04$, $P < 0.001$]. There were also significant interactions between time and group [$F(36, 336) = 4.37$, $P < 0.001$]. Bonferroni's pairwise comparisons of time × group interaction indicated that there were no differences between groups at baseline, day 1, day 3, and day 5. Withdrawal latencies in the *AAV8-V5-CA8WT* group were greater than the saline group at all time points after day 7 and after carrageenan application. Withdrawal latencies in the AAV8 empty vector and *AAV8-V5-CA8MT*

groups did not differ from saline treatment at all time points before and after carrageenan treatment.

On day 14, *AAV8-V5-CA8WT* treatment increased Hargreaves' thermal latency values by about 4 s compared with baseline values in the naive state (about 11 s compared with 7 s). Thermal latencies did not increase in saline, empty vector, or *AAV8-V5*-CA8MT-treated groups. Following carrageenan inflammation, the transduction of the mouse DRG with *V5-CA8WT* prevented a decrease in thermal thresholds below baseline values, with restoration of elevated thermal latencies of about 4 s above baseline by day 19. Conversely, SN administration of saline, virus containing empty vector, and *AAV8-V5-CA8MT* provided no protection from a decrease in thermal thresholds from baseline following carrageenan inflammation.

### Effect of morphine on thermal nociception before carrageenan inflammation

The raw baseline thermal latency for naive mice in Fig. 2 was ~7 s (mean ± SD: 6.87 ± 0.65), which were similar to the unadjusted baseline thermal latency for naive mice studied. Figure 2 shows normalized thermal latencies for intraperitoneal vehicle responses (saline) by subtracting the saline component. After normalization, we found significant increases in thermal withdrawal latencies from mice receiving 10, 30, and 60 mg/kg of morphine compared with saline-treated mice. Mice receiving the highest dose of morphine (60 mg/kg) had increases in thermal withdrawal latencies compared with mice receiving 1, 3, and 10 mg/kg of morphine, but not compared with mice receiving 30 mg/kg. On the basis of this analysis, we were able to assess the amount of parenteral morphine required to increase thermal withdrawal latencies in these naive mice and before carrageenan inflammation. In addition to normalizing the data, we also calculated maximal percentage effectiveness (%MPE) using the formula: (measured thermal latency − saline baseline latency)/[cutoff latency (20 s) − baseline latency] [13]. We found a progressive increase in %MPE in relation to the dose, in which the %MPE was 7.48% for the 1 mg/kg group, 20.17% for the 3 mg/kg group, 36.28% for the 10 mg/kg group, 78.86% for the 30 mg/kg group, and 86.20% for the 60 mg/kg group.

### Scaling of morphine from mice to humans based on the dose response of morphine on thermal nociception

The amount of morphine administered in mice to produce antinociception (increase in thermal response above baseline) is known as the mouse equivalent dose (Table 1). By obtaining mouse morphine equivalents, these data can be translated to a human equivalent dose by allometric conversion that depends on a formula that accounts for body surface area coefficients in humans and mice [16]. As shown in Table 1, we can extrapolate the mouse equivalent dose for thermal somatosensory responses to clinical oral morphine dosing in humans. Using this approach, we estimated that increases in thermal withdrawal latencies on the order of 4 s, as seen with *AAV8-V5-CA8WT* therapy, corresponds to a dose equivalent to about 10 mg/kg of intraperitoneal morphine (Fig. 2). We found that 10 mg/kg of systemic morphine in mice translates to human equivalent dose of 0.81 mg/kg, 48.65 mg of parental morphine, and 145.95 mg of oral morphine in a 60-kg adult (Table 1).

## Discussion

In a previous study, we noted that thermal antinociception in the noninflammatory state after *Car8* gene therapy persisted for at least 28 days [6]. In this report, we show that SN administration in naive inbred mice and DRG transduction by using *AAV8-V5-CA8WT* gene therapy produces profound analgesia as demonstrated by a maximal increase of thermal latencies about 4 s above baseline (Fig. 1). In addition, we show that *CA8* therapy produced antihyperalgesia by preventing a drop in thermal latencies below baseline in response to carrageenan inflammation along with restored thermal baselines to 4 s above baseline by day 19. These findings support the concept that *CA8* gene therapy provides prolonged analgesia before or after the onset of inflammatory pain.

As aforementioned, both *CA8* and morphine have 'paradoxical' effects on intracellular calcium release, even though both can produce antinociception. Morphine antinociception has been attributed to increased calcium release through ITPR1 binding of inositol 1,4,5-triphosphate (IP3) [7]. In contrast, we demonstrated that murine *AAV8-V5-Car8WT* showed inhibition of ITPR1-mediated cytosolic calcium release in conjunction with decreased thermal hypersensitivity [6]. We also found that greater DRG *Car8* expression antagonizes morphine analgesia concomitant with regulation of morphine-induced ITPR-mediated calcium release [8]. The disadvantage of μ opioids, such as of morphine, is the development of analgesic tolerance seen after morphine administration [14], which may involve pathways that increase intracellular calcium release such as IP3 binding to IP3 receptors [17]. Further studies are warranted to determine whether *CA8* therapy, which involves inhibition of ITPR1-mediated calcium release, could be advantageous in providing antinociception without the development of analgesic tolerance.

We sought to determine the amount of morphine equivalents needed to produce thermal antinociception and correlate that with thermal antinociception associated with *CA8* therapy. The degree of thermal antinociception in mice in response to morphine was assessed by dose–response analysis (Fig. 2). On the basis of allometric modeling by Reagan-Shaw *et al.* [16] among various species, the proper translation of animal dosing to human dosing entails the use of body surface area in addition to weight. Using the morphine dose–response data collected herein in naive male C57BL/6J mice, we show by

**Table 1 The effect of increasing parental doses of morphine on mouse hindpaw thermal withdrawal latency, with allometric conversion to human oral morphine dosing**

| | | | | | |
|---|---|---|---|---|---|
| Increase in mouse hindpaw thermal withdrawal latency (s) | 0.99 | 2.63 | 4.71 | 10.21 | 11.16 |
| Mouse equivalent dose of morphine (mg/kg) | 1 | 3 | 10 | 30 | 60 |
| Human equivalent dose of morphine (mg/kg) | 0.08 | 0.24 | 0.81 | 2.43 | 4.86 |
| Parental morphine dose in a 60-kg adult (mg) | 4.86 | 14.59 | 48.65 | 145.95 | 291.89 |
| Oral morphine dose in a 60 kg adult (mg) | 14.59 | 43.78 | 145.95 | 437.84 | 875.68 |

Mouse equivalent dose (MED) of morphine is obtained by determining the dose of morphine required to produce an analgesic effect as manifested by increases in mechanical or thermal thresholds above baseline. Human equivalent dose (HED) of morphine is obtained by dose translation from mouse to humans using $K_m$ factor coefficients. On the basis of allometric modeling by Reagan-Shaw *et al.* [16], the $K_m$ factor is derived by dividing the average body weight in kg of an animal species to its body surface area in $m^2$, in which $K_m$ mouse = 3 and $K_m$ human = 37. HED (mg/kg) = MED (mg/kg) × (mouse $K_m$/human $K_m$). Parental morphine dose in a 60-kg adult human is calculated after obtaining the HED. Oral morphine dose is approximately three times the parental morphine dose in a 60-kg adult.

allometric conversion that *CA8* analgesia from this experiment provided increases in thermal latency on the order of 4 s, which translates to morphine dosing equivalents of about 146 mg of oral morphine in a 60-kg adult human. These adult morphine quantities would exceed the recommended dosing maximum of 50–120 mg of daily oral morphine equivalents based on Centers for Disease Control [18] and Washington State Interagency Guidelines [19] on opioid dosing in chronic noncancer pain patients.

By establishing the relationship between morphine dosing and the degree of analgesia and antihyperalgesia associated with *CA8* gene therapy, we extrapolated the potential clinical significance of *CA8* gene therapy to morphine equivalents. However, there are several limitations with this strategy. We assessed morphine equivalents in a single inbred strain of male mice before inflammatory challenge. Nonetheless, the increases in thermal latencies observed after *AAV8-V5-CA8WT* therapy clearly persist after carrageenan inflammation, as compared with saline, empty vector, and MT. Thus, it appears that measuring morphine analgesic equivalents before the onset of carrageenan inflammation should still be relevant. In addition, inbred strains of mice differ in analgesic response by sex and strain, limiting the extrapolation of specific findings [20]. For example, C57BL/6 mice only achieve about 75% maximal percentage of thermal antinociception with morphine compared with BALB/cByJ mice, which had the highest degree of maximal percentage antinociception on the order of 90–100% [13]. Therefore, this strain appears relevant to test our general hypothesis.

## Conclusion

We determined that *AAV8-V5-CA8WT* SN injection produces profound analgesia in mice before and after severe inflammatory pain, as assessed in morphine equivalents. Further assessment of the safety profile of *AAV8-V5-CA8WT* therapy in mice will entail various assays to rule out tissue toxicity and neurobehavioral abnormalities. Future research is also needed in understanding the role of known analgesics such as morphine and clonidine in relation to CA8 analgesia, inflammation, and calcium release.

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### Conflicts of interest

There are no conflicts of interest.

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