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Characterization of gene expression in naturally occurring feline degenerative joint disease-associated pain

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ABSTRACT

Degenerative joint disease (DJD) associated-pain is a clinically relevant and common condition affecting domesticated cats and other species including humans. Identification of the neurobiological signature of pain is well developed in rodent pain models, however such information is lacking from animals or humans with naturally occurring painful conditions. In this study, identification of housekeeping genes (HKG) for neuronal tissue and expression levels of genes considered associated with chronic pain in rodent models were explored in cats with naturally occurring osteoarthritic pain. Fourteen adult cats were evaluated — seven without clinical signs of osteoarthritic pain, and seven with hind limb radiographic DJD and pain. Expression of an investigator-selected set of pain signaling genes (including *ASIC3*, *ATF3*, *COX2*, *CX3CL1*, *NAV1.7*, *NAV1.8*, *NAV1.9*, *NGF*, *NK1R*, *TNFα*, *TRKA*) in lumbar spinal cord dorsal horn and lumbar dorsal root ganglia tissues from clinically healthy cats and cats with DJD were studied using quantitative RT-PCR (qPCR).

HKG identified as the most stable across all tissue samples were many of the ribosomal protein genes, such as *RPL30* and *RPS19*. qPCR results showed *ATF3* and *CX3CL1* up-regulated in DJD-affected dorsal root ganglia compared to clinically healthy controls. In spinal cord, *CX3CL1* was up-regulated and *NGF* was down-regulated when DJD-affected samples were compared to healthy samples. Further work is needed to understand the neurobiology of pain in naturally occurring disease and what rodent models are predictive of these changes in more heterogeneous populations such as domestic cats.

Introduction

Feline degenerative joint disease (DJD) can be associated with mobility impairment in feline patients (Gruen et al., 2014, 2015; Lascelles, 2010). This impairment is believed to be due to pain associated with the DJD as mobility appears to be increased when pain relief is provided (Gruen et al., 2014, 2015; Lascelles, 2010). The approach to pain management can be divided into two basic approaches — to try analgesics known or thought to be effective in other conditions or other species, or to base the analgesic selection on a rationale evaluation of the neurobiological changes present in the target disease state in the target species. This latter approach could be described as making rationale analgesic choices on the neurobiological signature of the pain. Presently, nothing is known about the neurobiology of feline DJD pain.

One approach to understanding the neurobiology of pain is to look at differences in gene expression between normal and phenotypically well-defined diseased states. Limited work has been performed along these lines in dogs. Hegemann et al. (2005) measured gene expression levels of interleukin *(IL)-1α*, *IL-1ß*, *IL-2*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-12*, *IL-18*, *interferon gamma (IF-γ)*, *transforming growth factor beta (TGFß)*, and *tumor necrosis factor alpha (TNFα)* in synovial fluid collected from dogs with osteoarthritis and immune-mediated polyarthritis using semi-quantitative real-time PCR methods. They reported differences in gene expression levels

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for some of these genes when they compared affected and unaffected dogs, but did not relate the findings to the presence of pain — an important consideration given that common measures of disease, such as radiographic features, do not correlate well with pain levels. Other investigators have evaluated the changes in gene expression in canine osteoarthritic cartilage (Clements et al., 2006, 2009), but again, the results were not evaluated against whether or not there was a pain state present.

Quantitative real-time PCR (qPCR) studies have evaluated expression levels of various cytokines in feline allergic skin disease (Taglinger et al., 2008), feline chronic gingival stomatitis (Harley et al., 1999) and feline coronavirus infection (Gelain et al., 2006). However, no studies have been performed in cats with DJD to determine gene expression changes in relation to pain.

We were interested in understanding the gene expression changes at crucial points in the nociceptive signal (pain) generation and transmission pathway in the dorsal root ganglia (DRG) and the dorsal horn (DH) of the spinal cord. In this study, we first evaluated a panel of potential housekeeping genes to identify the most stable reference genes in DJD and healthy cats in DRG and dorsal horn as this had not been previously done. After the most stable reference genes were identified, a selection of genes previously associated with nociception in rodent models, and of interest to the authors, were examined using qPCR in the same samples to allow us to start characterizing the neurobiological signature of pain associated with DJD in cats. Identification of stable reference genes in pain-related tissues in felines is important because of the cat's use as a potential animal model for human conditions and the prevalence of aging cats in veterinary medicine.

Materials and methods

Animals

This study was approved by the NCSU Institutional Animal Care and Use Committee (IACUC; Approval No.10-133-O; Approval date: 27 October 2010). Fourteen domestic cats euthanased at a local animal shelter, and of known pain status via pre-euthanasia examinations, were used for sample collection. The investigators evaluated the cats prior to euthanasia to determine whether they had pain associated with hind limb joints. Cats were euthanased with an overdose of barbiturates for reasons unrelated to this study (population control) and the investigators had no input into which cats were euthanased. Immediately after euthanasia orthogonal digital radiographs of the lumbar axial skeleton and appendicular joints of the hind limbs were performed and evaluated for the presence of radiographic signs indicative of naturally occurring DJD as described previously (Lascelles, 2010). The tarsus, stifle and hip joints were opened and visually inspected for evidence of macroscopic lesions indicative of DJD as described previously (Freire et al., 2011) and scored as described previously (Lascelles et al., 2010). The spinal canal from the last thoracic vertebrae to the sacrum was opened and evaluated for macroscopic lesions indicative of intervertebral disc degeneration with disk prolapse and spinal cord compression. In summary, cats were considered 'pain positive' if there were signs of discomfort on manipulation of hind limb joints; there was gross evidence of DJD in those joints; there was muscle atrophy in the hind limbs that was not seen elsewhere in the body; and there were no grossly visible indications of intervertebral disk prolapse or spinal cord compression.

Seven cats were considered free of musculoskeletal pain on pre-euthanasia evaluation, and of free of naturally occurring appendicular joint DJD and spinal DJD, and were included in the normal group (no abnormalities seen on digital radiographs and on macroscopic inspection of appendicular joints and axial skeleton). Seven cats were included in the hind limb DJD group and showed radiographic signs indicative of moderate-severe DJD in one or multiple appendicular joints of the hind limbs. No evidence of spinal compressive lesions was present.

Tissue collection

The lumbar spine from mid body of third lumbar vertebrae to mid body of the fifth lumbar vertebrae (lumbar intumescence) was harvested and DRG of fourth, fifth and sixth spinal segments from both sides were dissected and individually stored. Right and left DH of the spinal cord segment harvested were dissected and individually stored (after initial exposure to RNA*later* for at least 48 h). Dissection was performed using a dissecting microscope (Olympus SZX16, 0.7x–11.5x). Looking at the transverse cut surface of the spinal cord, the white and grey matters can be differentiated and the dorsal horn can easily be identified. The dorsal and ventral aspects of the spinal cord are identifiable by location of the ventral median fissure and the cranial and caudal aspects are determined by looking at the orientation of the nerve rootlets. The dura mater was opened and the spinal cord was divided into right and left segments following the ventral median fissure. Another cut placed just dorsal to the central canal allowed the dorsal horn to be separated from the ventral. This cut was performed carefully to assure separation by identification of the grey matter corresponding to the dorsal horn on the transverse cut surface of the spinal cord (Supplementary Figs. S1–S4). The same DRG and DH segments of the spinal cord were sampled in normal cats for comparison. All tissue samples were stored in RNA*later* (Qiagen) at −4 °C for 24 h and at −20 °C thereafter until sample processing.

RNA extraction and quantification

Total RNA from DRG and DH was extracted using the Qiagen RNeasy kit and an on-column DNase digestion to remove genomic DNA. The manufacturer's protocol was followed through the chloroform extraction but instead of precipitating the RNA in 100% ethanol, 70% ethanol was added and the mixture applied to the RNeasy (Qiagen) column, following the RNeasy kit instructions through the end of that manufacturer's protocol. Quantity of the extracted RNA was measured on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and quality was evaluated by running the extracted RNA on a 1.2% agarose gel to determine the integrity of the 28S and 18S ribosomal subunits. Only samples showing a 28S:18S rRNA band intensity ratio of approximately 2 (as determined visually) were used in this study.

Quantitative real-time PCR

A High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA) was used to reverse transcribe 500 ng of total RNA per the manufacturer's protocol. Subsequently, reactions were diluted 1:20 to provide enough template for all genes to be evaluated. Primer sequences for *GUSB*, *HMBS*, *RPL17*, *RPS7*, *RPS19* and *YWHAZ* were obtained from Penning et al. (2007). The remaining housekeeping and target gene primers were designed using Beacon Designer Software (Premier Biosoft Intl., Palo Alto, CA) to be compatible with SYBR Green I and to span an intron in order to detect genomic contamination. PCR primer sequences for the remaining housekeeping genes and pain-related target genes are given in Table 1.

Quantitative PCR was performed in a volume of 20 μL, consisting of 1 μL of diluted cDNA, 400 nmol/L of forward and reverse primers, 10 nmol/L fluorescein, and 1× Power SYBR Green Master Mix (Applied Biosystems). A three-step amplification protocol was performed in an iCycler IQ (Bio-Rad). Each reaction included one cycle at 95 °C for 7 min, following by 40 cycles at 30 s at 95 °C, 30 s at 52 °C–62 °C for annealing, and extension for 30 s at 72 °C. Specificity of each reaction was assessed by melt curve analysis (80 cycles starting at 55 °C with an increase of 0.5 °C every cycle, with a dwell time of 10 s) and one amplicon from each primer pair was DNA sequenced to confirm identity. Reactions were performed in duplicate, C_t values were averaged for the replicates and negative controls were included to detect possible contamination. Any duplicate measurements more than $0.5C_t$ apart were repeated for that sample or removed from the analysis.

Standard curves were evaluated for each primer pair by combining equal amounts of cDNA from each specimen into a pool. The pool was then diluted 1:3, 1:9, 1:27, 1:81 and 1:243. Dilutions were evaluated in duplicate to calculate amplification efficiencies which ranged from 90% to 110%, depending on the type of tissue and primer pair.

Data analysis

BestKeeper (Pfaffl et al., 2004), geNorm (Vandesompele et al., 2002) and NormFinder (Ohl et al., 2006) were used to evaluate the potential reference genes and select the most stable in these particular tissues.

Evaluation of gene expression of pain-associated target genes

Fold changes between healthy and DJD affected samples were computed via normalization to the geometric mean of the selected housekeeping genes. The fold change calculations incorporated corrections for reaction efficiency using the method of Pfaffl et al. (2002). Changes in the expression of healthy and DJD samples were evaluated for statistical significance using a linear mixed model derived from the work of Steibel et al. (2009). Bayesian Information Criteria was used to select the best parameters to include in the model (those contributing to the lowest BIC score). Fixed factors included health status (healthy vs. DJD), reproductive status (sterilized vs. intact), and radiographic score for DJD. Random factors included variables for position of the sample (left vs. right), age, and sex of the cats. The model was established with duplicate samples from a given cat nested within the cat. All analysis was conducted with SPSS software (version 24.0, IBM) and an alpha (α) of 0.05 was used for all analyses.

Table 1
Primer and PCR amplification details for reference and target genes.

Gene	Annealing temperature (°C)	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Product size (bp)	NCBI accession number
Housekeeping genes					
ACTB	58	TCGCCGACAGGATGCAGAAG	AGGTGGACAGCGAGGCCAGG	129	AB051104.1
B2M	58	TGGCGCGTTTTGTGGTCTTGGT	TCTCTGCTGGGTGACGGGAGT	108	NM 001009876
RPL30	54	CTACGCCAGGACCGACGGGA	TGACTGCACGGCGGGTTCTT	170	NM_001128841.1
RPS9	56	TGATCCGCCAGCGCCATATCAG	GCATTCTTCCTCTTCACGCGACCA	148	ENSFCAT00000014052
Pain-related target genes					
ASIC3	56	GTCACCAAGACTCTGTCT	AGCCCAGAAGACACAGAT	82	ENSFCAT00000018953
ATF3	62	CCAAGTGCCGCAACAAGAAA	CCATTCTGAGCCCGGACAAT	178	XM_847382.2
COX2	56	TAAGATTGTGATTGAAGACTATGT	CAGTGGTAGAGTGTGTTAAAC	138	ENSFCAT00000005544
CX3CL1	62	CTTCCTCGGCCTCCTCTTCT	GGCACCAGGACGTACGAGTT	148	ENSFCAT00000004693
NAV1.7	53	CAATCTTCCGTTTCAATG	CAGTTCGTCAGAATAGTG	136	ENSFCAT00000000647
NAV1.8	58	GCTCGGGAACCTGGTGGTGC	TGCCTTGGGCCAGGGTAGCA	205	ENSFCAG00000003166
NAV1.9	60	CTGCCGAGACCGAGGCCAAG	CCCATGGCAACGAGAGCCTCC	85	ENSFCAT00000000236
NGF	56	GCAGGGCAGACCCGCAACAT	GCACCACCCGCCTCCAAGTC	141	AJ639860.1
NK1R	62	CCAACAAGATTTATGAGAAAGTG	GTAACGGTCAGAGGAGTC	146	ENSFCAT000000005986
TNFα	56	CAACTAATCAACCCTCTG	CTACTACATGGGCTACTG	79	ENSFCAT00000004500
TRKA	62	AAGAGCGGGCTCCGTTTC	TTAAAGGAGAGATTCAGGCGACT	77	ENSFCAT000000006915

bp, base pairs.

Results

Animals

Cats included in the DJD-pain group were two castrated males, three intact males and two spayed females. Mean age (±standard deviation, SD) was 12 (1.4) years old, and mean weight (±SD) was 3.6 (0.6) kg; median body condition score was 5/9. The characteristics of the cats are detailed in Table S1 (Supplementary data), and all were designated 'pain positive'. Bilateral hind limb appendicular joint DJD in one or multiple joints were present in five of seven cats and samples from right and left DH of the spinal cord and right and left DRG were analyzed. In the other two cats in the DJD-pain group, only one hind limb had one or multiple joints affected with DJD and samples from the nervous tissue ipsilateral to the affected side were collected. Cats studied included in the healthy group were two castrated males, three intact males, one spayed female and one intact female. Mean age (±SD) was 8.7 (3.4) years, and mean weight (±SD) was 4.2 (0.9) kg; median body condition score was 5/9. Samples were collected from right and/or left nervous tissues in these cats to match with the samples collected in the cats with DJD.

Stable housekeeping gene analysis

Ten potential housekeeping genes for DRG and DH collected from DJD-affected and unaffected cats were examined using the three gene expression reference gene programs. The ten genes evaluated in this study were selected based on previous work by Penning et al. (2007) that examined the stability of these genes in feline liver, kidney, dental roots, heart and mammary gland tissues but not in any central nervous system tissues. The genes examined were beta actin (*ACTB*); beta-2-microglobulin (*B2M*); beta glucuronidase (*GUSB*); hydroxymethylbilane synthase (*HMBS*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-zeta polypeptide (*YWHAZ*); and ribosomal proteins L17 (*RPL17*), L30 (*RPL30*), S7 (*RPS7*), S9 (*RPS9*) and S19 (*RPS19*).

Dorsal root ganglia (DRG) reference genes

Due to dissection errors, two DRG were missing from the sample set; however DRG representing all 14 cats were used to evaluate the reference gene panel using BestKeeper and NormFinder, as those programs allow for missing data. Only paired samples from 12 cats were able to be evaluated using geNorm as that program requires complete data.

All genes amplified in the majority of the samples examined but *B2M* had a SD > 1 and was removed as a potential reference gene based on the recommendation from the BestKeeper software. Based on BestKeeper results, *ACTB*, *RPS19* and *RPS9* were the most stable housekeeping genes in the DRG samples. Using results from geNorm, *RPS7*, *RPS9* and *ACTB* were the most stable. And NormFinder produced results that differed from both geNorm and BestKeeper, finding *RPL30*, *HMBS* and *GUSB* as the most stable genes in the DRG samples (Table 2). An additional feature of geNorm is the determination of the optimum number of reference genes needed for accurate normalization (Fig. S2a and S2b). The number of reference genes associated with an expression stability value of 0.15 or lower is considered the optimum number of housekeeping genes needed. For each of the tissues examined in the feline samples, the variation in normalization factor with two vs. three reference genes was <0.15, indicating the optimum

Table 2
Reference gene rankings for each tissue type using BestKeeper, geNorm and NormFinder.

Program	Reference gene ranked order (most stable to least stable)
Dorsal root ganglia	
BestKeeper	*ACTB* > *RPS19* > *RPS9* > *RPL30* > *HMBS* > *RPS7* > *GUSB* > *YWHAZ* > *RPL17*
geNorm	*RPS7* > *RPS9* > *ACTB* > *RPL30* > *GUSB* > *RPS19* > *HMBS* > *YWHAZ* > *RPL17* > *B2M*
NormFinder	*RPL30* > *HMBS* > *GUSB* > *ACTB* > *RPS9* > *YWHAZ* > *RPL17* > *B2M* > *RPS7* > *RPS19*
Dorsal horn	
BestKeeper	*HMBS* > *ACTB* > *YWHAZ* > *RPS9* > *RPL17* > *RPS19* > *RPL30* > *GUSB* > *B2M* > *RPS7*
geNorm	*HMBS* > *RPL17* > *ACTB* > *YWHAZ* > *RPS7* > *RPL30* > *RPS19* > *GUSB* > *RPS9* > *B2M*
NormFinder	*HMBS* > *ACTB* > *RPL17* > *RPS7* > *RPS19* > *YWHAZ* > *RPL30* > *RPS9* > *GUSB* > *B2M*

number of genes needed was two. However, since the three programs did not always agree on the two most stable genes, three were selected for each tissue type. Based on these results, the geometric mean of *ACTB*, *RPS9* and *RPL30* was used as the reference value in each of the DRG samples.

Dorsal horn reference genes

A total of 24 DH samples (12 from cats with DJD and 12 from healthy cats) were used to evaluate potential housekeeping genes in this tissue. All ten reference genes amplified and had SD <1 so all were included in the reference gene analyses. Determination of the most stable reference genes in DH was very consistent across the three evaluation programs, with *HMBS* being the most stable gene (Table 2). *ACTB* was the next most stable using BestKeeper and NormFinder and this gene was placed as the third most stable using geNorm. Based on these results, the geometric mean of *ACTB*, *HMBS* and *RPL17* was used as the reference value in each of the DH samples.

Pain-associated target gene analysis

After a set of stable reference genes were identified for each tissue type, 13 genes associated with pain in rodents were selected (based on current knowledge of genes involved in pain states (Foulkes and Wood, 2008) and their expression levels compared in the DRG from DJD-affected and healthy samples. We hypothesized that these genes would show increased expression in DJD cats. The selected genes were as follows: acid sensing ion channel 3 (*ASIC3*); activating transcription factor 3 (*ATF3*); calcitonin gene related peptide (*CGRP*); cyclooxygenase 2 (*COX2*); chemokine (C-X3-X motif) ligand 1 (*CX3CL1*); sodium channel, voltage-gated, alpha subunit types IX (*NAV1.7*), X (*NAV1.8*) and XI (*NAV1.9*); nerve growth factor (*NGF*); tachykinin receptor 1 (*NK1R*); substance P (*TAC1*); tumor necrosis factor alpha (*TNFα*); and tropomyosin receptor kinase A (*TRKA*). Although two primer pairs were designed for each target gene, no amplification was detected for *CGRP* and *TAC1*, so they were excluded from this study. When DJD-affected cats were compared to healthy control cats for the remaining 11 target genes, *ATF3* and *CX3CL1* were significantly up-regulated in the DRG (1.639-fold and 1.069-fold, respectively; Table 3).

Ten target genes were selected for examination in DH samples—*ASIC3*, *COX2*, *CX3CL1*, *NAV1.7*, *NAV1.8*, *NAV1.9*, *NGF*, *NK1R*, *TNFα*, and *TRKA*. *ASIC3*, *NAV1.7* and *TRKA* were excluded because their PCR efficiencies were outside the acceptable range of 90–110% and two genes (*NAV1.8* and *NAV1.9*) did not amplify in any of the DH samples. Of the five remaining genes, *CX3CL1* was up-regulated (1.878-fold) and *NGF* was down-regulated (0.457-fold) in the DJD affected samples (Table 3).

Table 3
Gene expression results for dorsal root ganglia and dorsal horn samples collected from degenerative joint disease-affected cats compared to clinically healthy control cats.

Gene	FC	FC bounds[a]	*P*	PCR efficiency (%)
Dorsal root ganglia				
ASIC3	0.842	0.659–1.075	0.269	99.8
ATF3	1.639	1.289–2.085	0.008[b]	90.0
COX2	0.754	0.542–1.050	0.237	100.9
CX3XL1	1.069	0.740–1.545	0.024[b]	110.0
NAV1.7	1.059	0.759–1.477	0.625	96.0
NAV1.8	0.953	0.700–1.297	0.372	91.4
NAV1.9	1.081	0.784–1.490	0.521	109.3
NGF	0.714	0.569–0.896	0.374	99.5
NK1R	0.901	0.688–1.180	0.306	100.0
TNFα	0.393	0.240–0.646	0.143	100.0
TRKA	0.589	0.440–0.789	0.834	108.6
Dorsal horn				
COX2	0.396	0.312–0.570	0.069	96.3
CX3CL1	1.878	0.359–2.597	0.018[b]	110.0
NGF	0.457	0.366–0.570	0.043[b]	95.8
NK1R	0.742	0.609–0.903	0.893	100.5
TNFα	1.177	0.892–1.553	0.858	101.4

FC, fold change.
[a] FC bounds (±1 standard error FC).
[b] $P < 0.05$.

Discussion

With quantitative PCR, multiple target genes may be evaluated to measure changes in expression. However, to accurately determine the relative expression levels, a reference is used to normalize the expression results for differences in cDNA quantity between different samples, enabling comparisons between target genes across disease states. BestKeeper, geNorm, and NormFinder provide three different approaches for examining potential genes to select as the most stable genes for a given set of conditions.

At least three other studies have identified suitable gene expression housekeeping genes in various feline tissues. Penning et al. (2007) found that most of the ribosomal genes they tested appeared to be suitable reference genes in the different feline tissues. Wensman et al. (2007) tested six potential housekeeping genes in different brain tissues and found that *HPRT*, *YWHAZ* and *RPS7* were the most stable. Kessler et al. (2009) tested 10 potential reference genes in neoplastic, endocrine, blood, liver, intestine and lymphoid tissues in healthy cats and found that *RPS7*, *ACTB* and *ABL* were the most stably expressed housekeeping genes. While none of these other feline studies examined central nervous tissues, our results are similar, with several of the ribosomal genes and *ACTB* being stable reference genes. In rat dorsal root ganglia and dorsal horn samples Piller et al. (2013) found that *ACTB* and *HPRT* and also *RPL29* and *RPL13A* were suitable housekeeping genes in DRG and DH samples, respectively, and Wan et al. (2010) found *RPL29*, *RPL3* and *ACTB* most stable in DRG samples isolated from a rat model of neuropathic pain. Based on these other studies, our findings that many of the ribosomal genes and *ACTB* are the most stable in feline DRG and DH samples are reasonable.

The target genes selected in this study were chosen based on results from work on skeletal pain conducted in rodents (e.g. Mantyh, 2014) and knowledge of genes involved in pain states (Foulkes and Wood, 2008) in an attempt to characterize the neurobiology of DJD-associated pain in the cat. They were also chosen because of therapeutics that are available, or in development, or because of their involvement in neuropathic pain states. mRNA levels of *CX3CL1*, also known as fractalkine, were higher in the DRG of DJD-affected cats, suggesting that these cats were experiencing a neuropathic pain state (Clark & Malcangio, 2014). In studies examining changes in gene expression in DRGs, the expression of activating transcription factor 3 (*ATF3*) is increased in the DRG after peripheral nerve injury (Shortland et al., 2006) and more recently has been shown to be increased also in models of OA in rodents (Thakur et al., 2012). *ATF3* is considered indicative of neuronal-damage and neuropathic pain. Similar results were identified in the DRGs isolated from the DJD-affected cats used in this study, where the expression of *ATF3* was increased 1.6-fold, and further suggests that cats with DJD may be experiencing neuropathic pain like states.

Very little other work has been performed in DRG from non-rodent species, but in horses with laminitis, immunohistochemical analysis of DRG from hind limb laminitic horses, comparing lumbar DRG with cervical DRG showed an increased expression of *ATF3*, and the authors suggested this may be indicative of neuropathic pain (Jones et al., 2007). In many experimental pain models the

expression of *COX2* has been found to be increased in the spinal cord in response to inflammation and injury (Vardeh et al., 2009) and contributes to pain, but we did not observe a significant difference in *COX2* when we compared DJD and healthy samples. The transcription factor ATF3 has been shown to negatively regulate *COX2* levels during acute inflammation in mice (Hellmann et al., 2015) and our results may indicate the same negative regulation is occurring in cats with DJD-pain. However, it is important to remember that our results reflect a single cross-sectional chronic time point. The mechanisms of pain likely change over time, and the time course of the rodent models tends to be very short, and may not reflect mechanisms at later stages of the pain state. However, considering again our findings around *COX2*, investigators have struggled to show clinical benefit of NSAIDs in cats with DJD-associated pain (Gruen et al., 2015).

In the DJD-affected cats, we found significant increased expression of *CX3CL1* in DH, similar to what we detected in the DRG samples. Studies in rodents have shown that CX3CL1 and its receptor CX3CR1 are an important signaling pathway involved in microglial contributions to chronic pain. Interestingly the fractalkine protein is membrane-bound until it is cleaved by different proteases, including cathepsin S. The cleaved, soluble form of fractalkine is associated with neuropathic pain, not the membrane-bound form (Clark & Malcangio, 2014). We also found *NGF* to be significantly differentially expressed in DH, but not in the expected direction of that reported in other pain models. Interestingly, in a study of a monoclonal antibody to nerve growth factor (anti-NGF mAb) (Gruen et al., 2016), the anti-NGF mAb produced robust and long-lasting improvements in activity and mobility, presumably due to a decrease in pain. This may illustrate how simply evaluating the expression of genes may not give the whole picture — for a comprehensive understanding of the neurobiology of pain, gene expression, translation into protein and post-translational modifications need to be evaluated.

Taken together, our results do point to increased expression of genes considered to be involved in neuropathic pain, and may be evidence of a neuropathic like pain state in cats with DJD-associated pain. We expected that several of the genes we selected (which have all been shown to play a role in pain in rodent models of arthritis) would show altered expression between the DJD-pain and healthy groups. There are several reasons for the relative lack of change across the genes we selected. Rodent models are just that — *induced models*, and these models may not reflect the neurobiology of pain in naturally occurring pain states. Indeed, this is one of the reasons that has been suggested for the lack of translation of basic pain research into effective new therapeutics (Lascelles et al., 2018). Another aspect of our study that should be factored into the interpretation of the results is that the tissues we collected contained varying types of cells and indeed tissue, and so we cannot directly infer that changes or lack of changes are due to any particular tissue type. We made every attempt to only use cats that definitely had DJD-pain or were healthy, however, we did not have detailed history on each cat, and our pain status was simply designated as 'yes' or 'no'. Future studies should endeavor to collect tissues from more highly phenotyped animals (with respect to pain status). This is particularly important as the neurobiology of pain probably varies over time and with features of the phenotype (e.g. excessive sensitivity present or not). The lack of changes in our study may reflect an underpowered study and the heterogeneity of the samples assessed. Despite the shortcomings, we believe our study points to the need to look more closely at the naturally occurring pain states and such investigations may lead to novel therapeutic targets. However, there is a danger of missing novel, relevant targets if the starting point is already biased to what is known from rodent models, as our study was.

Selection of appropriate stable housekeeping genes is extremely important and has not been previously done for feline nervous tissues. As rodent species continue to illustrate they are not appropriate models for different human conditions, other animal models with naturally occurring disease, such as the cat, will become more and more prevalent and appropriate reference genes will be needed for accurate gene evaluation studies.

Conclusions

Our approach of evaluating neurobiological changes (gene expression) in nervous system tissue from cats with naturally occurring pain appears feasible. The results of this small study point to increased expression of genes considered to be involved in neuropathic pain, and may be evidence of a neuropathic like pain state in cats with DJD-associated pain. Further work should be undertaken to confirm our results, and expand on these studies.

Conflict of interest statement

Funding for this work was provided by private donations to Translational Research in Pain Program from individuals concerned about the lack of effective therapeutics for cats with chronic pain. JB was employed on a Morris Animal Foundation grant, D08FE-043, working to develop an assessment tool for chronic pain in cats. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tvjl.2018.11.008.

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