Published in final edited form as:

Vet Anaesth Analg. 2017 March ; 44(2): 370–374. doi:10.1016/j.vaa.2016.05.003.

Relationship between the melanocortin-1 receptor (MC1R) variant R306ter and physiological responses to mechanical or thermal stimuli in Labrador Retriever dogs

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Abstract

Objective—Variants in the MC1R gene have been associated with red hair color and sensitivity to pain in humans. The study objective was to determine if a relationship exists between MC1R genotype and physiological thermal or mechanical nociceptive thresholds in Labrador Retriever dogs.

Study design—Prospective experimental study.

Animals—Thirty-four Labrador Retriever dogs were included in the study following public requests for volunteers. Owner consent was obtained and owners verified that their dog was apparently not experiencing pain and had not been treated for pain during the previous 14 days. The study was approved by the Institutional Animal Care and Use Committee.

Methods—Nociceptive thresholds were determined from a mean of three thermal and five mechanical replications using commercially available algometers. Each dog was genotyped for the previously described MC1R variant (R306ter). Data were analyzed using one-way ANOVA with *post hoc* comparisons using Tukey's test ($p < 0.05$).

Results—Thirteen dogs were homozygous wild-type (WT/WT), nine were heterozygous (WT/R306ter), and eight were homozygous variant (R306ter/R306ter) genotype. Four dogs could not be genotyped. A significant difference ($p = 0.04$) in mechanical nociceptive thresholds was identified between dogs with the WT/WT genotype (12.1 ± 2.1 N) and those with the WT/R306ter genotype (9.2 ± 2.4 N).

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Authors' contributions

TEP, KLM, SAG: study design, data collection, data analysis, manuscript preparation; NSB: data collection, data analysis, manuscript preparation; TLG, MHC: study design, data analysis, manuscript preparation.

Conclusion—A difference in mechanical, but not thermal, nociceptive threshold was observed between wild-type and heterozygous MC1R variants. Differences in nociceptive thresholds between homozygous R306ter variants and other genotypes for MC1R were not observed.

Clinical relevance—Compared with the wild-type MC1R genotype, nociceptive sensitivity to mechanical force in dogs with a single variant R306ter allele may be greater. However, in contrast to the reported association between homozygous MC1R variants (associated with red hair color) and nociception in humans, we found no evidence of a similar relationship in dogs with the homozygous variant genotype.

Keywords

dog; melanocortin-1 receptor; nociception

Introduction

Genetic variation of the melanocortin-1 receptor (MC1R) associated with red hair or yellow coat color has been shown to influence pain sensitivity in people and mice, respectively (Mogil et al. 2003). An increase in desflurane anesthetic concentration needed to prevent a response to noxious stimulation was demonstrated in women with MC1R variants as compared with women homozygous for the wild-type allele (Liem et al. 2004). In addition, MC1R variant genotypes have been associated with differences in sensation of both acute and inflammatory pain, but not neuropathic pain (Delaney et al. 2010). Homozygosity for a common loss-of-function mutation in the canine MC1R gene (R306ter, a premature stop codon substituted for arginine at amino acid 306) is strongly associated with yellow coat color in Labrador Retrievers, Golden Retrievers and other dog breeds (Everts et al. 2000). The objective of this study was to determine if MC1R genotypes among Labrador Retriever dogs are associated with differing sensitivities for responses to mechanical or thermal stimuli. We hypothesized that homozygous variant MC1R genotyped dogs would demonstrate greater sensitivity than wild-type dogs to mechanical and thermal stimuli.

Materials and methods

Recruitment of dogs

This study protocol was approved by Washington State University Institutional Animal Care and Use Committee (no. 04441-1). Dogs were enrolled following public requests for volunteers owning Labrador Retriever dogs believed to be pure-bred. Dogs were deemed healthy by a thorough history and physical examination. Owners verified that to the best of their knowledge their dogs were not in pain at the time of enrollment in the study and had not received treatment for pain in the previous 14 days. Owners gave written consent for their dogs' participation in the study.

Procedures

Each dog was physically examined and weighed, and the buccal mucosa was swabbed with a nylon cytology brush for collection of DNA. Samples were stored at room temperature until they were genotyped for the MC1R variant, which was performed after nociceptive

threshold determinations. Dogs were allowed to explore the testing room during attachment of the algometers to facilitate calming before testing for nociceptive thresholds. Nociceptive threshold for mechanical stimulation was determined first, followed by the threshold for thermal stimulation in each dog, for a total duration of approximately 90 minutes.

Mechanical and thermal nociceptive threshold determinations

Mechanical and thermal nociceptive thresholds were determined in unmedicated dogs by investigators unaware of the MC1R genotype for dogs with a black or chocolate coat. Based on previous work, yellow-coated dogs were predicted to have a homozygous variant (R360ter/R360ter) genotype. Investigators were trained by the vendor in the use of the commercially available mechanical force and thermal threshold algometers (TopCat Metrology, Inc., UK). The application of each device was noninvasive and involved a cuff attached to each thoracic limb (one cuff serving as a 'dummy') with remote activation of either mechanical or thermal stimuli. Dogs were not blinded to the device, and the measurements were started once the animal was no longer distracted by its presence. The measurements were all taken during a single session in an attempt to avoid learned or conditioned behavioral responses. All measurements were done by the same investigator (TEP).

Mechanical nociceptive threshold measurements were performed by positioning a 2 mm hemispherical blunt pin fixed in a rolling diaphragm actuator directly against the skin over the cranial aspect of the metacarpal region of one thoracic limb. Advancement of the pin was activated manually using a syringe connected to nondistensible tubing. A digital meter displayed the force exerted and provided instantaneous feedback to the operator, thereby guiding the steady rate of application of the force. Calibration of the device had been performed by the manufacturer up to a force of 25 newtons (N), with an accuracy limit of ± 0.5 N. The force applied to the actuator was ramped up at a rate of $0.8 \text{ N second}^{-1}$ until the animal responded by withdrawing the limb, looking at, shaking or biting at the cuff, or until the pin was maximally advanced. The pressure was released immediately on detection of any of these responses. Approximately 10 minutes elapsed between each test. A total of five measurements were recorded and the average force (in N) at the time of response detection was calculated.

Thermal nociceptive threshold measurements were performed by positioning a metal probe on the distal medial metacarpus of the thoracic limb opposite to the mechanical threshold test site. The hair at the test site was shaved ($0.5 \times 1.0 \text{ cm}$) to allow the probe to contact the skin directly and the probe was held in place with a wrap. A similar wrap was placed on the opposite thoracic limb with a similar pressure to minimize false-positive responses. The probe was calibrated prior to use with each dog. During testing, the probe was heated at a constant rate of $0.8 \text{ }^{\circ}\text{C second}^{-1}$ until the animal responded to the thermal stimulus by withdrawing the limb, looking at, shaking or biting at the cuff, or until the safety cutoff temperature ($50 \text{ }^{\circ}\text{C}$) was reached. The temperature was reduced immediately on detection of any of these responses. Ten minutes between each measurement was allowed for resting the dog and for the probe to cool to body temperature. Three measurements were recorded for each dog and the average value was calculated.

Determination of canine MC1R genotype

Genomic DNA was extracted from the buccal swab samples. The polymerase chain reaction (PCR) reaction contained approximately 20 ng genomic DNA template, 1× PCR buffer, 2 mM MgCl₂, 0.32 mM of each primer (Mc1r-E 5′-GAGATGCTGTCCAGTAGTCCC-3′ and Mc1r-D 5′-GGTCATGCTGAGCTGACAC-3′) and 0.05 U mL⁻¹ *Taq* DNA Polymerase (D4545; Sigma-Aldrich Corporation, MO, USA) in a final volume of 12.5 μL. A 1.3 kb product was generated using the following PCR conditions: 94 °C for 30 seconds for denaturation, 60 °C for 30 seconds for primer annealing, then 72 °C for 90 seconds for extension with 40 reaction cycles. Following PCR amplification, visualization of products on a 1.2% agarose/Tris Acetate-EDTA (TAE) gel was performed before treatment with ExoSAP-IT reagent (Affymetrix Life Science Reagents, OH, USA) in accordance with the manufacturer's instructions. Sequencing of the treated PCR products was performed using the primer nt773f 5′-CTTGACCTCTCACTCATGG-3′ and Big Dye 3.1 reagent mix (Applied Biosystems, Life Technologies, NY, USA). Sequence data were analyzed using Sequencher 5.2 software (GeneCodes Corp, MI, USA) which then allowed genotyping of the MC1R R306ter mutation at the 3′ end of the canine MC1R gene coding region.

Statistical analysis

Data were analyzed using GraphPad Prism Version 6.0 (GraphPad Software, Inc., CA, USA). Values for mechanical and thermal nociceptive thresholds were determined to be normally distributed by the D'Agostino & Pearson omnibus normality test. Data were analyzed following grouping by MC1R genotype and by coat-color phenotype using one-way ANOVA with *post hoc* comparisons made using Tukey's test. A *p*-value < 0.05 was considered statistically significant.

Results

Of the 34 dogs enrolled, four could not be genotyped using the collected samples and were lost to follow-up. Fourteen black-coated dogs, including one intact and six castrated males and seven ovariectomized females, were genotyped as WT/WT (*n* = 6) and WT/R306ter (*n* = 8). Eight chocolate-coated dogs (one intact and two castrated males and five ovariectomized females) were genotyped as WT/WT (*n* = 7) and WT/R306ter (*n* = 1). All eight yellow-coated dogs (one intact and three castrated males, one intact and three ovariectomized females) were R306ter/R306ter genotype. The ratios of males to females for each genotype were similar. Mean ± standard deviation (SD) values for mechanical nociceptive thresholds were as follows: WT/WT, 12.1 ± 2.1 N; WT/R306ter, 9.2 ± 2.4 N; R306ter/R306ter, 11.3 ± 3.1 N (Fig. 1). Significantly greater mechanical nociceptive thresholds were identified only for the wild-type (WT/WT), compared with heterozygous (WT/R306ter) genotypes (*p* = 0.04). Thermal thresholds were as follows: WT/WT, 43.6 ± 2.5 °C; WT/R306ter, 44.5 ± 3.8 °C; R306ter/R306ter, 44.4 ± 3.2 °C. Significant differences were not found for thermal thresholds between genotype groups (*p* = 0.77) or for either mechanical or thermal thresholds between groups based on coat-color phenotype (*p* = 0.6).

Discussion

Results of this study show decreased mean mechanical nociceptive thresholds for the heterozygous MC1R variant compared with the wild-type (WT/WT) in this population of Labrador Retriever dogs. The difference in mechanical nociceptive thresholds between these groups was small (about 3 N), suggesting that subtle differences in injury level and/or analgesic efficacy may be discriminated using this algometer. Involvement of MC1R in pain sensitivity and perception has been well established in mice and humans (Mogil et al. 2003; Delaney et al. 2010). While the specific mechanism underlying MC1R's involvement in pain perception has not been clearly defined, MC1R expression has been identified in brain areas that modulate nociception (Wikberg 1999). The results of the present study suggest that MC1R may also play a role in canine pain perception.

No significant difference between genotype groups was found for the thermal nociceptive threshold in this population of dogs. This is in contrast to studies of red-headed women with MC1R variants, who had a lower threshold for heat tolerance than women without MC1R variants (Liem et al. 2005).

There was complete conformity between the homozygous MC1R variant genotype and yellow-coated phenotype in our study population. This is in agreement with a previous study in which all yellow Labrador Retrievers tested were homozygous variant (R306ter/R306ter) for the MC1R genotype (Everts et al. 2000). However, our hypothesis was not supported, as there were no differences in thermal or mechanical nociceptive thresholds between homozygous MC1R variants (R306ter/R306ter) and the WT/WT or WT/R306ter genotypes in this small population of dogs. Thus, the relationship described in humans with red hair color having increased sensitivity to pain compared with the wild-type may not exist for yellow-coated Labrador Retrievers (homozygous MC1R variants). In dogs, coat color is determined by gene variants in addition to MC1R and it is unclear whether they have an impact on nociceptive sensitivity (Schmutz et al. 2002; Kerns et al. 2003). Future investigation using subpopulations of MC1R genotypes within another genotype that influences coat color in dogs may provide greater predictive power for clinically relevant differences in nociceptive sensitivity. The utility of this information in clinical trials involving analgesic efficacy in dogs could be significant.

The algometers used in the present study have been validated for the objective measurement of mechanical and thermal nociception in a variety of species, including dogs (Hoffmann et al. 2012). In another study of Retriever or Retriever-mix dogs, repeated sessions of nociceptive testing over a 2 week period led to decreasing values for the mechanical nociceptive threshold over time, presumably indicating a learned response (Coleman et al. 2014). However, in the present study, mechanical and thermal threshold measurements were performed within a single session of about 90 minutes and there was no trend in the directional change for each replicate response related to the order of testing. Thus, it is unlikely that a learned response introduced informational bias into this study.

A limitation of this study is that it was not possible to completely blind the investigators to the MC1R genotype of some dogs, as all yellow dogs were anticipated to have the R306ter/

R306ter genotype. However, it is unlikely that investigator bias influenced the results, as the significant difference between measured mechanical thresholds was found only between genotypes that cannot be predicted based on coat color. Furthermore, the number of animals in each group was relatively small and it is possible that the results are explained by a type 1 statistical error. Given the differences among breeds and the individual variations in nociceptive sensitivity, a larger study is required before conclusions can be made about the role of the MC1R gene in predicting pain sensitivity for dogs.

In conclusion, the results of this study suggest that single-allele MC1R variants may be associated with differences in nociception compared with the wild-type. However, no difference was observed between homozygous variants of MC1R (associated with yellow coat color) and other MC1R genotypes. These findings indicate that a clinical prediction of pain sensitivity based on the coat-color phenotype in Labrador Retrievers is unlikely.

Acknowledgments

The funds for this project originated from The Autzen Foundation Small Animal Endowment. MHC was supported by a US National Institutes of Health grant (GM102130) and the William R. Jones endowment to Washington State University College of Veterinary Medicine. The authors thank the staff at the Veterinary Clinical Pharmacology Laboratory at Washington State University, College of Veterinary Medicine for their technical assistance and the Washington State University College of Veterinary Medicine DNA Bank.

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Figure 1.

Mechanical nociceptive thresholds in Labrador Retriever dogs with different melanocortin-1 receptor (MC1R) genotypes. Shown are the mechanical nociceptive threshold data expressed as force in Newtons (N) plotted for individual dogs grouped by MC1R genotype [homozygous wild-type (WT/WT), circles; heterozygous variant (WT/R306ter), squares; and homozygous variant (R306ter/R306ter), triangles], and the mean $\pm$ standard deviations (horizontal lines) for each group. Significantly higher mean thresholds were observed in the wild-type (WT/WT) group than in the heterozygous (WT/R306ter) group ($p = 0.04$).