Quantitative Expression of the TRPV-1 Gene in Central and Peripheral Nervous Tissue in Horses

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ABSTRACT

The transient receptor potential vanilloid subfamily member-1 (TRPV-1), or vanilloid receptor, is a membrane ion channel highly expressed in nociceptive neurons. Over-stimulation of this receptor destroys the C-fibers responsible for chronic pain transmission, promoting long-lasting analgesia without motor impairment, which is a serious side effect for equine patients. To determine the TRPV-1 level of expression in the central and peripheral nervous system, hippocampus, hypothalamus, dorsal root ganglia (DRG), cervical spinal cord, and palmar digital nerve tissue were collected from 10 horses. After that, DRG cDNA prepared following mRNA extraction and reverse transcription was used in polymerase chain reactions (PCR) with cross-species primers designed to isolate cDNA encoding equine TRPV-1. The PCR products were cloned and submitted for sequence analysis. Once cDNA coding for TRPV-1 was isolated, equine-specific primers and probe were designed for TaqMan quantitative PCR. TRPV-1 mRNA levels were determined in the same harvested tissues and normalized against Beta-glucuronidase mRNA levels. Analysis of variance mixed procedure was applied to determine statistical difference in TRPV-1 mRNA levels. Statistical differences at $P \leq 0.05$ were analyzed by post hoc Tukey's studentized range test. Two polymorphic 263-bp PCR products were obtained. BLAST analysis demonstrated it to be between 92% and 98% identical to other species. TaqMan results showed significantly higher TRPV-1 mRNA level in the DRG when compared with all other tissues and in the hypothalamus when compared with palmar digital nerve. In conclusion, horses do have the TRPV-1 gene and level of expression varies among locations in the central and peripheral nervous system.

INTRODUCTION

The transient receptor potential vanilloid subfamily member-1 (TRPV-1) ion channel, also known as the vanilloid receptor (VR1), first described by Holzer in 1991[1] and first cloned in 1997[2] is a membrane cation sodium/calcium ion channel, highly expressed in the nociceptive neurons,[3] responsible for supplying polymodal thermal and inflammatory stimuli transduction.[4] Excessive stimulation of those receptors (for instance, after administration of TRPV-1-agonistic drugs like capsaicin or resiniferatoxin) causes severe calcium influx leading to cytotoxicity and neuronal apoptosis.[5] When resiniferatoxin is applied intrathecally, the result is a selective targeting and long-term removal of the vanilloid-expressing C-fiber and some Aδ-fiber cell bodies in the dorsal root ganglia (DRG).[6] This causes a disturbance of the neurotransmission from the cell body to spinal cord, thus impeding the modulation and perception of pain stimulation without interrupting mechanosensation, proprioception, or locomotor perception.[2,3] Analgesia without locomotor impairment is particularly important for species that are difficult to manage when locomotion is compromised, such as horses. Collectively, these receptors and their relatively selective expression suggest a promising role and attractive target for novel analgesic techniques for equine patients with chronic pain.

To the authors' knowledge, this is the first report regarding TRPV-1 gene expression and tissue quantification in horses. The TRPV-1 has been cloned from *Homo sapiens*,[7] *Rattus norvegicus*,[2] *Cavia porcellus*,[8] *Oryctolagus cuniculus*,[9] *Mus musculus*,[10] and *Canis familiaris*.[11] This information will probably influence the development of novel pain management strategies for the horse, especially if equine TRPV-1 is involved in similar pathways as described for other species. Further investigation using TRPV-1 agonists would be indicated.

The objective of this study was to confirm the presence of the TRPV-1 gene, responsible for encoding the receptor protein in various sites in the equine central and peripheral nervous system and verify if the gene expression varies among location.

MATERIAL AND METHODS

Collection and Processing of Equine Nervous System Tissues

Ten horses (8 Thoroughbred, 1 Arabian, and 1 Quarter horse) aged 7 ± 0.8 years old (mean ± SE), weighing 474.43 ± 33.3 kg, without musculoskeletal or neurologic disease were euthanatized using an intravenous bolus of pentobarbital sodium and phenytoin sodium (Beuthanasia-D Special, Schering-Plough Animal Health, Kenilworth, New Jersey, USA), for reasons other than this study under approval of the Louisiana State University–Institutional Animal Care and Use Committee. All the clinical evaluation and euthanasia was done under supervision of a trained clinician member of the Louisiana State University (LSU)–Equine Health Studies Program. Samples of the hippocampus, hypothalamus, DRG, cervical spinal cord, substantia nigra, and palmar digital nerve were immediately collected and preserved at -70°C in a mono-phase solution of phenol and guanidine thiocyanate (TRI REAGENT, Schering-Plough Animal Health, Kenilworth, New Jersey, USA). The total cellular RNA was isolated by chloroform extraction as per the manufacturers' instructions. RNA was re-suspended in 0.1% Diethyl Pyrocarbonate in water (DEPC water, Mo Bio Laboratories, Inc., Carlsbad, California, USA), analyzed by agarose gel electrophoresis, and quantitated by spectrophotometry.

All RNA samples (1.2 µg) were reverse transcribed to cDNA using Murine Moloney Leukemia Virus reverse transcriptase (MMLV-RT, Invitrogen, Carlsbad, California, USA), at 40°C for 1 hour.[12] This cDNA was employed as template cloned and quantitated by real-time polymerase chain reaction (RT-PCR) using the ABI PRISM 7700 sequence detection system (TaqMan, Applied Biosystems, Foster City, California, USA).

Identification of Partial TRPV-1 Sequence

Using an *Equus caballus* microsatellite sequence (AB033927) containing a suggested small piece of equine TRPV-1 DNA, an antisense primer was designed (Table 1 A) and used in conjunction with a primer designed from *Canis familiaris* TRPV-1 (Table 1 B) to isolate cDNA encoding equine TRPV-1 from DRG. The PCR reactions consisted of primers at 20 µM (1 µL of TRPV-1 forward plus 1 µL of TRPV-1 reverse), 5 µL of cDNA (prepared as described above), 1 µL of 50× polymerase (BD Advantage 2 polymerase mix, BD Biosciences, San Jose, California, USA), 1 µL of 10 mM 50× dNTP Mix (Invitrogen, Carlsbad, California, USA), 3 µL of 10× BD advantage 2 PCR buffer, and 18µL of PCR-grade water to complete a total volume of 30 µL per reaction, as per manufacturers' protocol. The PCR conditions consisted of an initial denaturation for 1 minute at 95°C, then 35 cycles of: 95°C for 30 seconds; 68°C for 1 minute; and a final extension at 68°C for 1 minute. The PCR products were cloned using Invitrogen's pTOPO cloning system[9] and ligations were transformed into *Escherichia coli* (TOP10 cells, Invitrogen, Carlsbad, California, USA) as per manufacturers' protocol. Recombinant plasmids were identified by PCR and grown overnight in 5 mL Luria Bertani broth containing 50 µg/mL kanamycin (Sigma-Aldrich, St. Louis, Missouri, USA). Plasmid DNA was isolated from these bacterial cultures using a Qiagen MiniPrep Spin Kit (Qiagen Inc, Valencia, California, USA) and submitted for DNA sequencing at the Biomedical and Molecular Medicine (BioMMed) facility within the Louisiana State University School of Veterinary Medicine. Sequence data were analyzed using the BLAST algorithm to compare them against the NCBI GenBank databases (www.ncbi.nlm.nih.gov/Genbank).

RT-PCR

To determine TRPV-1 mRNA level in various equine nervous system tissues, we employed 2 primers and a probe (Table 1 C, D, and E) designed from the cloned portion of equine TRPV-1 using Primer Express software (Applied Biosystems, Foster City, California, USA). The probe was labeled with the reporter dye FAM (6-carboxyfluoresceine) and the quencher dye TAMRA (6-carboxytetra methyl-rhodamine). Levels of the housekeeping gene Beta-glucuronidase (β-Gus) mRNA were also determined in the tissues and used for normalization of the TRPV-1 level in different tissues. The primers used to measure β-Gus mRNA levels are described in Table 1 F, G, and H.

A relative standard curve was constructed from DRG tissue by serially diluting (1:1) mRNA, then reverse transcribing it to cDNA. The highest point on the standard curve containing 1200 ng of total RNA was designated as a relative value of 1024 units. The first dilution containing 600 ng total RNA was designated as a relative value of 512 units. The dilutions were continued until 11 samples were prepared, with the lowest sample designated a relative value of 1. Cycle threshold values (CT) were obtained for TRPV-1 and β-Gus in all tissue samples, as well as for the standard curve. Linear regressions using the standard curves were carried out for both the TRPV-1 and β-Gus CTs as described in the ABI PRISM 7700 (Sequence Detection System User Bulletin #2, Applied Biosystems, Foster City, California, USA). Quantification of the TRPV-1 mRNA levels was achieved using the comparative threshold cycle method.[13]

Statistical Analysis

The interpolated values for TRPV-1 mRNA levels were normalized against those obtained for β-Gus mRNA by dividing the TRPV-1 CT value by that obtained for β-Gus. Thus the TRPV-1 mRNA level is expressed per β-Gus mRNA and presented as mean ± SE. In addition, the coefficient of variance among horses was calculated. Univariate analysis was used to determine normality of TRPV-1 mRNA levels among tissues type. The analysis of variance mixed procedure was used to determine statistical difference in TRPV-1 mRNA levels at $P \leq 0.05$ across tissues using SAS v.9.1 (SAS,

Table 1. Equine TRPV-1 primers and probe sequences. A and B: Nucleotide positions and sequences of primers for sequence determination of the equine TRPV-1. C, D, and E: Equine TRPV-1 primers and probe sequences for quantitative Taqman RT-PCR assays. F, G, and H: β-Gus primers and probe sequences for quantitative Taqman RT-PCR assays.

	Sequence (5'–3')	Position
A	GAT CCT GAG AGA CCT GTG TCG C	equineTRPV-1 forward
B	CGG TGA ACT CCA GGT GCG CCA T	canineTRPV-1 reverse
C	GGA AGC CAC GGG TAT AGT ACC	TRPV-1 – primers – forward
D	GTG GCC TCC ATG GTG TTC TC	TRPV-1 – primers – reverse
E	TGT TGG TCC AGC CCA TGG CCA G	TRPV-1 – probe
F	GAC ATC CGA GGG AAG GGC T	β-Gus – primer – forward
G	AGC CAA CGA AGC AGG TTG A	β-Gus – primer – reverse
H	GTC CTT CAC CAG CAG TGG CCA GTC T	β-Gus – probe

Raleigh, North Carolina, USA). When significant difference was observed, post hoc comparisons were made using Tukey's studentized range test to maintain Type I error at 0.05. For purpose of comparison, mean TRPV-1 mRNA level from hypothalamus, substantia nigra, hippocampus, spinal cord, and palmar digital nerve were summarized and compared as percentage of the DRG TRPV-1 mRNA level.

RESULTS

Isolation and Identification of the TRPV-1 Gene

Partial cds of the TRPV-1 gene were isolated from the equine DRG using the primers and probe described in Table 1. Two partial sequences containing 263 bp each were published in GenBank: "sequence A" with accession number DQ267482 (Figure 1A) and "sequence B" with accession number DQ267483 (Figure 1B).

The alignment of the 2 equine TRPV-1 partial sequences presented here shows a guanine/adenosine polymorphism at codon 123 and 133 in 50% of the subjects. The genotypic variation at codon 123 is not associated with amino acid changes (Figure 2). Conversely, the variance at codon 133 observed in the sequence A led to a polymorphism at the amino acid 545 where a valine was replaced with a methionine (Figure 2).

In a BLAST search against the GenBank public database, the 2 equine TRPV-1 partial sequences demonstrate the homology at the amino acid level to be: *Canis familiaris* = 98%; *Bos taurus* = 95%, *Homo sapiens* TRPV-1 = 92%, *Rattus norvegicus* = 91%, *Mus musculus* = 90%, *Oryctolagus cuniculus* = 92%, and *Cavia porcellus* = 92% (Figure 3).

Quantitative Tissue Detection of the Equine TRPV-1 mRNA Levels

The housekeeping gene β-Gus was expressed in all samples. TRPV-1 gene expression was also detected in all the samples analyzed. The ratio of TRPV-1 mRNA levels was normally distributed according to the univariate analysis. Post hoc Tukey's test detected significant differences in gene expression among the DRG and all other tissues evaluated ($P \leq 0.05$). Significant difference was also found in the TRPV-1 mRNA level between the hypothalamus and palmar digital nerve (Figure 4). No statistical difference in the TRPV-1 mRNA levels was found between hypothalamus, substantia nigra, hippocampus, and cervical spinal cord. A coefficient of variation in TRPV-1 mRNA level was observed among subjects: 56.67% in the DRG, 92.81% in the hypothalamus, 60.32% in the substantia nigra, 41.49% in the hippocampus, 57.34% in the spinal cord, and 90.86% in the digital nerve.

DISCUSSION

Presented here is an amino acid sequence closely homologous to TRPV-1 gene, which is part of the equine TRPV-1 orthologue, and also the differences in tissue expression of

Figure 1. *Equus caballus* TRPV-1 mRNA, partial cds isolated from DRG. Sequence A: GenBank accession no. DQ267482. Sequence B: GenBank accession no. DQ267483. Highlighted areas represent the nucleotide variance observed between sequences A and B.

Sequence A						
1	aatttagcgg	ccgcgaattc	gcccttgttt	gtggacagct	acagtgagat	gctcttcttt
61	gtgcagtcgc	tgttcatgct	ggggaccgtg	gtgctatact	tctgccaccg	caaggagtac
121	gtagcctcca	tgatgttctc	cctggccatg	ggctggacca	acatgctcta	ctatacccgt
181	ggcttccagc	agatgggcat	ctatgccgtc	atgatcgaga	agatgatcct	gagagacctg
241	tgtcgcttca	tgtttgtcta	cct			
Sequence B						
1	aatttagcgg	ccgcgaattc	gcccttgttt	gtggacagct	acagtgagat	gctcttcttt
61	gtgcagtcgc	tgttcatgct	ggggaccgtg	gtgctatact	tctgccaccg	caaggagtac
121	gtggcctcca	tggtgttctc	cctggccatg	ggctggacca	acatgctcta	ctatacccgt
181	ggcttccagc	agatgggcat	ctatgccgtc	atgatcgaga	agatgatcct	gagagacctg
241	tgtcgcttca	tgtttgtcta	cct			

Figure 2. TRPV1 amino acid sequences from *Equus caballus* sequence A (DQ267482), *Equus caballus* sequence B (DQ267483), *Homo sapiens* (BC132820), *Canis familiaris* (NM_001003970), *Rattus norvegicus* (NM_031982), *Mus musculus* (AY445519), *Oryctolagus cuniculus* (NM_001082166), and *Cavia porcellus* (AY729017). Highlighted area demonstrates the methionine (M) valine (V) variation at amino acid 545 from the horse sequence B.

Horse-A 508-LFVDSYSEMLFFVQSLFMLGTVVLYFCHRKEYVASMVFSLAMGWTNMLYYTRGFQQMGIYAVMIEKMILRDLCRFMFVYL-587
Horse-B . . . . . M . . . . .
Human . . . . . L . . . . . A . . . . . S . L . . . . . M . . . . L I
Dog . . . . . H . . . . . M . . . . .
Rat . . . . . I . . . . . V S . . . . . S Q . . . . . M . . . . .
Mouse . . . . . I . . . . . V S . . . . . S . . . . . M . . . . .
Rabbit . . . . . I . . . . . V S . . . . . S . . . . . M . . . . .
Guinea pig . . . . . L . . . . . A . . . . . S . L . . . . . M . . . . L I

this sequence in the central and peripheral nervous tissue. This study shows a polymorphism at the amino acid 545, in the equine sequences that are associated with a guanine/adenosine variation at codon 133, only observed in the sequence A. By comparing the TRPV-1 orthologues from *Homo sapiens* (accession number presented in Figure 3), *Canis familiaris*, *Rattus norvegicus*, *Mus musculus*, *Oryctolagus cuniculus*, and *Cavia porcellus* with the sequences A and B, it was observed that the methionine at the amino acid 545 is invariant across species. Furthermore, in a BLAST search against the equine genome database, it was observed that sequence B is currently held in GenBank for horses on chromosome 11.[14] It is possible that this polymorphism is a sequencing error. In addition, the relevance of the finding is controversial because 8 of 10 horses used in this experiment were Thoroughbred and the polymorphism was observed in only 50% of the subjects, leading us believe that the significance of the polymorphism observed in transcript A is low. However, to confirm allele frequency of this polymorphism, we suggest further investigation in a mixed population of horses using genomic PCR. Conversely, we observed no statistical difference between TRPV-1 mRNA from hypothalamus, substantia nigra, hippocampus, and spinal cord with a high coefficient of variance. Perhaps the high coefficient of variation is associated with the genotypic variation observed between horses and sequence A is a true polymorphism. If it is a true polymorphism, the consequence of this genotypic variation is unknown; the

Figure 3. Nucleotide sequences comparison of the 2 equine TRPV-1 sequences (Equine-A = GenBank accession number DQ26782 and Equine-B = accession number DQ26783) blasted against the nucleotide-binding sites of the human, dog, rat, mouse, rabbit, and guinea pig TRPV-1 available at NCBI library. The boxed areas represent conserved N-terminals sequence identity showing 70% threshold of obvious conservation among species. Note: in this alignment, a gap at codon 21 was inserted shifting the presented polymorphs to codons 124 and 134. Generated by BioEdit sequence alignment editor.

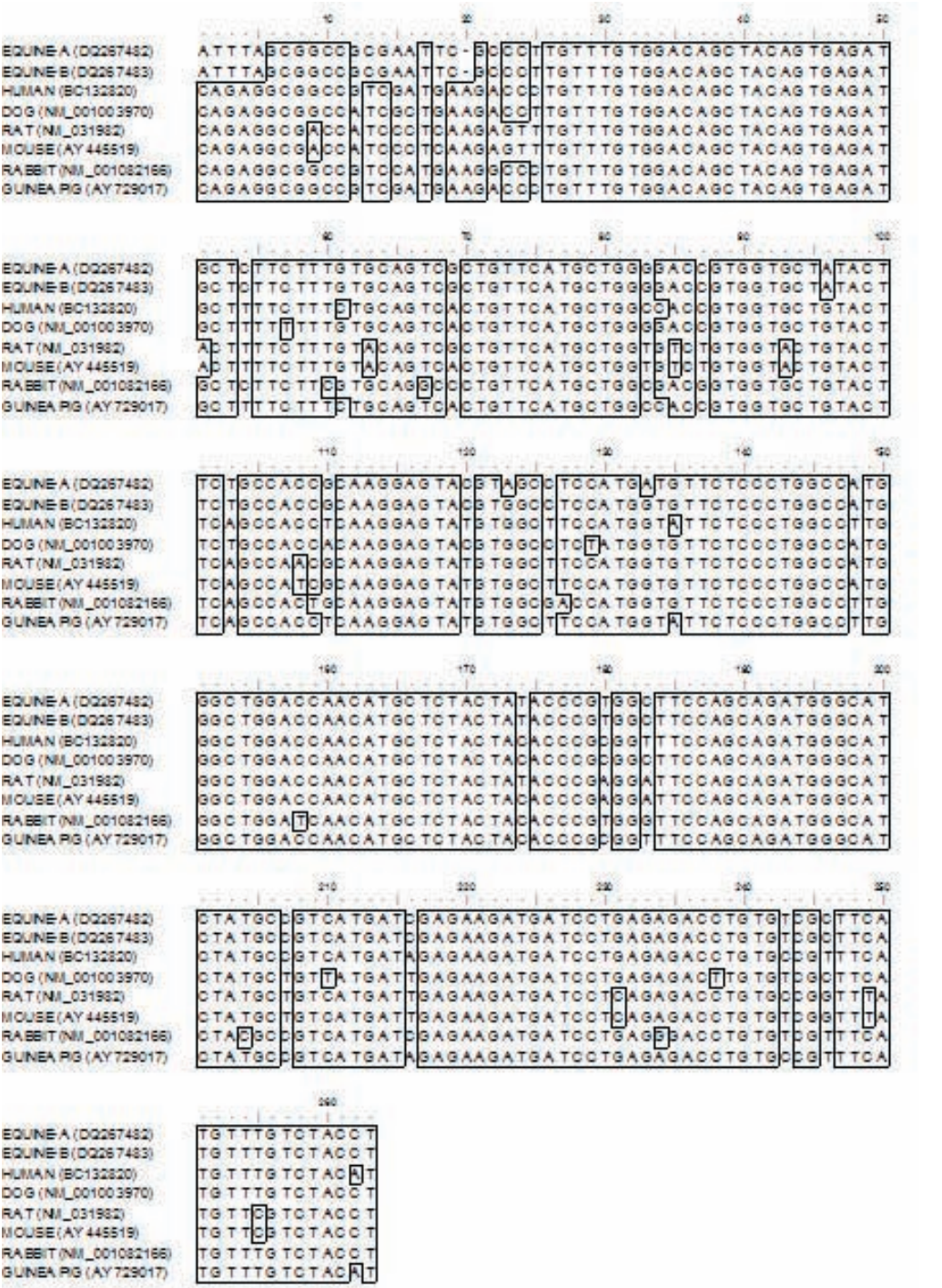
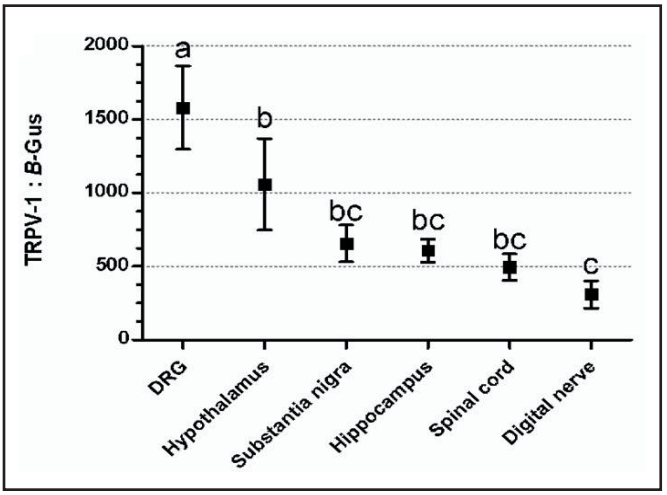


Figure 4. Tissue expression profiles for equine TRPV-1. Mean (± SE) ratio of TRPV-1 mRNA per β-Gus mRNA in nervous tissues. Means with the same subscript are not statistically different. DRG = dorsal root ganglia.



occurrence of a phenotypic variation is possible leading to a potential variation of the functional structure of the vanilloid receptor, which could interfere with the mechanism of action of vanilloid agonistic drugs in the horse. Possibly, we are describing here a new variant of the equine TRPV-1 gene. The high coefficient of variation can be also explained by sampling errors and tissue preparation, which could affect the overall quantitative expression results however the low CT values observed may imply appropriate sample quality, successful RNA extraction and cDNA transcription. The equine TRPV1 gene expression levels was also compared to the endogenous control β-Gus as an active reference to compare levels of gene expression in a specific tissue and to verify the sample quality during PCR analysis based on the CT values[18] where low CT value were observed indicating high level of gene expression.

The anatomical tissues examined here represent a wide array of functional tissues, including those associated with thermoregulation, pain pathway, and memory once the vanilloid receptor is a gated, nonselective cation channel[15] expressed mainly in the thermoregulatory center, nociceptors, and sensory neurons in the CNS of rats, dogs, humans, and guinea pigs.[16] A significant variation between tissue responses to TRPV-1 agonistic drugs implies that different tissues may have diverse receptor population and gene expression.[15] This study has shown that in horses there is indeed a high variance in level of TRPV-1 mRNA expression among the equine hypothalamus, hippocampus, DRG, substantia nigra, cervical spinal cord and palmar digital nerve. We observed a significant statistical difference between DRG and all other tissues and hypothalamus and palmar digital nerve, suggesting that in horses, the effects of TRPV-1 agonistic drugs will vary between peripheral or central administration. The high expression level in the DRG was expected because we based our study design on the results from a previous study with human orthologue of rat TRPV-1 where a higher gene expression of TRPV-1 was observed in the DRG.[7] It also explains why we chose DRG samples to identify the TRPV-1 gene sequence. Our results follow the path of other species showing that horse's DRG have a significant higher TRPV-1 mRNA level than all other locations analyzed here. From this result we speculate that either subarachnoid or direct injection of TRPV-1 agonist drugs into the nerve root would have a better effect than perineural injection in a peripheral nerve.

Comparing the TRPV-1 mRNA level expression among location, we observed that on average the level of expression from DRG is 33.0% higher than hypothalamus, 58.5% higher than substantia nigra, 61.5% higher than hippocampus, 68.7% higher than spinal cord, and 80.5% higher than palmar digital nerve.

The transient receptor potentials (TRP) family is subdivided into several subfamilies of ion channels including the vanilloid

subfamily (TRPV), which has 6 members; TRPV-1, TRPV-2, TRPV-3, TRPV-4, TRPV-5, and TRPV-6.[17] Due to sequence similarities, cDNA probes can be designed to cross-hybridize to different gene family members. It is important to note that our sequence data, when blasted against GenBank, showed homology only with TRPV-1 proteins. No TRPV-2, -3, -4, -5, or -6 proteins had any correlation with our isolated product, suggesting the isolated cDNA did indeed encode for equine TRPV-1. The equine PCR product showed a high degree of homology (92%-98%) to TRPV-1 genes identified from various species, supporting validity of the techniques used in this investigation. Even though we isolated a small part of the equine TRPV1 gene, the high degree of homology with other TRPV-1 orthologues and the lack of homology with any other gene led us to suggest that this sequence is a fragment of the equine TRPV-1 gene orthologue. Extrapolating the results from other species, the high degree of homology observed may suggest that TRPV-1 agonistic drugs[17] could produce analgesia through similar mechanisms as described in other species.

Our final objective is to elucidate clinically the effects of TRPV-1 agonistic drugs in horses suffering from chronic pain; however, acute pain right after administration of resiniferatoxin has been reported before and general anesthesia and analgesia is recommended to control the pain of injection.[6] This first stimulus is associated with Aδ-fibers agonistic effect of resiniferatoxin, and those fibers are present in the nervous system of any mammal species. Thus, even though we are unsure if horses will have the desired effect from TRPV-1 agonists, they will have the obnoxious stimuli. Considering this problem and avoiding animal suffering, we chose to begin with an in vitro study and after that, perform the clinical study only if the presence of the TRPV-1 receptor was confirmed.

Based on the results presented here, the authors conclude that horses do have the TRPV-1 gene with a significant level of expression variance among anatomical structures in the nervous system. Further investigation on the characterization of the protein encoded by TRPV-1 gene is still necessary. Now with the confirmation of the TRPV-1 gene in horses, we suggest that the TRPV-1 agonistic drugs should be evaluated clinically.

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