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Human Carbonic Anhydrase-8 AAV8 Gene Therapy Inhibits Nerve Growth Factor Signaling Producing Prolonged Analgesia and Anti-Hyperalgesia in Mice

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Abstract

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Carbonic anhydrase-8 (*Car8*; murine gene symbol) is an allosteric inhibitor of inositol trisphosphate receptor-1 (ITPR1), which regulates neuronal intracellular calcium release. We previously reported that wildtype Car8 overexpression corrects the baseline allodynia and hyperalgesia associated with calcium dysregulation in the waddle (*wdl*) mouse due to a 19 bp deletion in exon 8 of the *Car8* gene. In this report, we provide preliminary evidence that overexpression of the human wildtype ortholog of *Car8* ($CA8^{WT}$), but not the reported *CA8* S100P loss-of-function mutation ($CA8^{MT}$); inhibits nerve growth factor (NGF)-induced phosphorylation of ITPR1, TrkA (NGF high affinity receptor); and ITPR1-mediated cytoplasmic free calcium release *in vitro*. Additionally, we show that gene-transfer using AAV8-V5-$CA8^{WT}$ viral particles via sciatic nerve injection demonstrates retrograde transport to dorsal root ganglia (DRG) producing prolonged V5-$CA8^{WT}$ expression, pITPR1 and pTrkA inhibition, and profound analgesia and anti-hyperalgesia in male C57BL/6J mice. AAV8-V5-$CA8^{WT}$ mediated overexpression prevented and treated allodynia and hyperalgesia associated with chronic neuropathic pain produced by the spinal nerve ligation (SNL) model. These AAV8-V5-CA8 data provide a proof-of-concept for precision medicine through targeted gene therapy of NGF-responsive somatosensory neurons as a long-acting local analgesic able to prevent and treat chronic neuropathic pain through regulating TrkA signaling, ITPR1 activation, and intracellular free calcium release by ITPR1.

Keywords

Carbonic Anhydrase-8; hypersensitivity; Allodynia; Inflammatory Pain; Neuropathic Pain; Persistent Pain; Cytosolic Free Calcium; Inositol Trisphosphate; ITPR1; Nerve Growth Factor (NGF); Neurotrophic Tyrosine Kinase Receptor A (TRKA, NTRK1); Adeno Associated Virus (AAV8); Gene Therapy

INTRODUCTION

According to the IOM Report (2011) on Chronic Pain in America, chronic pain affects more than 100 million U.S. adults.[1] Our understanding of the natural history of persistent pain and the specific environmental and shared genetic factors thought to impact susceptibility to these pain disorders is lacking.[2] Moreover, effective mechanism-based treatments for persistent pain represent a major unmet need in medicine today. Systemic analgesics, including opioids, can be limited by side effects and are inadequate in relieving pain.[3] Regional anesthesia can be considered a potential therapy for these patients. Unfortunately, a major limitation in treating acute, subacute, and certain forms of chronic pain with regional techniques is the lack of available local anesthetics exceeding 12 hours in duration.[4–6] Moreover, local anesthetics block motor and desirable sensory functions. Agents with target selectivity and the potential for localized somatosensory selective prolonged effects (e.g., lasting weeks to months, or longer) represent a critical goal for ongoing translational research in regional anesthesia and pain medicine today.[4]

Many critical cellular functions are regulated by intracellular free calcium concentrations including neuronal excitability, neurotransmitter release, neurite outgrowth, apoptosis, and neuroplasticity associated with long-term adaptive responses.[7–13] Increased cytosolic free calcium contributes to chronic pain by activating CaMK IIα[14–16] and PKC; sensitizing and

depolarizing afferent neurons.[17–19] In addition, nuclear free calcium integrates communications between the synapse and the nucleus, thereby regulating 'spinal genomic responses' required for persistent pain.[20, 21] Any imbalance in these regulatory inputs associated with increased intracellular free calcium is likely to affect pain processing in the central or peripheral nervous system leading to altered pain thresholds.[22–24] Activation of metabotropic receptors produces IP3 ligand triggering entry into the cell of calcium through receptor-gated and voltage-gated calcium channels (such as N-methyl-D-aspartate receptors); all downstream of Inositol 1,4,5-trisphosphate (IP3) receptors (ITPRs).[25–28] IP3, ATP and calcium are significant co-regulators of ITPRs and thereby intracellular free calcium.[29, 30] Mouse ITPR1 contains the IP3 ligand-binding core, the 'modulatory' domain responding to intracellular regulators such as ATP, calcium and carbonic anhydrase-8 (Car8)[31, 32] and undergoes phosphorylation to enhance ATP-dependent calcium release by several protein kinases.[33–37] Not surprisingly, these molecular actions are known to play an vital role in persistent pain behaviors.[20] Calcium dependent mechanisms that lead to chronic pain may be persistent, difficult to reverse and resistant to current therapies. So far, no available treatments exist that would effectively reverse these mechanisms and produce specifically long lasting analgesia. However, agents that have the potential to shift the balance of cytoplasmic free calcium and thereby decrease neuronal and glial transduction/transmission for extended times, could potentially produce prolonged analgesia and greatly advance this field. In this context, gene therapies may be very promising as emerging novel therapeutic approaches.

Therapeutic gene transfer into somatosensory neurons, including sciatic nerve, dorsal root ganglia (DRG) and the spinal cord, represents a potentially transformational approach to treat chronic pain. Adeno-associated viral vectors (AAV) seldom induce immune responses or produce cytotoxicity, facilitating their targeted delivery of therapeutic genes for overexpression to somatosensory tissues.[38] AAV8 can be effectively delivered to the somatosensory pathway by different routes.[39–43] In our previous investigation, we demonstrated that Car8 is involved in persistent pain regulation via the ITPR1-intracellular free calcium-signaling pathway.[24] We showed that overexpression of murine Car8 wildtype protein downregulated pITPR1, inhibited ATP-stimulated intracellular free calcium release, and abolished mechanical and thermal hypersensitivity. Additionally, we showed that Car8 overexpression in the somatosensory system alleviated chronic inflammatory pain in mice. Herein, we provide the first preclinical proof-of-concept for gene therapy using AAV subtype-8 (AAV8)-mediated CA8 overexpression targeting somatosensory neurons. We show for the first time that CA8 overexpression inhibited NGF-stimulated ITPR1 and TrkA phosphorylation, and ITPR1-mediated calcium release *in vitro*. We also show AAV8 gene therapy delivered via sciatic nerve injection overexpressed V5-CA8^{WT} and inhibited DRG TrkA phosphorylation; produced prolonged mechanical and thermal analgesia and anti-hyperalgesia; and treated chronic neuropathic pain.

RESULTS

In vitro CA8 expression models

As a prelude to testing the functions of wildtype human $CA8^{WT}$ in the murine somatosensory system, we utilized two AAV vectors to assess whether $CA8^{WT}$ would express well in murine and human cell lines. A V5 sequence was fused to the C-terminal end of the *CA8*^{WT} cDNA so that exogenously introduced CA8 gene product could be distinguished from the endogenously expressed mouse ortholog with an anti-V5 antibody. These constructs included *AAV-V5-CA8*^{WT} and *AAV-V5*-CA8MT vectors. Human derived HEK293 and NBL cell lines were chosen for vector transfection on the basis of higher ITPR1 and of lower endogenous pITPR1 and Car8 levels. Immunostaining data in Fig. 1 shows that there is almost no detectable endogenous Car8 in NBL cells (Fig. 1A and E). In contrast, we observed high levels of ITPR1 in NBL cells (Fig. 1C and E). We also observed almost no detectable pITPR1 at baseline in NBL cells (Fig. 1D-E). In contrast, we observed high levels of TrkA expression at baseline in NBL (Fig. 1B and E). DAPI is used to stain all nuclei for normalizing immune-positive cells. Endogenous expression of CA8, ITPR1, pITPR1 and TrkA in HEK293 cells are similar as that in NBL cells (data not shown). Western blot analyses in Fig. S1 also demonstrate expression of CA8, TrkA and ITPR1 in HEK293 cells.

To explore whether *AAV-V5-CA8* are expressed in both human-derived and murine-derived neuronal and non-neuronal cells, we transfected human HEK293, NBL and murine N2A cells with *AAV-V5-CA8*^{WT}, *AAV-V5-CA8*^{MT} and control vectors. As expected, using quantitative real-time PCR we show that the relative transcript expression for the *V5-CA8*^{WT} and *V5-CA8*^{MT} constructs did not differ in NBL cells (Fig. 2A), HEK293 and N2A cells (data not shown); but the steady-state $CA8^{MT}$ protein expression on western blots was significantly lower than $CA8^{WT}$ protein due to rapid turnover of the $CA8^{MT}$ form in NBL (Fig. 2B), HEK293 and N2A cell lines (Fig. S2A-B). Similar results are demonstrated with immunocytochemistry to that observed with western blotting data. CA8 expression is easily observed after cells are transfected with *AAV-V5-CA8*^{WT} vector. In contrast, CA8 is difficult to detect after cells are transfected with *AAV-V5-CA8*^{MT} vector (Fig. 2C-F, S2C-I). Vector controls show no V5 signal in cultures (Fig. 2D, S2D and S2G). Both human cell lines and the N2A murine cell line exhibited high transfection efficiency (Fig. 2E, Fig. S2E and H, and Fig. S3). DAPI was used to stain all nuclei for normalizing V5-positive cells (Fig. S3). Our prior work demonstrates that overexpression of V5-Car8 can inhibit ITPR1 phosphorylation (pITPR1) in murine-derived N2A cells.[24] To further investigate if V5-CA8 overexpression in N2A cells also inhibits murine pITPR1, we used forskolin to stimulate N2A cells two days after *CA8* transfection. Western blots analyses show forskolin induces pIPTR1 in a dose-dependent manner (Fig. S4A). Overexpression of V5-CA8 inhibited forskolin-induced murine pITPR1 (Fig. S4B). We conclude that V5-$CA8^{WT}$ protein expression is significantly higher in both human and murine cell lines compared to the V5-$CA8^{MT}$ form. Our data implicate that the human protein (i.e., V5-$CA8^{WT}$) functions similar to the V5-$Car8^{WT}$ protein on murine ITPR1 and would be a suitable reagent for preclinical studies in rodent pain models facilitating further preclinical testing in preparation for therapeutic development.

CA8 overexpression down regulates ITPR1 phosphorylation and inhibits NGF-induced free calcium release *in vitro*

ITPR1 translates IP3 signaling into $Ca2^{+}$ signaling and thereby plays a key role in many cellular functions.[25, 44] Forskolin increases cyclic adenosine monophosphate (cAMP) to phosphorylate ITPR1 via protein kinase A (PKA). Car8 regulates affinity of the ITPR1 intracellular calcium release channel for its IP3 ligand. This allosteric regulator of ITPR1 reduces calcium release from the ER and thereby modulates excitatory calcium signaling.[31, 32] To explore whether the interaction of CA8 with ITPR1 will affect its phosphorylation and thereby affect intracellular free Ca2+, we tested whether V5-$CA8^{WT}$ overexpression inhibited the phosphorylation of ITPR1 (pITPR1) by NGF in human HEK293 and NBL cells. NGF-induced increases of ITPR1 phosphorylation (pITPR1) in NBL (Fig. 3A) in a dose-dependent manner and overexpression of V5-$CA8^{WT}$ inhibited the increase of NGF-induced pITPR1 in murine NBL cells, but the V5-$CA8^{MT}$ expressing and empty vectors did not impact pITPR1 (Fig. 3B). $CA8^{WT}$ inhibition of pITPR1 in NBL cells suggests that it may alter ITPR1 mediated intracellular free Ca++ release. Therefore, we next tested the dose-response of NGF stimulated calcium release in NBL and HEK293 cells (Fig. 4A-I and S5A-I). Increasing NGF (1, 10 and 100 ng/mL) produced increasing calcium steady state intracellular calcium, compared to vehicle. The overexpression of $CA8^{WT}$ apparently inhibited the effects of even the highest NGF dose tested (10 ng/mL) but V5-$CA8^{MT}$ could not inhibit NGF-induced calcium release (Fig. 4J and S5J). These findings suggest that NGF signals through ITPR1 to induce calcium release and V5-CA8 inhibits NGF-stimulated ITPR1 activity and thereby reduces steady-state intracellular free calcium levels.

NGF calcium release is ITPR1-dependent

We next tested whether NGF-induced calcium release *in vitro* is ITPR1 dependent. ITPR-specific antagonist 2-aminoethoxydiphenyl borate (2-APB) inhibited the response to 10 ng/mL of NGF in both NBL and HEK293 cultures (Fig. 5 and Fig. S6) in a dose-dependent manner. These findings suggest that NGF signaling is predominantly through ITPR1 and not ryanodine receptor.

Overexpression of CA8 in DRG reduces SNL-induced phosphorylation levels of TrkA and ITPR1

To explore if exogenous CA8 can block phosphorylation of TrkA and ITPR1 following lumbar level 5 (L5) spinal nerve ligation (SNL), we analyzed DRG immunofluorescence of V5, pTrkA and pITPR1 Day 21 after SNL. *AAV8-V5-CA8* viral particles were injected into sciatic nerve (SN) 3 days before or after SNL (pre-SNL or post-SNL). Using immunohistochemistry, the percentage of V5-positive cells in pre-SNL $AAV8\text{-}V5\text{-}CA8^{WT}$ group is clearly higher than that in post-SNL $AAV8\text{-}V5\text{-}CA8^{WT}$ group in L5 DRG (Fig. 6). L4 DRG in Pre-SNL $AAV8\text{-}V5\text{-}CA8^{MT}$ group showed rare V5-positive cells, however the percentage of V5-positive cells was similar between pre-SNL $CA8^{WT}$ group and post-SNL $AAV8\text{-}V5\text{-}CA8^{WT}$ group and there were no V5-positive structures in contralateral DRG (data not shown). Overexpression of $V5\text{-}CA8^{WT}$ inhibits SNL-induced phosphorylation of TrkA and ITPR1 in L5 DRG in both pre-SNL and post-SNL groups, in comparison with post-SNL $V5\text{-}CA8^{MT}$ (Fig. 6 and S7).

Overexpression of V5-CA8^{WT} in DRG produces prolonged analgesia, preventing and treating neuropathic pain

We next tested whether if V5-CA8 could prevent or reverse neuropathic pain using the SNL model in mice. Surprisingly, we found that when *AAV8-V5-CA8^{WT}* producing viral particles (Fig 7 A and B, blue bars) were injected into SN 3 days after SNL, mechanical thresholds returned to baseline as early as Day 10. Moreover, *AAV8-V5-CA8^{WT}* produced analgesia (starting from Day 24 through Day 38) by significantly raising mechanical thresholds *above* baseline. However, when *AAV8-V5-CA8^{MT}* producing viral particles (Fig 7 A, red bars) were injected 3 days after SNL, this failed to produce any effect on mechanical responses through Day 24, with a slow return to baseline on Days 31-35 (Days 28-35 post-SNL). When *AAV8-V5-CA8^{WT}* encoding viral particles (Fig S8 A, green bars) were injected into SN 3 days before SNL, mechanical thresholds returned to baseline as early as Day 10, once again producing significant analgesia on Days 31-38. Similar findings were observed for thermal responses (S8 B) after sciatic nerve injections of *AAV8-V5-CA8^{WT}* and *AAV8-V5-CA8^{MT}*. Before viral SN injections, the average baseline withdrawal threshold was about 2 gms (2.16 ± 0.06 gms) and thermal latency in naïve mice was about 8 secs (8.03 + 0.12 secs). Differences in withdrawal responses were found between groups {F (2, 21) = 29.5, P=7.8E-7 mechanical threshold; F (2, 21) = 28.6, P=1E-5 thermal latency} and across the 8 time points {F (8,168) = 38.8, P=1.9E-34 mechanical threshold; F(8, 168)=18.7, P=1E-20 thermal latency}. There were also significant interactions between time and group {F (16, 168) = 3.9, P=1E-6 mechanical threshold; F (16,168) = 2.7, P=6.5E-4 thermal latency}. Bonferroni's pairwise comparisons of time*group interaction indicated that there were no differences between all groups at baseline, Day 1 and D3. Thermal withdrawal latencies in the AAV8-V5-CA8WT group were greater than the *AAV8-V5-CA8^{MT}* group at all time points after Day 10 and greater than Baseline for the *AAV8-V5-CA8^{WT}* group on Day 31 post-SNL. Mechanical thresholds in the *AAV8-V5-CA8^{WT}* group were greater than the *AAV8-V5-CA8^{MT}* group at all time points after Day 17 and beyond. Both thermal withdrawal latencies and mechanical thresholds in the pre-SNL *AAV8-V5-CA8^{WT}* treated group show significantly anti-hyperalgesia and/or analgesia from Day 7 or beyond (Fig S8).

DISCUSSION

In this report, we demonstrate several novel and important findings related to the role of CA8 in regulation of the ITPR1 pathway in chronic pain. First, we show *in vitro* that overexpression of the human ortholog of murine *Car8* (*CA8^{WT}*) inhibits NGF-stimulated phosphorylation of Serine-1755 of murine ITPR1. Second, we demonstrate that NGF-induced calcium release in NBL and HEK293 cells is inhibited by transfection with AAV vectors encoding *V5-CA8^{WT}* but not *V5-CA8^{MT}*. Third, we show in NBL and HEK293 cells that NGF-induced calcium release is almost entirely suppressed by ITPR1-specific inhibitor 2-aminoethoxydiphenyl borate (2APB) suggesting NGF signals almost exclusively through ITPR1 in these cells. Fourth, we demonstrate that SN injection of *AAV8-V5-CA8^{WT}* viral particles can undergo retrograde transport and transduce DRG somatosensory neurons producing long-lasting expression. Fifth, we show overexpression of CA8 in DRG by sciatic injection inhibits phosphorylation of TrkA and ITPR1. Sixth, we show that sciatic

nerve injection of *AAV8-V5-CA8^{WT}*, but not *AAV8-V5-CA8^{MT}* viral particles produce significant long-acting analgesia and anti-hyperalgesia in C57BL/6J male mice. Finally, we show *AAV8-V5-CA8^{WT}* but not *AAV8-V5-CA8^{MT}* viral particles can prevent or treat SNL induced neuropathic pain. Collectively, these findings are consistent with the actions of CA8 regulating the neuronal ITPR1-cytosolic free calcium pathway downstream of NGF signaling associated with chronic pain.

CA8 inhibits ITPR1-mediated calcium release by regulating TrkA and ITPR1 phosphorylation downstream of NGF

Nerve growth factor[45] with activation of its TrkA receptor are sufficient to cause persistent pain.[46, 47] We also show that NBL cells express TrkA (NGF high affinity receptor) and when transfected with *AAV-V5-CA8^{WT}* NGF-induced pITPR1, pTrkA and calcium release were inhibited, but not after transfection with *AAV-V5-CA8^{MT}*. Similarly, we show that SN injection 3 days after SNL with *AAV-V5-CA8^{WT}* but not *AAV-V5-CA8^{MT}* inhibits L5 DRG pTrkA. We believe that this is the first demonstration of CA8 downstream of NGF/TrkA and CA8 inhibition of NGF signaling *in vitro* and *in vivo*. We also confirmed that the observed NGF signaling is mediated by ITPR1 using ITPR-specific antagonist 2APB, which inhibited calcium release by NGF in a dose-dependent manner.[48] These findings confirm TrkA-dependent NGF signaling requires TrkA phosphorylation (pTrkA) and is associated with ITPR1 activation (pITPR1) and cytoplasmic Ca^{++} release, each inhibited by CA8 overexpression. After peripheral nerve injury, a positive feedback loop may lead to overproduction of NGF and inflammatory mediators, promoting the upregulation of alpha1A-adrenoceptors expression increasing the production of pro-inflammatory mediators with noradrenaline release. Consequently, nociceptive afferents become primed to respond to adrenergic mediators during continuing inflammation.[49] If these mechanisms are relevant to sympathetically maintained pain (e.g., such as cutaneous neuromas, post-herpetic neuralgia, Causalgia and amputation stump pain),[50] they may be amenable to CA8 gene therapy. This is a promising approach similar in concept to the anti-NGF therapies in late-stage clinical trials for cancer pain, severe osteoarthritis, and low back pain.[51–54] However, anti-NGF therapeutics are currently systemic reagents and significant drug-related adverse events have been described for the class.[55, 56] Using precision medicine with targeted overexpression of CA8 via gene therapy could potentially avoid systemic anti-NGF side effects while providing long-lasting analgesia and anti-hyperalgesia.

Many critical cellular functions are regulated by cytoplasmic free calcium concentrations including synaptic plasticity, neurotransmitter release, neuronal excitability, and neuroplasticity.[7, 10] Many neuronal biological functions, including memory and pain are dependent to synaptic plasticity.[57] It is well known that intracellular signals, including intracellular calcium regulate the activation of pain receptors.[58, 59] Pain transmission and perception are affected by physiological as well as pathophysiological modulators of cytosolic free calcium. Generally, alterations of intracellular free calcium in nociceptive neurons will result in a modulation of pain signals. Intracellular free calcium is increased by entry from the extracellular space and/or by consequent Ca2+ release from the intracellular stores. Increased intracellular calcium activates adenylate cyclase and subsequently increases intracellular cAMP, which activates the PKA.[60, 61] Similarly, a forskolin-induced rise of

intracellular cAMP also subsequently activates (phosphorylates) PKA.[60, 61] Activated PKA increases the sensitivity of ITPR1 for IP3 and induces calcium release from endoplasmic reticulum stores. Increased [Ca2+]i also activates PKC and CaM-kinase.[62, 63] Interestingly, prior publications implicate PKC in persistent pain associated with the depolarization of afferent neurons and enhanced currents activated by noxious thermal stimuli.[18, 57, 64] CaMKIIα also plays an essential role in the initiation of persistent pain.[14, 15] CaMKII-dependent ATP sensitive potassium channel (K_{ATP}) activation following elevated intracellular free calcium may limit excitotoxicity and cell injury.[65] K_{ATP} channel opening results in decreased neuronal excitability, reduced neurotransmission, and possibly analgesia and anti-hyperalgesia. Previous data shows that K_{ATP} regulation is altered by nerve injury.[66] Specifically, activation of K_{ATP} channels via intracellular free calcium and the CaM/CaMKII pathway are suppressed by nerve injury, resulting in neuronal hyperexcitability. Therefore, neuronal hyperexcitability represents a molecular lynchpin of pathologic neuroplasticity associated with chronic pain. Based on the significant baseline analgesia and hyperalgesia produced in our CA8 preclinical gene therapy model of neuropathic pain, it will be critical to understand in the future how CA8 impacts the Calcium/CaM/CaMKII pathway and K_{ATP} activation in this model.

Increased calcium can also have far reaching effects on cellular functions through effects on nuclear functions. Recent reports show that calcium transients in the nuclei of spinal dorsal horn neurons are associated with a reprogramming of the transcriptome including many pain-related genes.[21] The impact of CA8 on the transcriptome and long-term potentiation is another relevant area for future investigation.

AAV8 demonstrates adequate retrograde transport and DRG transduction after sciatic nerve injection to treat neuropathic chronic pain

We choose AAV8 viral particles using the SN route of injection and observed very high expression in small to medium sized primary sensory neurons as well as glial cells, suggesting AAV8 maybe a desirable serotype for delivering this gene construct for treating chronic pain via this route.[24] Foust et al., (2008) previously showed intravenous or intraperitoneal injections of neonatal mice with AAV8-GFP produced infrequent labeling of lower motor neurons across all time points and injections.[67] In contrast, they showed widespread labeling of dorsal horn fibers and posterior columns, consistent with dorsal root ganglion transduction. These findings suggested AAV8 might be useful for targeting somatosensory pathways important in chronic pain.[67] AAV8 delivered by lumbar puncture showed robust transgene expression in DRG neuronal nuclei and axonal projections into the dorsal horn. Immunohistochemical studies show AAV8 transduction of nociceptive neuronal cells staining for peptidergic marker vanilloid receptor subtype 1; small peptidergic neuron markers calcitonin gene-related peptide and substance P; and the nonpeptidergic neuron marker isolectin-B4 (IB4).[68] Despite known limitations in retrograde transport for AAV, our findings extend the above studies by showing adequate retrograde transport and transduction of DRG somatosensory neurons with the AAV8 viral particles using the SN injection route. While prolonged expression was demonstrated in our experiments, it is unclear if repeat injections would be necessary for chronic indications. It is well known that repeat AAV

injections may lead to inactivity through immune surveillance.[69] Host immune response and more efficient DRG transduction may be limitations to the current AAV approach.[69]

The role of primary afferents in maintaining persistent pain: Implications of CA8 gene therapy for neuropathic pain

It is now well accepted that pain processing and transmission in primary afferent fibers depend on ion channel function.[70, 71] Ion channels determine neuronal excitability and transmitter release by regulating resting membrane potential. Neuronal hyperexcitability (peripheral sensitization) associated with both inflammatory and neuropathic pain can result from alterations in ion channel expression. Intense ongoing efforts are focused on developing analgesic molecules that modulate ion channels in primary afferents.[71] Primary afferents are compose of mostly unmyelinated (C fibers) that can be further characterized as peptidergic (TrkA) and non-peptidergic fibers. One mechanism of primary afferent sensitization may depend on TrkA phosphorylation medicated by NGF, which was shown to upregulate expression of peripheral nerve sodium channel Nav1.7 (SCN9A) through release of intracellular calcium stores and activation of phospholipase C-gamma.[72] Prototypical nociceptors that respond to a wide variety of noxious stimuli, including thermal, mechanical and chemical insults are polymodal C-fibers. A-fibers can also contribute to persistent pain. Shortland et al. (1997) identified sprouting and aberrant functional synapses in lamina II after SN injury (CCI or SNL L5 lesion), but sprouting was not observed in uninjured A-fibers or C-fibers.[73] Nonetheless, deafferentation of A-beta and A-delta primary afferents associated with CCI nerve injury led to spontaneous discharge in adjacent uninjured afferents, likely critical in maintaining neuropathic pain.[74] Subtypes of primary afferents differ in their profile as potential therapeutic targets and maybe selectively addressed with precision medicine to produce optimal analgesia for selected indications by using promoter specific expression systems.[75] Thus, primary afferents represent attractive targets for regional long acting local analgesic delivered by gene therapy.

Summary

In the present study, we demonstrate that the human carbonic anhydrase-8 (CA8) overexpression inhibited NGF-induced pITPR1 and [Ca2+]i increases in NBL and HEK cells. We also show, once again that sciatic nerve injection of *AAV8-V5-CA8*^{WT} produced profound analgesia in mice[3] and effectively prevented and treated mechanical and thermal hyperalgesia after SNL in mice. Collectively, our data demonstrate that CA8 functions in mouse-derived cell lines and the mouse somatosensory nervous system, to inhibit murine ITPR1-mediated calcium release and pain processing. This work provides the first preclinical therapeutic validation for using CA8 as an allosteric inhibitor of ITPR1 to produce prolonged analgesia; prevent and treat neuropathic pain; all suggesting broad potential applications of this long acting local analgesic approach.

MATERIALS AND METHODS

Animal preparations and care

All procedures related to animal use and care were pre-approved by the University of Miami Institutional Animal Use and Care Committee. All C57BL/6J inbred male mice used in our

experiments were 2-3 months of age, weighing 20–35 grams. All animals were housed in a virus/antigen-free facility at a controlled temperature and humidity. A 12 hour light/dark cycle was provided along with water and food *ad libitum*. These animals randomly assigned to groups using random number tables. All samples were de-identified so the investigator performing an experiment was masked to the group and condition.

Mouse models of neuropathic pain

AAV8-V5-CA8^{MT} injections were used as negative controls, which was shown to produce an unstable protein with rapid proteasome degradation of CA8 leading to an effective null mutant.[76] To produce a spinal nerve ligation (SNL) model of neuropathic pain, the L5 transverse lamina was removed to exposure L4 and L5 spinal nerves after male C57BL/6J mice anesthetized as described. The L5 spinal nerve was then isolated and tightly ligated with 6-0 silk thread as previously described.[77]

Neurobehavioral testing

A quiet room maintained at a temperature of 23–25 °C was used to conduct all behavioral tests. Before collecting data, these mice were trained for 5 consecutive days to adapt to the experimental environment. Prior to each set of measurements, all mice were allowed to acclimate to the testing apparatus for a minimum of 60 min. A set of repeat measurements were used to familiarized all mice with each testing paradigm before collecting baseline measurements. An investigator blind to animal grouping performed behavioral tests essentially as described elsewhere.[24]

Generation of AAV8 viruses expressing wildtype or mutant CA8 with V5 tag using AAV-MCS vectors

CA8 wildtype (WT) gene cDNA was purchased from Origene (Cat. No. RC210228). An Eppendorf Recycler gradient (Model 5331) was used to amplify the WT gene which was cloned into pcDNA3.1/V5-His A between the BamHI and XhoI (NEB) restriction sites (Invitrogen Life Technologies, Carlsbad, CA) using (TTTGGATCC*GCCACC* ATGGCGGACCTGAGCTTC) as the forward primer and (TTTCTCGAGCTGAAATGCAGCTCTAATGACTC) as the reverse primer. The V5-CA8 construct was then cloned between the BamHI and BglII restriction sites of the pAAV-MCS vector, one component of AAV Helper-Free System (Agilent Technologies, Santa Clara, CA), after amplification from pcDNA3.1/V5-His A using (CTCGGATCC*GCCACC*ATGGCGGAC) as forward primer and (TTTGTCGACTCACGTAGAATCGAGACCGAG) as reverse primer. The S100P mutation was introduced into the CA8 cDNA (MT) by using the GENEART Site-Directed Mutagenesis System (Invitrogen Life Technologies, Carlsbad, CA) with the forward primer: AGTCAAAATCAGTTCTTCCGGGAGGACCATTGC and the reverse primer: GCAATGGTCCTCCCGGAAGAACTGATTTTGACT. The S100P mutation is associated with proteasome-mediated degradation that represents a null mutation comparable to the causal Car8 deletion mutation of the waddles mouse.[82]

The Miami Project Viral Vector Core, University of Miami Miller School of Medicine produced the recombinant AAV8-V5-CA8 viral particles, as described elsewhere.[24] The

purified AAV particles were titrated for genome contents using real-time qPCR method. Titers were in the range 1-3E^{14} GC (Genome Copy) per mL.

Sciatic nerve injections of AAV8-V5-CA8 viral particles

Male mice were anesthetized by intraperitoneal injection of ketamine, xylazine and acepromazine (VEDCO, Saint Joseph, Mo). After sciatic nerve exposure, 1.5 μl 2.65E+14 genome copies/ml viral particles of *AAV8-V5-CA8^{WT}* or *AAV8-V5-CA8^{MT}* were injected into the sciatic nerve through a 35-gauge needle using a NanoFil syringe (World Precision Instruments, Sarasota, FL). The injection site was approximately 45 mm from the tip of the third toe. The needle remained at the injection site for 1 additional min before it was slowly removed.

Tissue and cell culture preparation

Male mice (2-3 months) were anesthetized as described elsewhere.[24] Spinal cord and DRG were dissected from the L4 and L5 levels, and put in the same fixative for 2–4 h, then transferred to 20% sucrose overnight or until the tissues sank to the bottom of the vessel. The tissue was embedded with OCT (Andwin Scientific Inc., Schaumburg, IL) on dry ice. Leica 1900 Cryostat (Leica Microsystems Inc., Buffalo Grove, IL) was used to cut 16 μm sections and mounted to slides for immunofluorescence staining. Western blots were run with fresh spinal cord and DRG tissues dissected directly from mice for western blot. The vendor supplied validation of cell lines. N2A (ATCC CCL-131), HEK293 (ATCC CRL-1573) and NBL (ATCC CRL-1622) were plated in 24-well plates on poly-D-lysine-, laminin-coated glass coverslips for immunocytochemistry, in 6-well plates for western blot. Cells were seeded at a density of 2×10^5 cells/ml. Cultures were set up in 6-well plates in 2 mL per well, and in 24-well plates at 0.5 mL. The cultures were incubated in a water-saturated atmosphere containing 5% CO_2/95% air at 37°C in Gibco DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1X cellgro penicillin-streptomycin (Fisher Scientific, Pittsburg, PA).

Transfections

HEK293, NBL and mouse N2A cells were plated in 6-well plates at 4×10^5 cells and 24-well plates at 1×10^5 cells per well to obtain 90% confluent layer after 24 h and transfected, as described elsewhere.[24] Cells were used for measurement of mRNA and protein expression using real-time PCR, immunocytochemistry and western blot after 48 h incubation.

Immunocytochemistry and imaging

Tissue immunostaining was performed, as previously described.[84] Tissue sections and cell cultures were fixed by 4% PFA in PBS for 30 min, permeabilized in 0.3% Triton X-100 for 1.5 h at room temperature, and blocked in 4% normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 min. Primary antibodies specific for CA8, V5, vinculin, TrkA, pTrkA (Abcam, Cambridge, MA), pITPR1 and ITPR1 (Cell Signaling Technology, Danvers, MA). Antibodies were allowed to incubate with sections and cell cultures overnight at 4°C after dilution in PBS containing 0.1% Triton X-100. Sections and

cultures were washed three times for 10 min each in PBS, and incubated with Alexa Fluor 488 and/or Alexa Fluor 594-conjugated second antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at RT. Sections and cultures were mixed with the 2nd antibody to assess the total number of cells, and images were acquired as described elsewhere.[24]

Western blot assays

HEK293, NBL and N2A cultures were homogenized in RIPA buffer with a mixture of proteinase and phosphatase inhibitors (Sigma). Protein samples were generally separated on 4–15% SDS polyacrylamide gels and transferred to nitrocellulose membrane. For big protein p-ITPR1 western blot, 6% Tris-HCl gel in transfer buffer were used to increase efficiency. Western blots were prepared, handled and analyzed essentially as presented elsewhere[24] using anti-CA8 (Santa Crutz, Santa Crutz, CA), anti-V5 (Invitrogen), anti-pITPR1 (Cell Signaling Technology), vinculin, TrkA (Abcam, Cambridge, MA) and anti-β-actin (Sigma). Density analysis was performed using UN-SCAN-IT, standardized to β-actin, and a one-way ANOVA was used for statistical analysis.

RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) and real time PCR (qPCR)

Total RNA was extracted from cultured N2A, NBL and HEK293 cells essentially as described elsewhere.[24] qPCR was used to amplify exogenous CA8-V5 with the forward primer: ACCTTGCAGTGAAGGTGTCA and the reverse primer: CCGAGGAGAGGGTTAGGGAT. GAPDH was used as internal control with the forward primer: GGATTTGGTCGTATTGGGCG and the reverse primer: ATCGCCCCACTTGATTTTGG. qPCR and analysis as described elsewhere[24] using $CA8^{WT}$ and $CA8^{MT}$ versions and these results were normalized to the expression of GAPDH. Efficiency of each primer pair was determined via standard curve and used to determine relative quantities of starting mRNA amounts.

Calcium assay methods

Human HEK293 (ATCC CRL-1573), or SH-SY5Y (NBL) neuroblastoma (ATCC CRL-2266) cells were transfected with AAV-V5-$CA8^{WT}$, AAV-V5-$CA8^{MT}$ vectors or treated as sham 48 h prior to assay, according to the manufacturer's instructions. Twenty-four hours before assay, 100K to 250K cells/mL in 1 or 0.5 mL 12 or 24-well plates respectively and then split onto 15 mm glass coverslips (Propper, Long Island, NY) coated with poly-lysine overnight and laminin for 2 h at RT. On the day of the assay, cells were rinsed with perfusate buffer (Buffer 1),[85] then incubated in Buffer 1 containing 0.1 μM Fura-2/AM (Molecular Probes) and 0.012% pluronic F-127 (Sigma) for 45 min at RT (21-22OC) and washed twice with Buffer 1. After another 10 min incubation in Buffer 1 at RT, coverslips were transferred to a RC-42 LP open bath chamber in the QE-1 platform, loaded onto a Leica DMI 6000 B inverted microscope and perfused with Ca2+ free Buffer 2 (Buffer 2, all concentrations in mM: 130 NaCl; 4.7 KCl; 2.3 MgSO4; 5 Glucose; 20 HEPES; 10 EGTA; 1.2 KH2PO4).[86] Cells were allowed to equilibrate for 2-5 min before recording on Leica Application Suite 1.1.0.12420 (Leica, Germany) was initiated. Cells from 2-3 different cultures were visualized every 5s for 7-15 min while perfused with Buffer 2; or

Buffer 2 containing NGF or 2-APB/NGF (Sigma), as described elsewhere.[85] Six consistent points of peak or base line values of F340/F380 from a special coverslip were collected and averaged as one sample (N=1). Intracellular free calcium concentration [Ca2+]I were calculated, as described elsewhere.[85]

Quantification and statistics

To quantify immunoreactive staining of CA8, ITPR1, pITPR1, TrkA, pTrkA and V5 expression in the DRG, the percentages of positive neurons in the L5 and L4 DRG from four nonadjacent sections were determined as described previously.[72] We have previously shown that SN injection of AAV8 is highly selective for small to medium sized DRG neurons.[24] Briefly, the DRGs were serially sectioned at 16 μm. The percentage of positive DRG neurons was estimated by calculating the average total number of V5 positive cells divided by the total number of DAPI positive nuclei from 3-4 sections of each animal. Results represent 3 to 4 mice in each group. Quantitative evaluations were made by an investigator masked to the arrangement of DRG sections analyzed. Groups were compared using Student's t-test, or ANOVA, followed by Fisher's PLSD test and data presented as mean ± SEM. The criterion for statistical significance was $P < 0.05$.

The sample size was N=8 per group for all *in vivo* experiments. For data presented in Figure 7, a Tukey's multiple comparison post-hoc test of a two-way repeated-measures ANOVA (treatment X time) was used to analyze these data (IBM SPSS Statistics 24). 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 correction. The number of mice used per assay group was based on power analyses using the observed variation in prior AAV8 assays; and to ensure sufficient tissues to measure the planned post-mortem endpoints. We estimated that N = 8 animals per group would provide 95% power at P=0.05. No animals were excluded in our analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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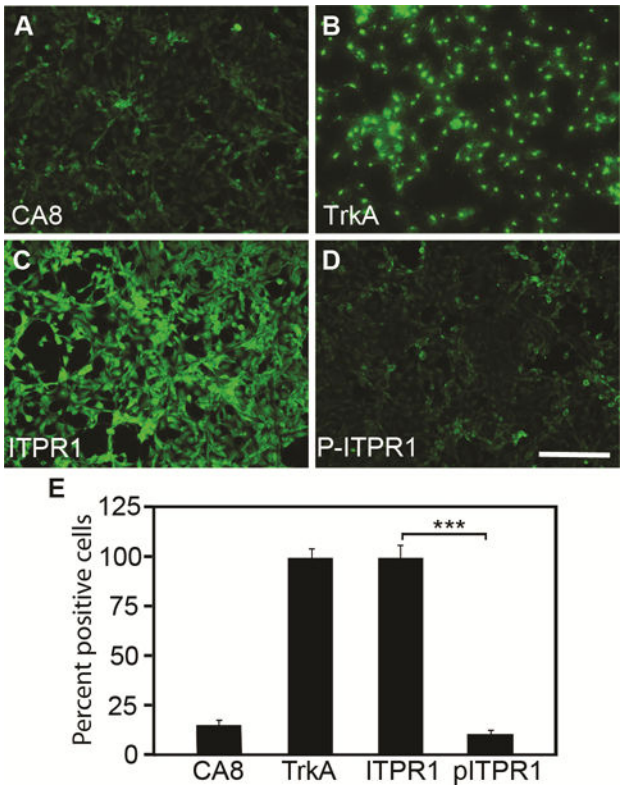

Figure 1. Endogenous expression of CA8, TrkA, ITPR1 and pITPR1 in cell lines

Baseline expression of CA8, TrkA, ITPR1, and pITPR1 are shown for NBL cells using immunohistochemistry (A-D). Quantitation (E) was relative to DAPI staining, which was used to normalize positive staining cells. We observed low levels of CA8 and pITPR1 relative to IPTR1. N=4 from 2 independent cultures in duplicate. Scale bar: 100 μM. (Error bars are SEM; ***P<0.001; Student's *t*-test).

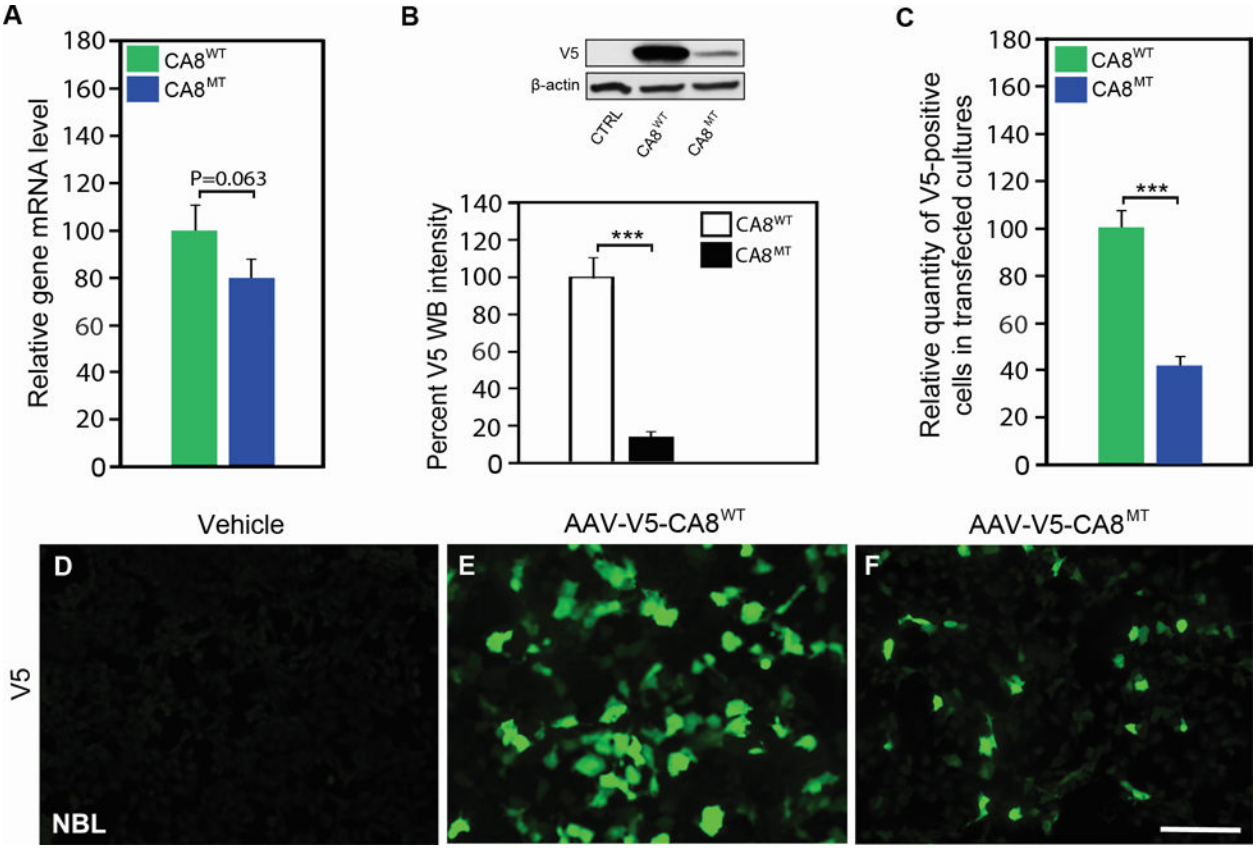

Figure 2. Expression of V5-CA8^{WT} and V5-CA8^{MT} in NBL cell culture

NBL cultures were untransfected, or transfected with *AAV-V5-CA8^{WT}* or *AAV-V5-CA8^{MT}* encoding vectors expressing CA8 fused to V5 tag as wildtype or the S100P point mutation. There was no difference in expression of *CA8^{WT}* as compared to *CA8^{MT}* as measured by qPCR (A). In contrast, there was little detectable CA8^{MT} protein detected in comparison to CA8^{WT} by western blotting (B). Immunofluorescence data (C, D-F) corroborates our western data showing more positive V5 staining NBL cells after transfection with CA8^{WT} (E) as compared with the CA8^{MT} (F) or untransfected NBL cells (D). N=4 from 2 independent cultures in duplicate. Scale bar: 100 μM. (Error bars are SEM; ***P<0.001; Student's *t*-test).

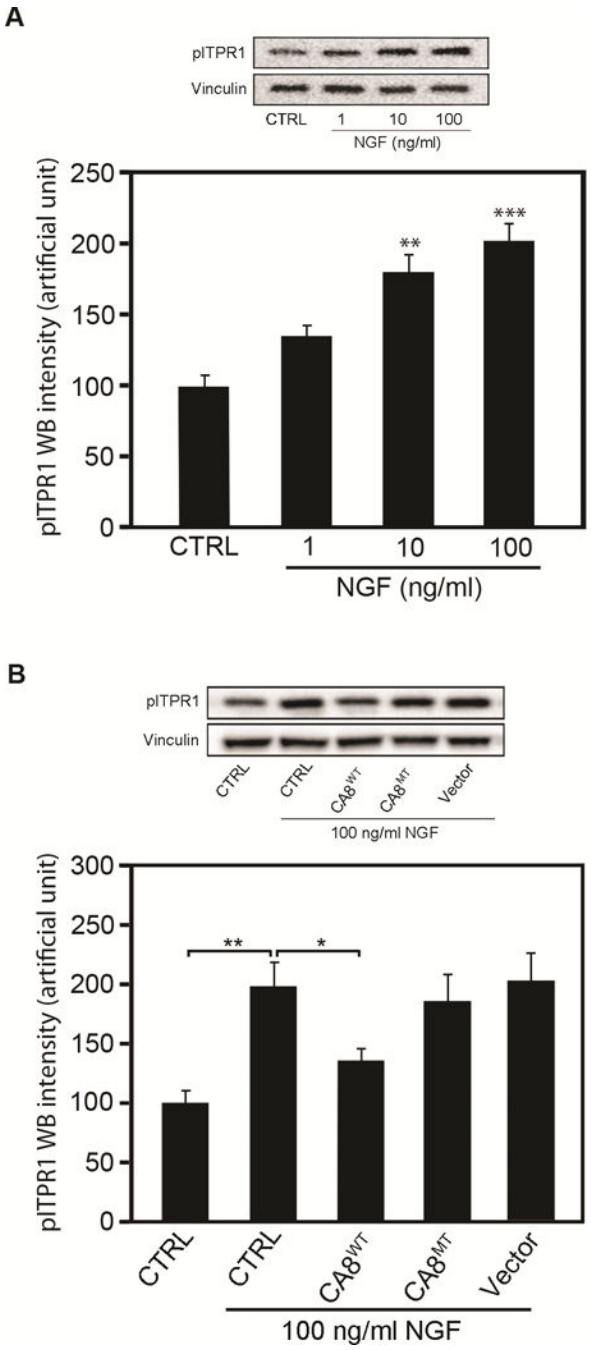

Figure 3. CA8^{WT} and CA8^{MT} Overexpression in cell cultures inhibit NGF-induced ITPR1 phosphorylation (pITPR1)

NBL cells were transfected with *AAV8-V5-CA8^{WT}* or *AAV8-V5-CA8^{MT}* encoding viral particles. The cultures were collected 48 h after transfection for western blots. Western blotting analyses of pITPR1 demonstrate dose-dependent NGF increase in pITPR1 levels in NBL cultures (A). Overexpression of V5-CA8^{WT} protein significantly reduced NGF-induced pITPR1 increases in NBL cultures (B) but transfection with vectors overexpressing V5-CA8^{MT} failed to effect pITPR1 levels (B). N=6 from 2 independent cultures in triplicate.

(Error bars are SEM; *P<0.05; **P<0.01; ***P<0.001; Student's *t*-test or one-way ANOVA.)

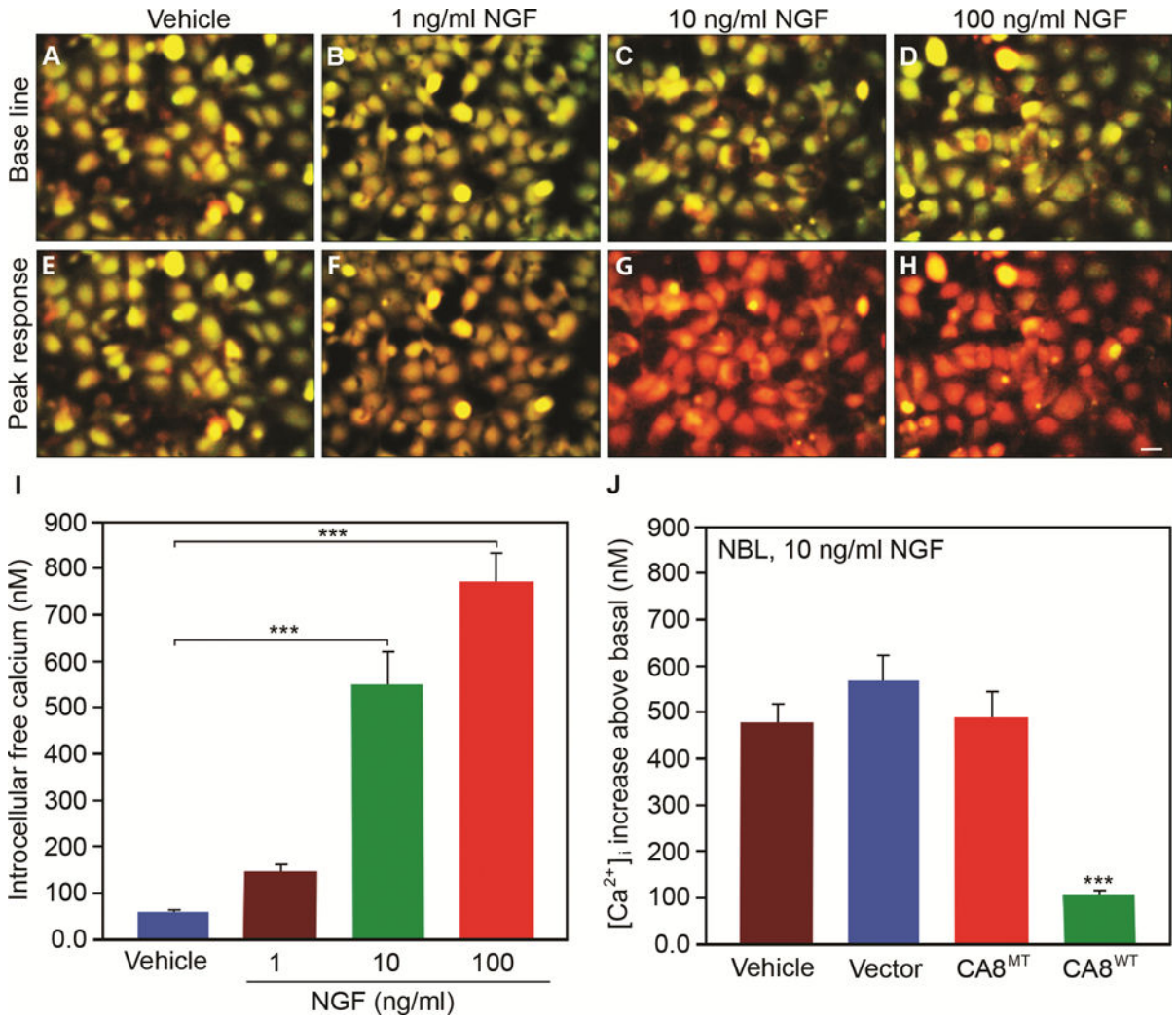

Figure 4. NGF induces release of cytoplasmic free calcium in a dose-dependent manner and inhibition by CA8^{WT} overexpression *in vitro*

NGF induced intracellular free calcium release as shown by fura-2 labeling in representative NBL cell cultures in a dose-dependent manner from 1ng/mL to 100 ng/mL as compared with vehicle control (A-I). Panels E-H show the peak response for each condition. Panel J shows NBL cells transfected with *V5-CA8^{WT}* encoding vector inhibited NGF-induced (10 ng/mL) cytoplasmic free calcium release (green bar), but *V5-CA8^{MT}* encoding vector, empty vector and vehicle could not. N=9 from 3 independent cultures in triplicate. Scale bar: 50 μM. (Error bars are SEM; ***P<0.001; One-way ANOVA.)

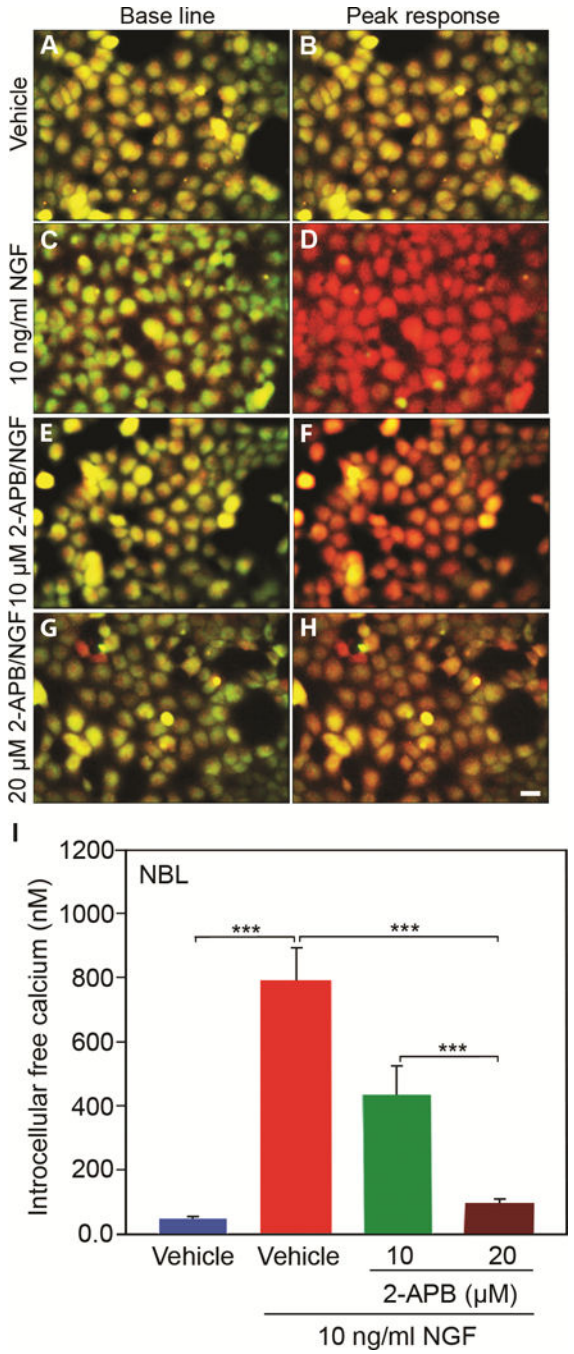

Figure 5. NGF induced calcium release is ITPR1-dependent in NBL cells

NGF (10ng/mL) stimulated intracellular calcium release was inhibited in a dose-dependent manner by ITPR1 selective inhibitor 2-APB in NBL cells. N=9 from 3 independent cultures in triplicate. Scale bar: 50 μM. (Error bars are SEM; ***P<0.001; One-way ANOVA.)

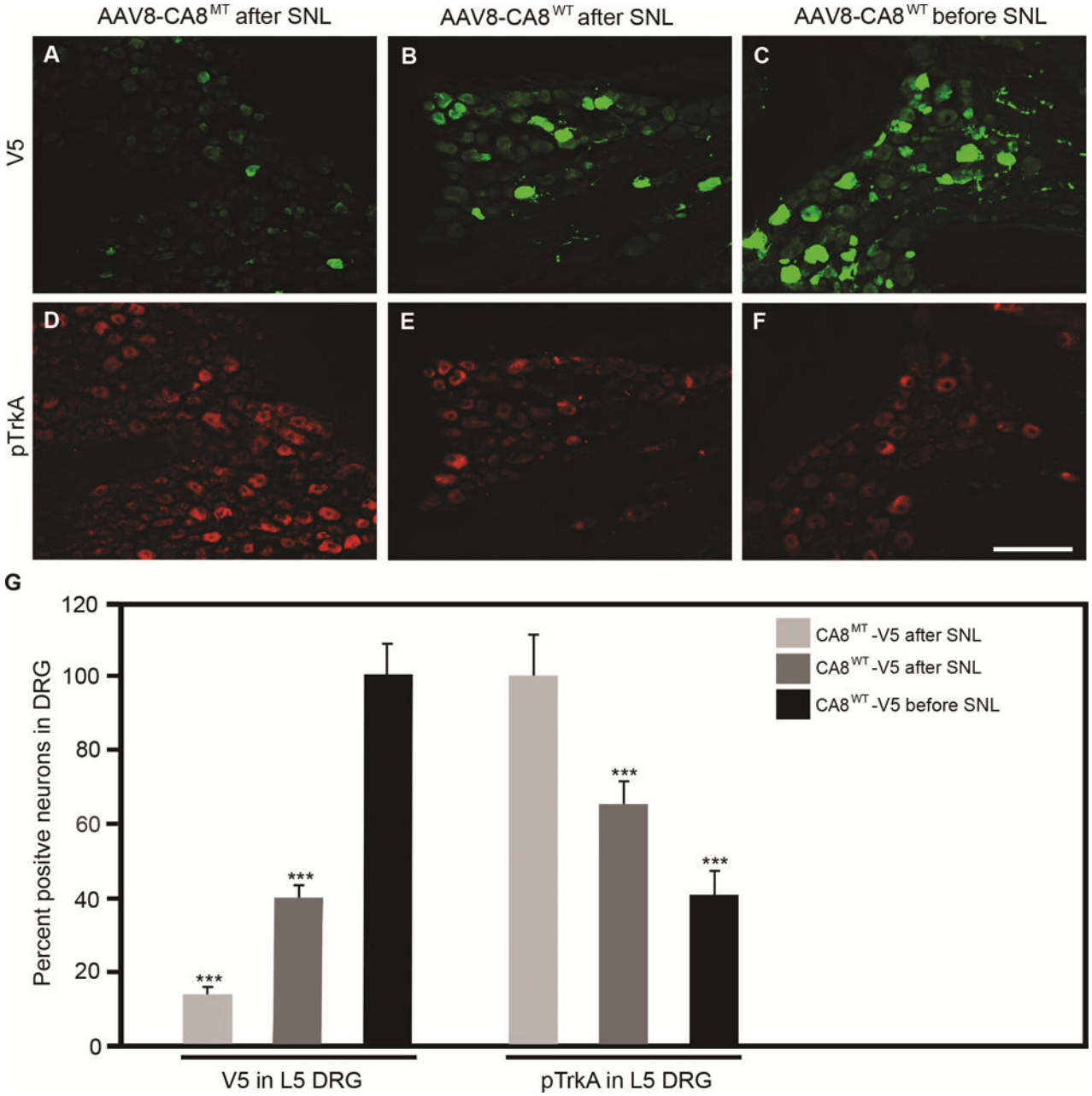

Figure 6. AAV8-mediated V5-CA8 gene transfer via sciatic nerve injection transduces L5 DRG and inhibits spinal nerve ligation (SNL)-induced TrkA phosphorylation

AAV8-V5-CA8^{WT} and *AAV8-V5-CA8*^{MT} viral particles (1.5μl, 2.65E+14 genome copies/ml) were injected into the sciatic nerves 3 days either before, or after SNL. L5 DRG were harvested on D21 after SNL and immunohistochemistry (IHC) was used to measure V5-CA8^{WT}, V5-CA8^{MT} (A-C) and pTrkA (D-F). SNL L5 DRG V5 IHC was increased significantly at the end of the experiment with SN injection before and after SNL with *AAV8-V5-CA8*^{WT}, but not after SN injection with *AAV8-V5-CA8*^{MT} viral particles. There was a significant reduction in pTrkA IHC detected with SN injection before and after SNL with *AAV8-V5-CA8*^{WT}, but not after SN injection with *AAV8-V5-CA8*^{MT}. Quantitation

was relative to DAPI staining which was used to normalize positive staining cells. N=4. Scale bar: 100 μM. (Error bars are SEM; ***P<0.001; One-way ANOVA.)

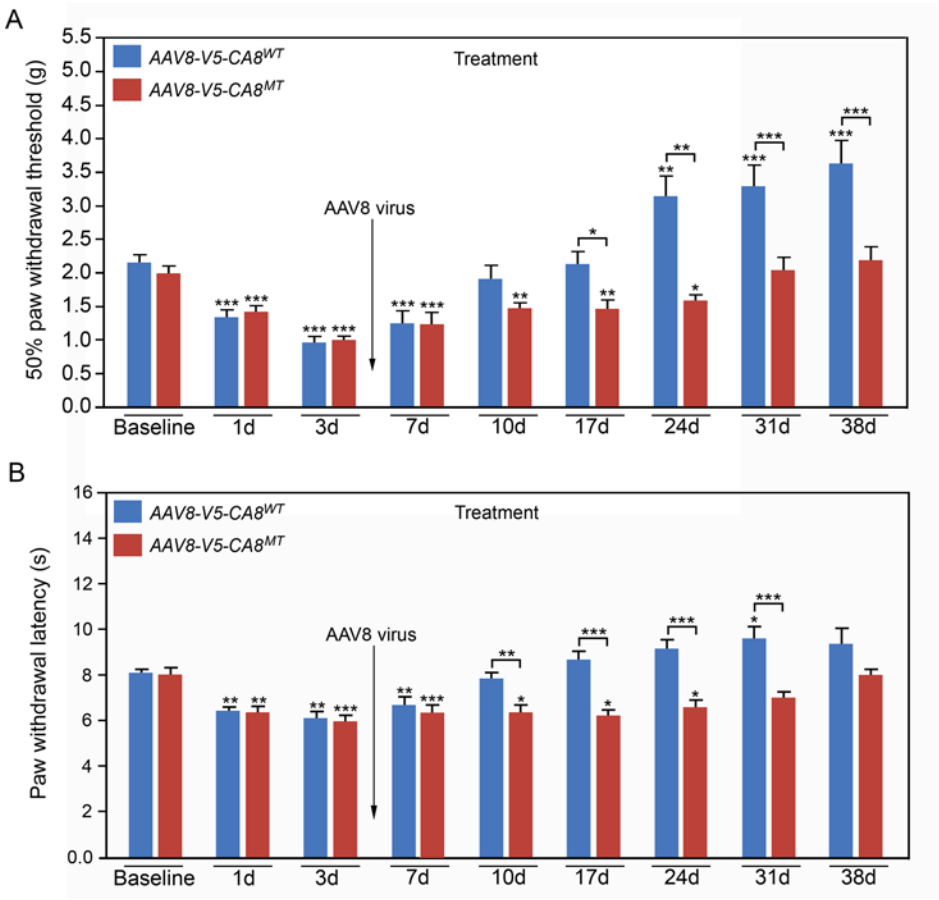

Figure 7. AAV8-mediated V5-CA8 gene transfer via sciatic nerve injection treats chronic neuropathic pain associated with the SNL model

AAV8-V5-CA8^{WT} and *AAV8-V5-CA8*^{MT} encoding viral particles (1.5μl, 2.65E+14 genome copies/mL) were injected into the sciatic nerves 3 days after SNL. Mechanical withdrawal thresholds (A) were tested by von Frey fibers and thermal paw withdrawal latencies (B) were tested using the Hargreaves method. *AAV8-V5-CA8*^{WT} inhibited SNL-induced hyperalgesia starting at Day 10 and beyond after virus injection for both mechanical (A) and thermal behaviors (B); but *AAV8-V5-CA8*^{MT} did not. N = 8. (Error bars are SEM; *P<0.05; **P<0.01; ***P<0.001 by two-way repeated-measures ANOVA in SPSS)