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Car8 dorsal root ganglion expression and genetic regulation of analgesic responses are associated with a cis-eQTL in mice

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Abstract

Carbonic anhydrase-8 (*Car8* mouse gene symbol) is devoid of enzymatic activity, but instead functions as an allosteric inhibitor of inositol trisphosphate receptor-1 (ITPR1) to regulate this intracellular calcium release channel important in synaptic functions and neuronal excitability. Causative mutations in *ITPR1* and carbonic anhydrase-8 in mice and humans are associated with certain subtypes of spinal cerebellar ataxia (SCA). SCA mice are genetically deficient in dorsal root ganglia (DRG) Car8 expression and display mechanical and thermal hypersensitivity and susceptibility to subacute and chronic inflammatory pain behaviors in mice. In this report we show that DRG *Car8* expression is variable across 25 naive-inbred strains of mice, and this cis-regulated eQTL (association between rs27660559, rs27706398, and rs27688767 and DRG *Car8* expression; $P<1\times10^{-11}$) is correlated with nociceptive responses in mice. Next, we hypothesized that increasing DRG *Car8* gene expression would inhibit intracellular calcium release required for morphine antinociception and might correlate with antinociceptive sensitivity of morphine and perhaps other analgesic agents. We show that mean DRG *Car8* gene expression is directly related

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Authors' contributions: TW designed and ran the DRG eQTL studies and data analyses. GYZ, YK and MO ran the NBL studies on morphine response and Car8 antagonism. LD, WM, SBS, ESF, KDS, DME, GYZ and UU assisted with manuscript preparation, provided critical scientific review, revisions, and editing. ERM contributed to data analyses, reviewed and revised the manuscript. RCL designed the overall study, supervised the studies, setup collaborations, analyzed data, and wrote the manuscript.

to the dose of morphine or clonidine needed to provide a half-maximal analgesic response (r=0.93, P<0.00002; r=0.83, P<0.0008, respectively), suggesting that greater DRG *Car8* expression increases analgesic requirements. Finally, we show that morphine induces intracellular free calcium release using Fura 2 calcium imaging in a dose-dependent manner; and *V5-Car8*^{WT} overexpression in NBL cells inhibits morphine-induced calcium increase. These findings highlight the 'morphine paradox' whereby morphine provides antinociception by increasing intracellular free calcium, while Car8 and other antinociceptive agents work by decreasing intracellular free calcium. This is the first study demonstrating that biologic variability associated with this cis-eQTL may contribute to differing analgesic responses through altered regulation of ITPRI-dependent calcium release in mice.

Keywords

Carbonic Anhydrase-8; eQTL Mapping; Pain Genetics; Dorsal Root Ganglia; Analgesic Response; Antinociception; opioid analgesia; intracellular calcium regulation

Unlike most other members of the carbonic anhydrase super gene family, carbonic anhydrase-8 (Car8) lacks enzymatic activity to hydrate CO_2.(Sjoblom et al. 1996) Instead Car8 functions to lower the affinity of inositol 1,4,5-trisphosphate receptor type-1 (ITPR1) for its inositol 1,4,5-trisphosphate (IP3) ligand, inhibiting ITPR1 phosphorylation, channel activation and intracellular calcium release.(Zhuang GZ 2014) Car8 thereby modulates calcium release, calcium signaling and neuronal excitability.(Hirasawa et al. 2007; Hirota et al. 2003) Importantly, phospholipid signaling has recently been implicated in the regulation of nociception,(Neely et al. 2012) consistent with the potential importance of this candidate gene in pain perception associated with its regulation of ITPR1.(Komalavilas and Lincoln 1994; Luo et al. 2012; Wagner et al. 2003)

Spinal Cerebellar Ataxia (SCA) is a rare genetic disorder frequently associated with debilitating symptoms. Causative mutations in *ITPR1* and *Car8* are both associated with SCA disorders in mice and humans.(Jiao et al. 2005; Marelli et al. 2011; Turkmen et al. 2009) Using health related quality of life instruments (EQ-5D, and PHQ) in 526 SCA patients from a European multicenter study at 17 institutions, Schmitz-Hubsch et al., reported problems in mobility (86.9% of patients), usual activities (68%), pain/discomfort (49.4%), depression/anxiety (46.4%), and self-care (38.2%).(Schmitz-Hubsch et al. 2010) Because these human data link this pathway to pain and its comorbidities including anxiety, depression and difficulties with self-care, we tested the hypotheses that this candidate gene is variably expressed in the DRG, which underlies differences in nociceptive behaviors and analgesic responses in mice.

We have previously shown SCA mice, which are Car8 null mutants, demonstrate thermal and mechanical hypersensitivity at baseline; and are susceptible to inflammatory pain. (Zhuang et al. 2015) We further demonstrated that overexpression of Car8 in nociceptors after gene transfer in SCA mice down regulates ITPR1 activation (Ser-1755 phosphorylation, pITRP1), decreases steady-state cytoplasmic free calcium, inhibits ATP-stimulated calcium release, and abolishes mechanical allodynia and thermal hyperalgesia. In addition, we demonstrated inflammation-induced hyperalgesia and a relative reduction of

Car8 protein to ITPR1 activation (phosphorylation) as a potential mechanism of inflammatory pain that was reversed by overexpression of the Car8 wildtype protein in mice. These discoveries established a critical role for Car8 in nociception and subacute and chronic inflammatory pain-related behaviors in mice.(Zhuang et al. 2015)

Herein, using dorsal root ganglion (DRG) microarray analyses from 25 naïve inbred strains of mice, we show that *Car8* represents a cis-regulated eQTL on chromosome 4 and DRG *Car8* expression is correlated with nociceptive thermal responses across inbred strains. We further show that the relationship between *Car8* expression and half-maximal analgesic responses of (morphine and clonidine), are strongly correlated with *Car8* DRG gene expression in 11 inbred strains ($P<0.001$). Finally, we show the dose-response to morphine-induced calcium release in NBL cells is shifted to the right by Car8 overexpression. These discoveries represent an important, newly revealed variably expressed gene and pathway that underlies clinically relevant phenotypic differences pain behaviors and analgesic responses in mice.

RESULTS

Carbonic Anhydrase 8 (Car8) is a pain susceptibility gene that is cis-regulated in mouse DRG

Association analyses were used to identify loci controlling variable *Car8* gene expression in the lumbar DRG supplying the sciatic nerve.(Su et al. 2004; Wu et al. 2008a) Using the efficient mixed model algorithm to generate association analyses of microarray data from 25 inbred strains (Figure 1, top panel), we identified *Car8* as an eQTL ($P<1\times10^{-11}$) (Figure 1, middle panel). This eQTL was localized to the *Car8 locus* on chromosome 4 of the mouse, suggesting variants in the vicinity of the *Car8* locus regulate DRG expression of this gene (cis-regulation) (Figure 1, bottom panel). We further demonstrate that *Car8* gene expression correlated with withdrawal latency to noxious thermal stimulation.(Grubb et al. 2014; Mogil et al. 1999) Cis-regulatory elements (e.g., enhancers, gene promoters, etc.) control physiology and development and are localized to the region of the gene they regulate. Variants that affect the functions of these regulatory DNA sequences are important contributor to phenotypic diversity within and between species and help explain genetic mechanisms of phenotypic diversity.(Wittkopp and Kalay 2012)

The significance of variable DRG Car8 expression on pain behaviors and analgesic responses

To characterize the relationship between *Car8* DRG expression and analgesic response, we assessed mean DRG *Car8* expression using 11 inbred strains of mice previously characterized for analgesic response to multiple agents, as shown in Table 1. We found very strong correlations between mean DRG *Car8* expression levels and the dose of analgesic to provide half-maximal thermal antinociception in genetic mouse strains.(Wilson et al. 2003) In this analysis, three analgesics were compared, which include morphine, clonidine (alpha 2 agonist) and u50,488 (mixed opioid receptor agonist).

Male DRG *Car8* expression most tightly correlated with the dose of morphine required to produce a half-maximal antinociceptive response in these same inbred strains (r=0.93, P<0.00002). This correlative relationship suggests that higher DRG *Car8* expression is associated with higher dose requirements of each analgesic in mg/kg to produce the half-maximal antinociception. This finding corroborates our hypothesis that increasing DRG *Car8* expression would correlate with the antinociceptive sensitivity of morphine and perhaps other analgesics due to decreased ITPR1-mediated calcium release. A similar relationship was demonstrated for half-maximal antinociception for clonidine (r=0.83, P<0.0008), suggesting male DRG *Car8* expression also increases clonidine analgesic requirements. As shown previously, these effects are likely to occur through Car8 inhibition of neuronal ITPR1 activation by phosphorylation at Ser-1755 (pITPR1), important to calcium channel activation resulting in reduction of cytoplasmic free calcium release critical to neuronal excitability and the analgesic actions of these agents (Aoki et al. 2003; Zhuang et al. 2015).

Car8 overexpression inhibits morphine-induced calcium release in NBL cells

We overexpressed the wildtype or mutant *Car8* transcripts to assess the impact on morphine-induced calcium release in NBL cells. Calcium imaging was performed one day after NBL cultures were transfected with V5-tagged wildtype transcript (*AAV8-V5-Car8*^{WT}) or mutant transcript (*AAV8-V5-Car8*^{MT}), which is devoid of Car8 due to rapid degradation.(Jiao et al. 2005; Marelli et al. 2011; Turkmen et al. 2009) Morphine dose-response data show the EC_{50} is approximately 9 micromolar (Figure 2A). The baseline of intracellular free calcium i[Ca^{2+}] was 78 nM. Morphine (10 micromolar) was then used to stimulate NBL cultures infected with *AAV8-V5-Car8*^{WT} (*Car8*^{WT}), *AAV8-V5-Car8*^{MT} (*Car8*^{MT}) or empty vector (Figure 2B). *Car8*^{WT} was able to attenuate morphine-induced calcium release in NBL cells, but not *Car8*^{MT} or empty vector. Immunohistochemistry shows 41% of DAPI positive NBL cells are also V5 positive (Figure 3A–C) after *Car8*^{WT} *infection*, and only 8.9% of DAPI positive NBL cells are V5 positive after *Car8*^{MT} infection (Figure 3D–F). Western blotting confirms robust endogenous mu opioid receptor expression in NBL cells (Figure 3G). These data demonstrate that Car8 overexpression is able to inhibit morphine-induced calcium release in NBL cells consistent with the mechanism of decrease analgesic (morphine and clonidine) response.

DISCUSSION

eQTL regulation of Car8 and analgesic responses

We identified a cis-regulated eQTL for *Car8* in mice correlated with nociceptive behavior. Cis-regulatory elements (e.g., enhancers, gene promoters, etc.) control physiology and development and are localized to the region of the gene they regulate. Based on known functions of Car8 described in our previous animal studies,[4] and in these eQTL studies disclosed herein; we show (1) that DRG *Car8* expression correlates with analgesic responses of morphine and clonidine (Figure 1); (2) the dose required to produce half-maximal antinociceptive responses across inbred strains of mice for these analgesics is increased with increased DRG *Car8* expression (Table 1); (3) morphine induces calcium release in vitro in a dose-dependent manner (Figure 2A); and (4) Car8 overexpression in NBL cells inhibits

morphine-induced calcium release (Figure 2B and Figure 3). These data suggest for the first time overlapping and opposing mechanistic actions, specifically that increased *Car8* expression antagonizes analgesic actions of both mu opioid and α2-receptor agonists. Nonetheless, as shown previously, increasing *Car8* expression provides analgesia via the inhibition of ITPR1 phosphorylation and the reduction of intracellular calcium release by allosteric inhibition of IP3 binding to ITPR1. (Zhuang et al. 2015)

Wilson et al., showed high correlations in strain sensitivity to antinociception with various analgesics including morphine, clonidine, and k-opioid receptor agonist U50,488), thereby suggesting common genetic mechanisms alter signal transduction of G-protein coupled receptors (GPCR's).(Wilson et al. 2003) As shown in Table 1, *Car8* DRG expression correlated with the amount of morphine, U50,488 (k-opioid agonist) and clonidine to produce half-maximal antinociception, signifying that higher *Car8* expression is associated with higher dose requirements of each of these analgesic molecules. These findings suggest a negative correlative relationship with morphine antinociceptive sensitivity, perhaps based on opposing effects of these molecules on intracellular calcium regulation by ITPR1. Since increased dosage of morphine and clonidine are needed to produce the same degree of antinociception, analgesic treatment with the $Car8^{WT}$ this treatment should not only produce analgesia,(Zhuang et al. 2015) but also antagonize calcium release and antinociception by analgesic molecules.

Mechanisms of Car8 regulation of analgesic responses

Interestingly, morphine, a μ-opioid agonist exerts analgesia through the IP3 mediated release of intracellular calcium,(Aoki et al. 2003) thereby corroborating our hypothesis of opposing effects between *Car8* expression and morphine on IP3 mediated calcium release. Unexpectedly, drugs targeting al-receptors, but not α2-receptors, are linked to PLC/IP3/PKC signaling and intracellular calcium release and should be antagonized by Car8 inhibition of ITPR1-mediated calcium release.(Kreydiyyeh et al. 2000; Zhang et al. 2006) These effects are likely due to α2-receptor activation and IP3-mediated activation of ITPR1 and intracellular calcium release. This should be testable by assessing the actions of Car8 on α1-receptor agonists. In contrast, drugs targeting α2-receptors stimulate Na+-K+ ATPase in isolated rat jejunal, but not villus cells.(Kreydiyyeh et al. 2000) The functions of neuronal Na+-K+ ATPase are complex. Brain Na+-K+ ATPase are thought to regulate electrochemical gradients important in membrane potential and the generation of action potentials, and the uptake and release of neurotransmitters.(Zhang et al. 2006) Moreover, Na+-K+ ATPase acts as a signal transducer through direct interactions with ITPR1 via a highly conserved N-terminal binding motif, triggering calcium release.(Zhang et al. 2006) Based on our findings, we anticipate that Car8 may antagonize α2-receptor agonist mediated ITPR1-dependent intracellular calcium release via the direct actions of Na+-K+ ATPase on ITPR1, thereby antagonizing the analgesic actions of clonidine and potentially other α2-receptor analgesics.

Study Limitations

One limitation to this study is that we could only assess the relationship between mean DRG *Car8* expression and half-maximal analgesic responses from male mice.(Wilson et al. 2003)

The role of sex is an important variable in pain perception and behaviors. In this study, males were more sensitive to drug-induced antinociception than females.(Wilson et al. 2003) Additionally, Wilson showed a significant main effect of sex only for clonidine antinociception. The prevalence of female patients seeking treatment for chronic pain is greater than 80%, however the explanations and mechanisms of this phenomenon are controversial.(Mogil 2012) Possible biological explanations include the role of estrogen receptors regulating opioid receptors and the effects of estrogen on PKC signaling on nociceptive neurons.(Hucho et al. 2006; Liu et al. 2011) While many researchers have removed the variable of sex by studying only male rodents, it appears that future translation would benefit from defining those specific biologic pathways important in sex differences in pain behaviors and analgesic responses.(Mogil 2012)

Conclusions

Herein, we show for the first time in naive mice that lumbar DRG *Car8* expression is variable, and that a cis-eQTL is associated with this variable DRG *Car8* expression. Moreover, Car8 regulates morphine-induced ITPR1-mediated calcium release important to nociceptor responses. Furthermore, DRG *Car8* expression is tightly correlated with thermal nociceptive behaviors and analgesic responses to morphine and clonidine. Finally, we show that morphine induced intracellular free calcium increases in a dose-dependent manner; and $Car8^{WT}$ protein overexpression in NBL cells inhibits calcium release by morphine as the potential mechanism of Car8 regulation of analgesic responses.

MATERIALS AND METHODS

eQTL methods

All animal experiments were performed after the appropriate institutional committee approved the experiments and all experiments conformed to the relevant international, national, and/or institutional guidelines regulatory standards. Male 8-week-old mice from 25 inbred strains were purchased from the Jackson Laboratory and habituated for one week prior to tissue collections. The twenty-five inbred strains of mice used were AKR/J; BALB/cByJ; 129S1SvlmJ; BTBRT+tf/J; SJL/J; C57BL/6J; NZW/LacJ; BUB/BnJ; CBA/J; KK/HIJ; SM/J; PL/J; C58/J; RIIIS/J; NZO/HILtJ; DBA/2J; C3H/HeJ; NOD/LtJ; C57BR/cdJ; FVB/NJ; P/J; MRL/MpJ; SWR/J; A/J; and NON/LtJ. All dissections and gene expression experiments were conducted as reported elsewhere.(Simonetti et al. 2013; Su et al. 2004; Wu et al. 2008b) Lumbar DRG were collected (3 lumbar DRG from each side innervating the sciatic nerves in 3 animals and pooled) after sacrifice by cervical dislocation without anesthesia avoiding anesthetic effects on gene expression. RNA was purified, amplified and labeled using the Affymetrix one-cycle target labeling kit. Samples were hybridized to Affymetrix GeneChip 430v2 whole-genome mouse arrays and data were processed using the gcRMA algorithm.(Wu et al. 2008a) For mouse the accession ID is NCBI Taxon ID 10090). To identify cis-regulated genes the EMMA algorithm(Kang et al. 2008) was applied in the R statistical package to gene expression data from 25 inbred strains that exhibited greater than 3-fold difference of expression between all strains. Known and imputed dense SNP data were downloaded from http://phenome.iax.org/db/q?rtn=snps/download and http://compgen.unc.edu/wp/ and pooled. *Car8* gene expression values (probe set ID 1427482_a_at)

were correlated to three nociceptive response data sets in the Jackson Laboratory Mouse (MPD) MPD: 48001, MPD: 2204, and MPD: 2201 using the Spearman's rank correlation test built into the R statistical package.

Relationship between analgesic responses and relative DRG Car8 expression

We tested the hypothesis that increasing mean DRG *Car8* gene expression values (probe set ID 1427482_a_at) would inhibit analgesic response and that there would be a direct correlation between mean *Car8* DRG expression and half-maximal analgesic responses in eleven inbred strains of male mice.(Wilson et al. 2003) Pearson correlation coefficients were calculated using Excel and P-values calculated using a one-tailed Student's t-test.

Calcium assay methods

On assay days, cells were incubated with culture media containing 1 μM Fura-2/AM (Molecular Probes) and 0.012% pluronic F-127 (Sigma) for 25 min at 37°C and washed twice with culture media. After another 25 min incubation in culture media at 37 °C, coverslips were transferred to a RC-42 LP open bath chamber in the QE-1 platform with buffer 1 (all concentrations in mM): 130 NaCl; 4.7 KCl; 1.3 CaCl2, 1 MgSO4; 5 Glucose; 20 HEPES; 10 EGTA; 1.2 KH2PO4, loaded onto a Leica DMI 6000 B inverted microscope and perfused with Ca^{2+} free buffer (Buffer 2, all concentrations in mM: 130 NaCl; 4.7 KCl; 2.3 MgSO4; 5 Glucose; 20 HEPES; 10 EGTA; 1.2 KH2PO4).[Boehmerle, 2007, Chronic exposure to paclitaxel diminishes phosphoinositide signaling by calpain-mediated neuronal calcium sensor-1 degradation] Cells were allowed to equilibrate for 1–2 min before recording on Leica Application Suite 1.1.0.12420 (Leica, Germany) was initiated. Cells were visualized every 2 s for 3 – 5 min while buffer 2 alone or buffer 2 containing morphine at 0.5, 1, 5, 10, 50, 100 or 500 μM were perfused onto cells. A Leica High Speed Filter Changer with 340 nm and 380 nm filters were used to produce dual excitation wavelengths for fura-2. Fluorescence emission images at 510 nm were collected with a Leica HCX PL FLUOTAR L 40×/0.6 DRY objective and a DFC 365FX Digital Camera System. In each field, 25–35 cells were randomly circled for measurement of F340/F380. The background was assessed at a location nearby cells and the ratio of F340/F380 was close to 1. Background fluorescence of F340 and F380 measured with buffer 2 alone was subtracted before calculating the F340/F380 ratio. Each group was composed of 5-to-9 coverslip from at least 2 different cultures. Three consistent points of peak or baseline values of F340/F380 from a special coverslip were collected and averaged as one sample (N=1). The ratio of F340/F380 was converted to free intracellular calcium concentration $[Ca^{2+}]i$ by the formula: $[Ca^{2+}]=Kd*(R-Rmin)/(Rmax-R)*$ F380max/F380min.(Grynkiewicz et al. 1985) where Kd is 224nM, R is the measured as the ratio of F340/F380 nm; Rmin and Rmax are the ratios in absence of Ca^{2+} or when Fura-2 is saturated by Ca^{2+}, and was determined by incubating cells in zero Ca^{2+} recording buffer with 0.2 mM EGTA and 8uM ionomycin; or treating cells with 8uM ionomycin in recording buffer containing 10 mM Ca^{2+}. F380max and F380min are the fluorescence intensity of 380nm excitation at zero Ca^{2+} and saturated Ca^{2+}. Data were presented as mean ± SEM. Differences between groups were compared using or ANOVA, followed by Fisher's PLSD test. The criterion for statistical significance was $P<0.05$.

Immunocytochemistry imaging

The immunostaining was performed as described previously (Zhuang et al., 2010). Briefly, 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 2% normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA) for 20 min. Primary antibodies specific for V5 (Abcam, Cambridge, MA) were diluted in PBS containing 0.1% Triton X-100 and allowed to incubate cell cultures overnight at 4°C. Cultures were washed three times for 10 min each in PBS, and incubated with Alexa Fluor 488-conjugated second antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1h at room temperature. Cultures were washed again in PBS and coverslips with stained cells face down to mount on slides using Gel/Mount anti-fading mounting media (Biomeda, Foster City, CA). DAPI (Sigma) was mixed with 2nd antibody for total cells number counting. Images were acquired using an inverted microscope (DMI 6000B, Leica, Germany). Gamma, gain and exposure levels were set for control sections and kept constant for all other culture coverslips within an experiment.

Western blotting

NBL 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. The blots were blocked with 5% milk in PBS with 0.1% Tween 20 for 1h at room temperature and incubated overnight at 4°C with primary antibody. The blots were then incubated for 1 hr at room temperature with HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and bands were visualized using Pierce SuperSignal substrate (Thermal Scientific, Rockford, IL). The anti-mu opioid receptor (Sigma) primary antibody was used.

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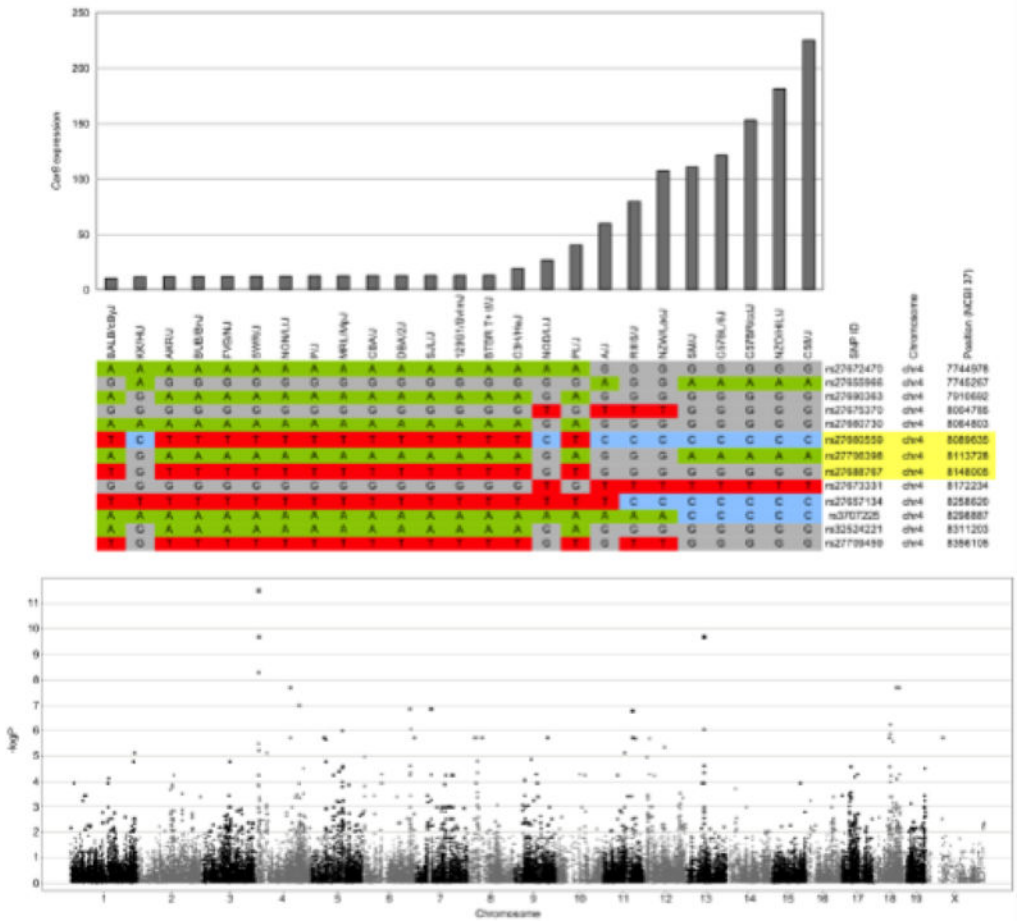

Figure 1. Relative DRG expression, and eQTL analysis of *Car8*, as a function of inbred strain

(A) The relative expression of *Car8* is plotted (grey bars) for each inbred strain along with haplotypes in the region covering *Car8*. (B) The haplotypes for thirteen variable single nucleotide polymorphisms (SNPs) are indicated by their allele, SNP ID, chromosomal location, and colored to indicate haplotype groupings. The *Car8* locus is shown in yellow. (C) Genome wide association analysis of DRG mRNA gene expression patterns (e-QTL analysis) reveals a highly significant locus on Chr4 (−logP value=11.5) in the Manhattan plot. The locus marked by SNP rs27660559, delineates a strong association between gene expression pattern and the genomic locus for *Car8* indicating cis-regulation of gene expression. Alternating black and grey bands denote each chromosome number.

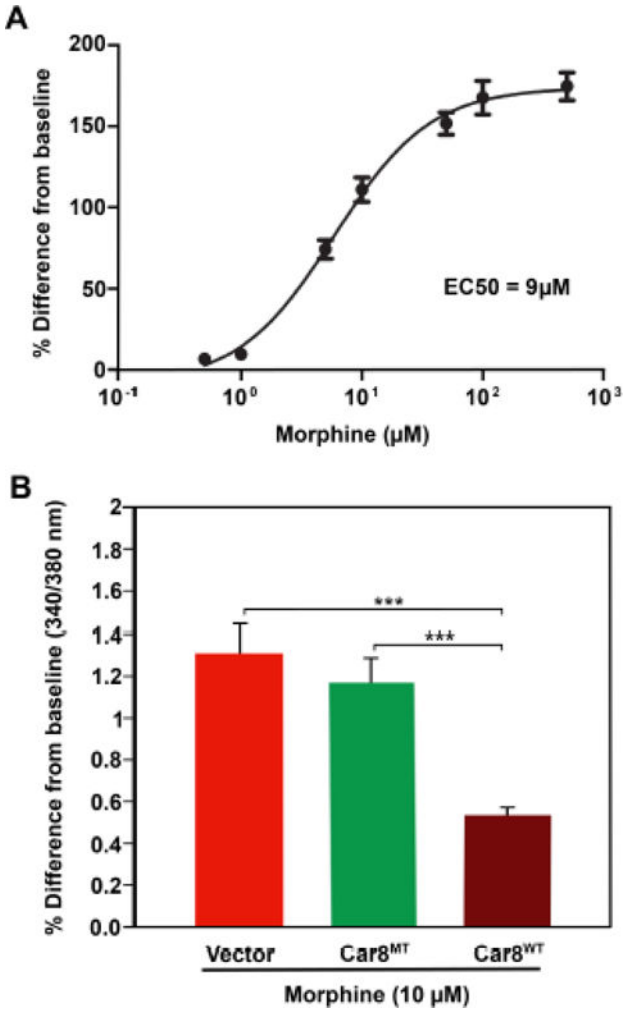

Figure 2. Morphine-induced calcium release in NBL cells is attenuated by Car8 overexpression

Morphine induces increased intracellular free calcium in NBL cells. Intracellular free calcium was analyzed using Fura 2 imaging. (A) Morphine induces increase of intracellular free calcium denoted on the Y-axis as % difference from baseline in a dose-response manner denoted on the X-axis as morphine dose (micromolar). The EC_{50} to morphine was approximately 9micromolar. (B) The response to 10 micromolar morphine was attenuated after NBL cells were infected with *AAV8-V5-Car8*^{WT}, but not after infection with *AAV8-V5-Car8*^{MT} or empty vector. N=6 from 2 independent cultures. ***P<0.001; ANOVA.

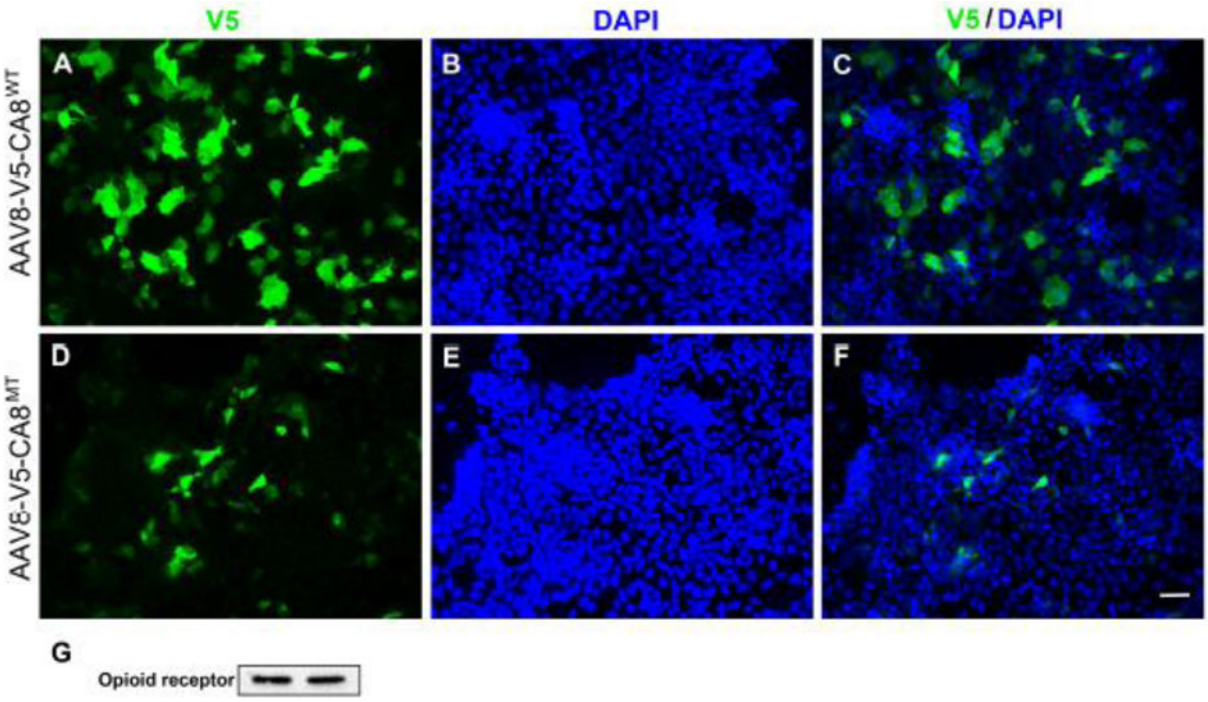

Figure 3. Immunocytochemistry and western blotting confirm Car8 and mu opioid receptor expression in NBL cells

Immunocytochemistry demonstrates levels of V5-tagged Car8 after infection with *AAV8-V5-Car8*^{WT} (A–C), and *AAV8-V5-Car8*^{MT} (D–F). Western blotting shows endogenous mu opioid receptor expression in NBL cells that are responsive to morphine (G). Scale bar = 100 μm.

Table 1

Relationship between half-Maximal analgesic response and mean DRG *Car8* expression

dRg were dissected from male inbred strains: AKR/J, BALB/cByJ, 129S1SvlmJ, C57BL/6J, CBA/J, SM/J, C58/J. RIIIS/J, DBA/2J, C3H/HeJ, and A/J mice and processed for gene expression.(Wu et al. 2008b) *Car8* gene expression was measured and correlated with antinociceptive data on three analgesics as published.(Wilson et al. 2003) Correlations were made comparing mean DRG *Car8* expression with half-maximal analgesic responses (dose, in mg/kg).

Inbred Strains	Bas line Latency (seconds)	Morphine Antinociception* (mg/kg)	Clonidine Antinociception* (mg/kg)	U50.488 k-opioid Antinociception* (mg/kg)	CarB DRG Expression (1427482)
AKR/J	2 85	15.3	0.2	31.7	11.592
BALB/cByJ	3,64	16.8	0.27	22.9	9.858
129S1SvlmJ	3.17	10.5	0.16	24.5	12.526
C57BL/6J	2.12	23.3	1.53	54.6	120.81
CBA/J	2,29	13.8	0,4	50.7	12.301
SM/J	2.3	24.7	1.08	36.1	110.453
C58/J	2.56	38.5	1.31	56.7	224.934
RIIIS/J	3.17	13.7	1.21	25.1	79.177
DBA/2J	2.8	11	0.22	24.1	12.319
C3H/HeJ	2.4	11.1	0.31	43.9	19.065
A/J	3.16	14.2	0.2	27.1	59.462
Pearson	**−0.37**	**0.93**	**0.83**	**0.59**	
P-value	**NS**	**P<0.001**	**P<0.01**	**P<0.05**	

*Table 1 from Wilson et al., J Pharmacol Exp Ther 2003